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SYNTHESIS OF A CLEAVABLE HETEROBIFUNCTIONAL PHOTOLABELLING REAGENT: RING-LABELLED 3-[(4-AZIDOPHENYL)DITHIO]PROPIONIC ACID-^{14}C

VARADARAJAN RAMASWAMI, Department of Chemistry, Carnegie-Mellon University, Pittsburgh, PA 15213 USA.

DAVID A. TIRRELL,* Department of Polymer Science and Engineering, University of Massachusetts, Amherst, MA 01003 USA

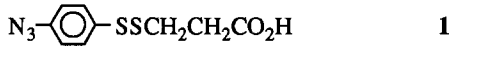
SUMMARY

An efficient synthesis of ring-labelled 3-[(4-azidophenyl)dithio]propionic acid-^{14}C is described. Chlorosulfonation of uniformly ring-labelled acetanilide-^{14}C followed by reductive dimerization of the sulfonyl chloride with HI afforded 4-acetamidophenyl disulfide. Hydrolysis and diazotization then gave 4-azidophenyl disulfide, which was converted to the title compound via the sulfur transfer reagent N-(4-azidophenylthio)phthalimide. The overall yield of 3-[(4-azidophenyl)dithio]propionic acid-^{14}C was 22%. 3-[(4-Azidophenyl)dithio]propionic acid-^{14}C is a cleavable heterobifunctional photolabelling reagent of potential utility in studies of biomembrane structure and intermacromolecular interaction.

KEY WORDS: 3-[(4-azidophenyl)dithio]propionic acid, photolabelling reagent, biomembrane, synthesis, carbon-14.

INTRODUCTION

Cleavable, heterobifunctional photolabelling reagents have been used in a range of studies of biomembrane organization and intermacromolecular interaction (1). Among the most useful of these reagents are derivatives of 3-[(4-azidophenyl)dithio]propionic acid (APDP, **1**).



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Kiehm and Ji introduced in 1977 the use of ethyl 4-[(4-azidophenyl)dithio]butyrimidate **2** to effect photochemical crosslinking of erythrocyte membrane proteins (2). The N-hydroxysuccinimidyl

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*To whom correspondence should be addressed.

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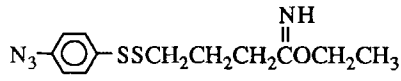

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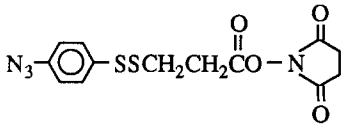
ester of APDP (3) has since been used by Vanin and Ji (3) to crosslink concanavalin A oligomers, by Baenziger and Fiete (4) to prepare photoactivatible glycopeptides for site-specific labelling of lectins,



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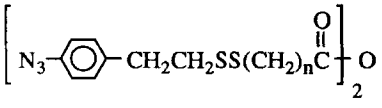
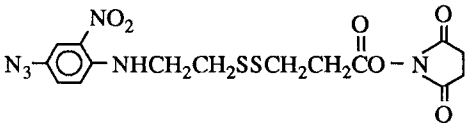
by Zarling and coworkers (5) to examine the association of the Sendai viral envelope with surface polypeptides on newly infected cells, and by Jung and Moroi (6) to crosslink platelet glycoproteins. Brunner and Richards (7) have used analogous reagents (4) to prepare photoactive lecithin deriva-



4 n = 2, 7

tives, and Hynes and coworkers (8) have employed N-succinimidyl 3-[(2-nitro-4-azidophenyl)-2-aminoethyldithio]propionate 5 to examine the binding of gelatin and fibronectin.



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In many of these experiments, identification and analysis of the products of photoinduced crosslinking require the use of radiolabelled probes. In the present paper, we describe an efficient synthesis of ring-labelled ^{14}C-APDP. Positioning of the ^{14}C-label on the ring allows transfer of radioactivity from the macromolecule of interest (bound via the carboxylic acid function of **1**) to the biological target, in two discrete steps: initial reaction of the photogenerated nitrene followed by mild reduction of the disulfide linking group. The following sections provide detailed procedures for the preparation of ^{14}C-APDP, as well as comments on the factors that determine the efficiency of each reaction step.

EXPERIMENTAL

Materials and Measurements

Acetanilide-^{14}C (uniformly labelled on the ring) was purchased from Pathfinder Laboratories, St. Louis, MO. Chlorine gas (research grade) was obtained from Linde Division of Union Carbide

Corporation. Chloroform-d (99.96% d), 4-aminophenyl disulfide (recrystallized three times from ethanol/water), N,N-dimethylformamide (DMF, gold label), phenyl disulfide (99+%), hydriodic acid (51% solution), 3-mercaptopropionic acid (distilled under reduced pressure, b.p. 110-111°C/15 mm), methanol (HPLC grade), methylene chloride-d_2 (99.96% d), phthalimide (gold label), potassium bromide (infrared spectral grade), sodium nitrite (ACS reagent grade), tetrahydrofuran-d_8 (gold label), triethyl amine (gold label, distilled over CaH_2, b.p. 88°C) were purchased from Aldrich Chemical Company. Benzene (distilled over 4A molecular sieves, b.p. 80°C), calcium sulfate (anhydrous) chloroform (spectrophotometric grade), diethyl ether (anhydrous), dimethylsulfoxide (DMSO, re-agent grade), ethyl acetate (reagent grade), hydrochloric acid (analytical reagent), methylene chloride (reagent grade), pentane (distilled over CaH_2, b.p. 36°C), petroleum ether (distilled over CaH_2, b.p. 50-60°C), silica gel (E. Merck Reagent for chromatography), sodium bisulfite (analytical reagent), sodium chloride (reagent grade), sodium carbonate (99%, ACS reagent grade) were purchased from Fisher Scientific Company.

^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded at 300 MHz and 75 MHz respectively on a Varian XL-300 spectrometer. Proton spectra at 80 MHz were recorded on an IBM-Bruker NR80 instrument. ^{13}C NMR spectra were recorded with broad band decoupling. Chemical shifts for ^1H and ^{13}C spectra are reported in parts per million downfield from tetramethylsilane (TMS). Infrared measurements were recorded on either a Perkin Elmer Model 580 or a Model 1320 spectrometer. Fourier transform infrared spectra were recorded on an IBM 32 spectrometer. Ultraviolet spectra were recorded on Varian Carey 219, Perkin Elmer Lambda 5 or Beckman DU-7 spectrophotometers. Melting points were recorded on a Thomas-Hoover capillary apparatus and are uncorrected.

Preparations

4-Acetamidobenzenesulfonyl Chloride-^{14}C. Acetanilide (133.2 mg, 0.985 mmol) was added to 367 mg (2.71 mmol, 100 mCi) of acetanilide-^{14}C, ring labelled, dissolved in 2 mL of dry methylene chloride in a 5 mL screw cap vial. Small volumes (0.2 mL) of this solution were transferred via pipet into a 5 mL round-bottomed flask fitted with gas inlet and outlet tubes. The solution was evaporated under a gentle stream of N_2, leaving behind a thin solid coating on the bottom of the flask. This procedure was repeated until all of the acetanilide-^{14}C had been transferred into the flask. The screw cap vial and the pipet were rinsed with an additional 2 mL of methylene chloride, which was also transferred into the flask and evaporated under a stream of nitrogen. The film of acetanilide-

^{14}C was then dried under vacuum in a desiccator for 1 hr. The flask was then placed in an ice bath and cooled to 10-12°C. Chlorosulfonic acid (3.0 mL, 5.26 g, 45.1 mmol) was then added very slowly from a pipet under a flow of dry N_2. After all the chlorosulfonic acid had been added, the mixture was heated to 60-65°C for 3.5 hours under N_2 to complete the reaction. The reaction was complete when no more HCl gas was evolved. The viscous liquid was cooled to 10°C and poured into ca. 30 g of ice water to quench unreacted chlorosulfonic acid. 4-Acetamidobenzenesulfonyl chloride precipitated as a white solid which was filtered, washed with 5-10 mL of cold water and then dried under vacuum. The crude product weighed 590 mg (69% yield). Thin layer chromatography (silica gel, chloroform:ethyl acetate 2:3) showed a single spot at $R_f = 0.6$. TLC and ^1H NMR spectra were identical to those of an authentic sample of 4-acetamidobenzene sulfonyl chloride. ^1H NMR (300 MHz, $CDCl_3$) δ: 8.3, 7.7 (AB quartet, 4H), 7.5 (broad singlet, 1H), 2.6 (singlet, 3H).

4-Acetamidophenyl Disulfide-^{14}C. 4-Acetamidobenzenesulfonyl chloride-^{14}C (590 mg, 2.62 mmol) was placed in a 10 mL round-bottomed flask. Hydriodic acid (3.0 mL of a 51% aqueous solution, 5.26 g, 45.1 mmol) was added slowly while the temperature of the reaction mixture was maintained at or below 10°C. The reaction mixture was stirred for 30 min, a reflux condenser was attached and the temperature was raised to 95°C. After 2.5 hr the reaction mixture was cooled to 10°C and solid sodium bisulfite was added until all the iodine formed as a side product was reduced to sodium iodide (a pale yellow suspension and the absence of dark I_2 crystals). 4-Acetamidophenyl disulfide-^{14}C precipitated as a pale yellow solid. The precipitate was filtered, washed with cold water, and dried under vacuum (359 mg, 85% yield). Thin layer chromatography (silica gel, ethyl acetate) and ^1H NMR spectra were identical to those of an authentic sample of 4-acetamidophenyl disulfide. ^1H NMR (300 MHz, THF-d_8) δ: 9.1 (broad singlet), 7.6, 7.4 (AB quartet), 2.0 (singlet).

4-Aminophenyl Disulfide-^{14}C. 4-Acetamidophenyl disulfide-^{14}C (359 mg, 1.08 mmol) was placed in a 25 mL round-bottomed flask. Hydrochloric acid (20 mL, 6M) was added with stirring at ambient temperature. A reflux condenser was attached and the reaction mixture heated to 90-100°C. After 24 hr the reaction mixture was cooled to 10°C and 9 M aqueous NaOH was added (CAUTION) to bring the reaction mixture to pH 8.0. 4-Aminophenyl disulfide-^{14}C (239 mg, 89% yield) precipitated as a yellow solid upon standing for 15-30 min at room temperature. Thin layer chromatography (silica gel, ethyl acetate:petroleum ether 7:3, $R_f = 0.6$) and ^1H NMR spectra were identical to those of an authentic sample of 4-aminophenyl disulfide. ^1H NMR (300 MHz, $CDCl_3$) δ: 7.25 (doublet), 6.61 (doublet), 3.78 (broad singlet).

4-Azidophenyl Disulfide-^{14}C. 4-Aminophenyl disulfide-^{14}C (239 mg, 0.96 mmol) was

dissolved in 5 mL of 2 M HCl in a 10 mL round-bottomed flask cooled in an ice bath in the dark. Solutions containing sodium nitrite (264 mg, 3.84 mmol) in 2 mL distilled water, and sodium azide (374 mg, 5.76 mmol) in 2 mL distilled water were prepared and cooled to 10°C. The sodium nitrite solution was added slowly via pipet. The reaction mixture was allowed to stir for several minutes, after which time the sodium azide solution was added rapidly to the solution of the diazonium salt. The reaction flask was then wrapped in aluminum foil and placed in the freezer overnight. 4-Azidophenyl disulfide-^{14}C precipitated and was filtered and dried under vacuum (269 mg, 92% yield). The crude product was recrystallized from n-hexane to afford 346 mg (85%) of pure 4-azidophenyl disulfide-^{14}C. Thin layer chromatography (silica gel, benzene:hexane 4:1, $R_f = 0.5$) and ^{1}H NMR spectra were identical to those of an authentic sample of 4-azidophenyl disulfide. ^{1}H NMR (300 MHz, CD_2Cl_2) δ: 7.45 (doublet) 6.95 (doublet).

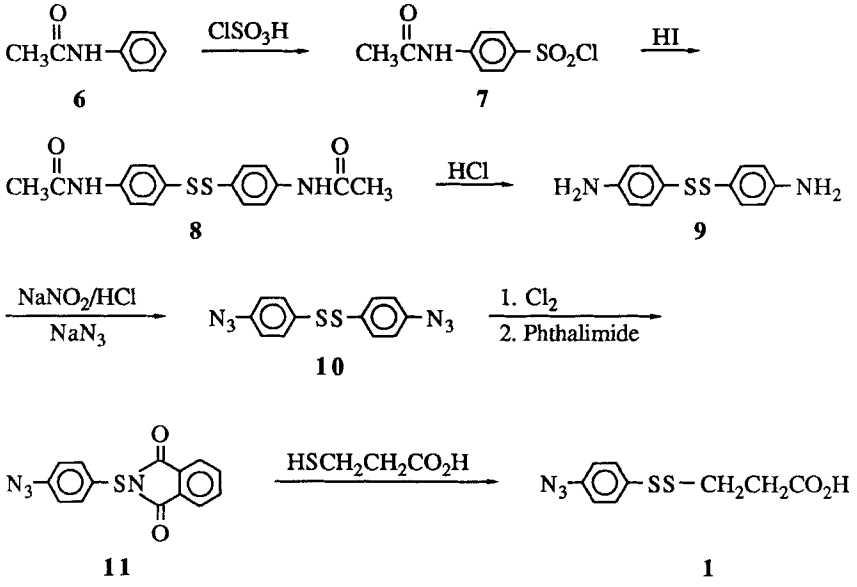
N-(4-Azidophenylthio)phthalimide-^{14}C. 4-Azidophenyl disulfide-^{14}C (246 mg, 0.822 mmol) was suspended in 5 mL of dry pentane in a 10 mL round-bottomed flask and cooled to 10°C in an ice bath in the dark. Chlorine gas from a lecture bottle was bubbled very slowly through the solution. The reaction progress was monitored by thin layer chromatography (silica gel, chloroform). 4-Azidophenyl disulfide-^{14}C ($R_f = 0.8$) disappeared and 4-azidophenylsulfenyl chloride-^{14}C ($R_f = 0.25$) appeared within 2 min. Chlorine flow was stopped before the appearance of a second spot ($R_f = 0.5$) which is the result of further chlorination of the sulfenyl chloride. The resulting solution was used directly in the next step of the sequence. A 5 mL two-necked round-bottomed flask fitted with a reflux condenser and addition funnel was charged with 330 mg (2.24 mmol) of phthalimide, 277 μL (379 mg, 3.74 mmol) of triethylamine and 2.5 mL of N,N-dimethylformamide. 4-Azidophenyl sulfenyl chloride-^{14}C in hexane prepared as described above was added dropwise with stirring. The temperature was maintained below 30°C during the addition. After the addition was complete, the solution was stirred for 2.5 hr and the hexane was evaporated under a stream of nitrogen. The solution was poured into ice water and the precipitated N-(4-azidophenylthio) phthalimide-^{14}C was extracted twice with ether (15 mL). The ether solution was dried over anhydrous $CaSO_4$, and on evaporation yielded 489 mg (90%) of the crude product. Thin layer chromatography (silica gel, chloroform:methanol 10:1) showed a spot ($R_f = 0.6$) which was identical to that of an authentic sample of N-(4-azidophenylthio)phthalimide. The crude product also contained phthalimide and N-chlorophthalimide as side products, but was used in the next step of the sequence without purification.

3-[(4-Azidophenyl)dithio]propionic acid-^{14}C. N-(4-azidophenylthio)phthalimide-^{14}C

(ca. 489 mg, 1.64 mmol) and 3-mercaptopropionic acid (150 μL, 183 mg, 1.72 mmol) were dissolved in 15 mL of N_2-purged benzene in a 25 mL round-bottomed flask fitted with a reflux condenser. The solution was heated to reflux under N_2 for 16 hr. Cooling of the reaction mixture in an ice bath caused precipitation of phthalimide, which was removed by filtration. Evaporation of the solvent under a flow of N_2 left crude 3-[(4-azidophenyl)dithio]propionic acid-^{14}C. The crude product was recrystallized from n-hexane, filtered and dried to leave 150 mg (35%, 27 mCi/mmol) of pure 3-[(4-azidophenyl)-dithio]propionic acid-^{14}C. Thin layer chromatography (silica gel, chloroform:methanol 9:1) showed a single spot ($R_f = 0.3$) identical to that of an authentic sample of 3-[(4-azidophenyl)dithio]propionic acid. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 2.78 (triplet), 2.98 (triplet), 7.0, 7.5 (AB quartet).

RESULTS AND DISCUSSION

Uniformly ring-labelled 3-[(4-azidophenyl)dithio]propionic acid-^{14}C (^{14}C-APDP, **1**) of specific activity 27 mCi/mmol was prepared from acetanilide-^{14}C in an overall yield of 22% (Scheme I). Chlorosulfonation of uniformly ring-labelled acetanilide-^{14}C followed by reductive dimerization of



6 **7** **8** **9** **10** **11** **1**

the sulfonyl chloride **7** with HI afforded 4-acetamidophenyl disulfide **8**. Hydrolysis and diazotization then gave 4-azidophenyl disulfide **10**, which was converted to **1** via the sulfur transfer reagent N-(4-azidophenylthio)phthalimide **11**. The following paragraphs describe our efforts to optimize this procedure on cold acetanidide. The Experimental Section provides detailed instructions for the efficient synthesis of **1** in uniformly ring-labelled form.

4-Acetamidobenzenesulfonyl Chloride. Table I summarizes the results of optimization of the chlorosulfonation of acetanilide. Acetanilide was treated with neat $ClSO_3H$ for 3.5 hr at 60-5°C, with the molar ratio of the acid to acetanilide varied from 6 to 24. Yields were consistently 60-70% when freshly distilled $ClSO_3H$ was used. Reaction for 18 hr at 50-5°C afforded reduced yields (entries 6 and 7). Higher yields of **7** were obtained with highly purified (3X recrystallized) acetanilide, but because of the limited purity of the commercially available acetanilide-^{14}C (tan crystals), optimization was performed with once-recrystallized starting material. In the synthesis of ^{14}C-labelled **7**, acetanilide-^{14}C was used as received from Pathfinder Laboratories.

Table I

OPTIMIZATION OF THE SYNTHESIS OF 4-ACETAMIDOBENZENESULFONYL CHLORIDE[a]

Expt.	$ClSO_3H$ g (mmol)	$ClSO_3H$ / **1** (mol/mol)	Yield mg (%)
1	2.63 (22.6)	6.2	520 (61)
2	3.50 (30.0)	8.2	552 (65)
3	5.36 (46.0)	12.6	544 (64)
4	7.01 (60.1)	16.5	608 (71)
5	10.5 (90.1)	24.1	522 (61)
6[c]	2.63 (22.6)	6.2	330 (39)
7[c]	5.26 (45.1)	12.4	506 (59)

[a] 0.50 g acetanilide was treated with neat $ClSO_3H$ for 3.5 hr at 60-5°C, unless otherwise noted.
[b] Average of three runs.
[c] 18 hr at 50-5°C.

4-Acetamidophenyl Disulfide. 4-Acetamidobenzenesulfonyl chloride was converted to 4-acetamidophenyl disulfide by treatment with hot aqueous HI (Table II). Attempted reductions of **7** with Zn/H_2SO_4 or $NaBH_4$ failed. On the other hand, nearly quantitative conversion of **7** to **8** was accomplished in 2.5 hr at 95°C with a 4- to 5-fold excess of HI.

Table II

OPTIMIZATION OF THE SYNTHESIS OF 4-ACETAMIDOPHENYL DISULFIDE

Expt.	**7** mg (mmol)	HI g (mmol)	HI / **7** (mol/mol)	Time (hr)	Temp (°C)	Yield mg (%)
1	330 (1.41)	1.02 (7.98)	5.65	14	75	124 (50)
2	506 (2.16)	1.78 (13.9)	7.80	14	75	239 (60)
3	657 (2.80)	1.27 (9.96)	3.55	2.5	95	330 (71)
4	542 (2.32)	1.27 (9.96)	4.20	2.5	95	275 (71)
5	522 (2.23)	1.27 (9.96)	4.40	2.5	95	335 (90)

4-Aminophenyl Disulfide. Conversion of the acetamide to the free amine was readily accomplished in ca. 90% yield by treatment of **8** with a large excess of hot 6N HCl. Use of H_2SO_4 (6-9 N) afforded yields of 30-60%.

4-Azidophenyl Disulfide. Our procedure for the conversion of **9** into **10** was based on that of Vanin and Ji (3). 4-Aminophenyl disulfide was diazotized with $NaNO_2$/HCl, and the intermediate diazonium chloride converted to the azide by treatment with NaN_3 in the dark. The success of this reaction was strongly dependent on acid strength and on the molar amounts of $NaNO_2$ and NaN_3 (Table III). We were able to improve upon the reported yield for this reaction (62% (3)) by use of the conditions listed in entry 8 of Table III. *Recrystallization of **10** at this stage was critical to the overall success of the synthesis; use of crude **10** resulted in consistent failure of the subsequent chlorination step and total loss of product.* The contaminants that interfere with chlorination must accumulate from previous steps in the preparation of **10**; commercial samples of **10** chlorinate smoothly. In any case, a single crystallization of the crude azide from hexane is sufficient to avoid this problem.

N-(4-Azidophenylthio)phthalimide. Conversion of **10** to **11** proved to be the most critical step in the synthesis of APDP. 4-Azidophenyl disulfide was treated with Cl_2 in pentane, and the intermediate sulfenyl chloride was trapped with phthalimide in DMF to provide **11** (Table IV). The chlorination is sensitive both to water and to metallic impurities (e.g., to metal syringe needles or cannulae, or to the aluminum foil that might be used to shield to reaction flask from light). When the reaction was run on a scale of less than 500 mg, the rate and duration of Cl_2 flow were critical in de-

Table III

OPTIMIZATION OF THE SYNTHESIS OF 4-AZIDOPHENYL DISULFIDE

Expt.	**9** mg (mmol)	HCl	$NaNO_2$ mg (mmol) ml H_2O	NaN_3 mg (mmol) ml H_2O	$NaNO_2$ / **9** (mol/mol)	NaN_3 / **9** (mol/mol)	Yield mg (%)
1	250 (1.0)	12N, 6 ml	100 (1.5) 1	130 (2.0) 1	1.0	2.0	no significant product
2	250 (1.0)	12N, 6 ml	138 (2.0) 1	130 (2.0) 1	2.0	2.0	130 (46)
3	25 (0.1)	12N, 2 ml	27.5 (0.4) 0.25	39.0 (0.6) 0.50	4.0	6.0	no significant product
4	25 (0.1)	6N, 2 ml	27.5 (0.4) 0.25	39.0 (0.6) 0.50	4.0	6.0	2-3.5[a] (10-15)
5	25 (0.1)	2N, 1.2 ml	27.6 (0.4) 0.25	39.0 (0.6) 0.25	4.0	6.0	18-19[b] (82-86)
6[c]	232 (0.93)	2N, 5 ml	258 (3.74) 1.0	362 (5.57) 1.0	4.0	6.0	260 (93)
7[d]	222 (0.89)	2N, 5 ml	258 (3.74) 1.0	362 (5.57) 1.0	4.0	6.0	242 (87)
8	210-235 (0.81-0.95)	2N, 5 ml	265 (3.84) 2.0	368 (5.66) 2.0	4.5	6.5	232-268[a] (91-94)

[a] Average of three runs.

[b] Average of four runs.

[c] Fast addition of NaN_3 solution.

[d] Slow addition of NaN_3 solution.

termining the products of the reaction. The progress of the chlorination was monitored by thin layer chromatography, and the sulfenyl chloride appeared as a single spot at $R_f = 0.3$ (c.f. Experimental Section). In reactions run with high Cl_2 flow rates, a second spot at $R_f = 0.5$ also appeared. The formation of this by-product was difficult to control, and appearance of the second spot on TLC was

invariably accompanied by failure of the reaction with phthalimide and by the formation of complex mixtures of products. In the optimal procedure, chlorination was stopped just before the appearance of the second spot (less than 2 min). This procedure was highly reproducible, and gave good yields of crude **11** in a form suitable for direct conversion into **1** without the need for purification.

Table IV

OPTIMIZATION OF THE SYNTHESIS OF N-(4-AZIDOPHENYLTHIO)PHTHALIMIDE

Expt.	**10** mg (mmol)	Phthalimide mg (mmol)	NEt_3 mg (mmol)	DMF ml	Pentane ml	**11** mg (%)
1	1900 (6.32)	1850 (12.64)	1280 (12.64)	75	25	2600 (70)
2	30 (0.1)	29.5 (0.2)	20.2 (0.2)	1.5	2	29[a] (50)
3	30 (0.1)	29.5 (0.2)	40.4 (0.4)	1.5	2	30[b] (50)
4	30 (0.1)	29.5 (0.2)	20.2 (0.2)	1.5	2	42[a] (70)
5	265 (0.88)	323 (2.2)	201 (2.0)	2.5	5	455.2 (87)[c]
6	246 (0.82)	330 (2.24)	201 (2.0)	2.5	5	489 (>100)[c]

[a] Average of four runs.

[b] Average of two runs.

[c] Crude wet product.

3-[(4-Azidophenyl)dithio]propionic Acid. The sulfur transfer reagent **11** was converted smoothly and reliably to APDP in ca. 75% yield by treatment with 3-mercaptopropionic acid in benzene. The progress of the reaction was monitored by TLC (cf. Experimental Section) and the reaction was stopped upon complete consumption of **11**. After removal of the precipitated phthalimide, the solvent was evaporated under an N_2 stream and the product was recrystallized from hexane. The infrared, ^1H NMR and ^{13}C NMR spectra of the product were consistent with the expected structure, and TLC ($CHCl_3$:methanol, 10:1) showed a single spot. ^{14}C-APDP prepared as described in the

Experimental Section was identical by these criteria to a sample of cold APDP prepared from commercial 4-aminophenyl disulfide.

The use of ^{14}C-APDP in membrane labelling experiments will be described in future papers.

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