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Photoreactive Derivatives of Corticotropin. 2. Preparation and Characterization of 2-Nitro-4(5)-azidophenylsulfenyl Derivatives of Corticotropin†

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ABSTRACT: Two new photoreactive arylsulfenyl chlorides, 2-nitro-4-azidophenylsulfenyl chloride (2,4-NAPS-Cl) and 2-nitro-5-azidophenylsulfenyl chloride (2,5-NAPS-Cl), have been synthesized and used for the selective modification of corticotropin (ACTH). Both reagents reacted rapidly with *N*-acetyltryptophanamide and ACTH under acidic conditions. The NAPS derivatives of ACTH were purified by partition chromatography and characterized by absorption spectra, amino acid analysis, and peptide mapping. The spectral changes caused by photolysis as well as the kinetics of photolysis are described. Tritiated 2,5-NAPS-ACTH was attached covalently to a pituitary protein fraction FI by photolysis. The photolabeling of FI was blocked in the presence of excess ACTH.

In order to prepare photoreactive derivatives of the pituitary hormone corticotropin (ACTH),[1] we have investigated the synthesis and use of arylsulfenyl chlorides incorporating an azido group. Arylsulfenyl chlorides have been found to be specific mild reagents for the modification of tryptophan and cysteine residues of polypeptides in acidic media (Scoffone et al., 1968; Fontana et al., 1968; Canova-Davis & Ramachandran, 1976). Tryptophan is converted into a derivative with thioether function in the 2 position of the indole nucleus, and cysteine is converted to an unsymmetrical disulfide. In aqueous alkaline solution, sulfenyl chlorides react with α-amino groups of amino acids and peptides as well as ϵ-amino groups of lysine residues (Zervas et al., 1963). Hence, a variety of photoreactive derivatives of polypeptides and proteins may be obtained by selective modification with arylsulfenyl chlorides containing azido groups. Our initial studies revealed that the preparation of 2-nitro-4-azidophenylsulfenyl chloride (2,4-NAPS-Cl) was more difficult than the synthesis of 2,4-dinitro-5-azidophenylsulfenyl chloride (DNAPS-Cl). Therefore, we synthesized DNAPS-Cl and used it for the modification of ACTH. The preparation and characterization of DNAPS-Trp^9-ACTH and its use in photoaffinity labeling have been described in the previous article (Canova-Davis & Ramachandran, 1980) and elsewhere (Ramachandran et al., 1979, 1980). However, these studies showed that the DNAPS group is unstable. The presence of a nitro group adjacent to the azido group may also decrease the efficiency of photoaffinity labeling because of the formation of benzofuroxan derivatives (Bailey & Case, 1958). It became apparent that a more stable photoreactive arylsulfenyl chloride would be highly useful in preparing photoaffinity labels for ACTH receptors in particular and for the introduction of photoreactive groups into proteins in general. We have developed procedures for

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[1] Abbreviations used: ACTH, corticotropin; NAPS, 2-nitro-4-azidophenylsulfenyl; DNAPS, 2,4-dinitro-5-azidophenylsulfenyl.

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the synthesis of 2,4-NAPS-Cl as well as the isomer 2-nitro-5-azidophenylsulfenyl chloride (2,5-NAPS-Cl). Both reagents have been used to selectively modify tryptophan in model compounds and ACTH. These results are described in this article.

Materials and Methods

Porcine ACTH was isolated in this laboratory as described in the preceding article, which also contains descriptions of general analytical procedures used in this work.

Synthesis of 2-Nitro-4-azidophenylsulfenyl Chloride (2,4-NAPS-Cl). Bis(2-nitro-4-aminophenyl) Disulfide (I). Sodium sulfide nonahydrate (12 g, 50 mmol) in 50 mL of 95% ethanol was heated in a round-bottomed flask until the sulfide dissolved. Then 1.6 g of finely divided sulfur was added, and heating was continued until the sulfur dissolved, forming a brownish red solution of sodium disulfide. Meanwhile, 10.5 g (67 mmol) of 4-fluoro-3-nitroaniline (Aldrich) was dissolved in 30 mL of hot 95% ethanol and added to the sodium disulfide solution. The mixture was refluxed for 18 h and cooled to 3 °C. The precipitated product was collected and washed with water and 95% ethanol. Finally, it was triturated with boiling 95% ethanol and filtered to yield lustrous reddish brown crystals (5.1 g, 45%): mp 211–213 °C; TLC [silica gel G, $CHCl_3$–acetone (3:1 v/v)] R_f 0.27. Anal. Calcd for $C_{12}H_{10}N_4O_4S_2$ (338.24): C, 42.56; H, 2.98; N, 16.52. Found: C, 41.4; H, 3.03; N, 15.9.

Bis(2-nitro-4-azidophenyl) Disulfide (II). The disulfide I (625 mg, 1.85 mmol) was dissolved in concentrated H_2SO_4 (10 mL) and cooled to −10 °C (acetone–dry ice bath) with vigorous stirring. Sodium nitrite (290 mg, 4.2 mmol) in water (1.5 mL) was added dropwise over 15 min, and stirring was continued another 45 min. Ice-cold water (50 mL) was added, followed by sodium azide (290 mg, 4.5 mmol) in water (1.5 mL dropwise. After all the azide was added, the mixture was stirred for another 15 min at −10 to 0 °C. The product was filtered, washed with ice-cold water, and dried to yield 667 mg of II (92.5% yield). II was recrystallized from acetone, mp 157 °C dec: homogeneous on TLC on silica gel G, R_f 0.95 in $CHCl_3$–acetone (3:1 v/v), and R_f 0.60 in $CHCl_3$–methanol (1:1 v/v); IR (Nujol) 2112–2303 cm^{-1}, N_3 asymmetric stretch. Anal. Calcd for $C_{12}H_6N_8O_4S_2$ (390.24): C, 36.92; H, 1.55; N, 28.71. Found: C, 37.06; H, 1.67; N, 28.59.

2-Nitro-4-azidophenylsulfenyl Chloride (2,4-NAPS-Cl). II was converted to 2,4-NAPS-Cl by chlorinolysis with sulfuryl chloride by the procedure of Lawson & Kharasch (1959). II (380 mg, 0.97 mmol) was suspended in CCl_4 (2.5 mL) in a 10-mL flask with a reflux condenser. Sulfuryl chloride (Matheson Coleman and Bell, 0.16 mL) and pyridine (0.02 mL) were added. The mixture was refluxed for 1 h, and another 0.2 mL of sulfuryl chloride was added in four equal portions at 30-min intervals. An additional 0.02 mL of pyridine was added, and the reaction was continued for 8 h. The hot reaction mixture was treated with charcoal (Norit A, 20 mg), filtered, and evaporated to dryness. The residue was crystallized from petroleum ether to yield 162 mg (36%) of 2,4-NAPS-Cl: mp 91 °C; TLC [silica gel G, benzene–petroleum ether (1:1 v/v)] R_f 0.80; IR (Nujol) 2080–2040 cm^{-1}, N_3 asymmetric stretch. Anal. Calcd for $C_6H_3N_4O_2SCl$ (230.6): C, 31.25; H, 1.31; N, 24.29. Found: C, 31.46; H, 1.42; N, 24.06.

Preparation of the 2,4-NAPS Derivative of N-Acetyltryptophanamide (III). To a solution of *N*-acetyltryptophanamide (Aldrich, 100 mg, 0.41 mmol) in 3 mL of glacial acetic acid was added 86 mg (0.37 mmol) of 2,4-NAPS-Cl, and the reaction was allowed to proceed at room temperature for 24 h in the dark. The reaction mixture was poured into 30 mL of ice water. The precipitate was filtered and dried in vacuo. Crystallization from ethyl acetate–benzene yielded 100 mg of (61%) III: mp 175 °C dec; TLC [silica gel G, $CHCl_3$–methanol (6:1 v/v)] R_f 0.95; IR (Nujol) 2105 cm^{-1}, N_3 asymmetric stretch. Anal. Calcd for $C_{19}H_{17}N_7O_4S$ (439.3): C, 51.93; H, 3.90; N, 22.31. Found: C, 51.77; H, 3.94, N, 22.55.

Synthesis of 2-Nitro-5-azidophenylsulfenyl Chloride (2,5-NAPS-Cl). 3-Fluoro-4-nitroaniline (IV). 3-Fluoroaniline (Aldrich) was nitrated according to the method of Hodgson & Nicholson (1941), and IV was separated from the isomer 2-nitro-3-fluoroaniline by stream distillation: mp 163–165 °C. Anal. Calcd for $C_6H_5N_2O_2F$ (156.1): C, 46.16; H, 3.23; N, 17.95. Found: C, 46.14; H, 3.25; N, 17.82.

2-Nitro-5-aminophenylbenzyl Sulfide (V). In a round-bottomed flask equipped with a reflux condenser were placed 4.8 g (31 mmol) of IV, 15 mL of methanol, 4 mL (34 mmol) of benzyl mercaptan, and 3 mL of triethylamine. The mixture was refluxed with stirring for 16 h and then cooled to 0 °C. The yellow precipitate was filtered, washed with ice-cold methanol, and dried to yield 5.4 g (68%) of V: mp 155 °C. Recrystallization from methanol gave yellow needles: mp 156 °C. V was homogeneous by TLC on silica gel G in benzene, R_f 0.25. Anal. Calcd for $C_{13}H_{12}N_2O_2S$ (260.2): C, 59.98; H, 4.65; N, 10.76. Found: C, 59.75; H, 4.65; N, 10.70.

2-Nitro-5-azidophenylbenzyl Sulfide (VI). V (3.4 g, 13.1 mmol) was dissolved in 60 mL of glacial acetic acid and 12 mL of concentrated sulfuric acid (Smith & Brown, 1951). The solution was cooled to 5–10 °C and diazotized by the slow addition of 1.6 g (14.3 mmol) of isopentyl nitrite (Eastman Kodak). After the solution was stirred for 1 h at 5–10 °C, 150 mL of water was added. Sodium azide (1.6 g, 26.2 mmol) in 30 mL of water was added dropwise and stirred for 1 h in an ice–water bath. The brownish yellow precipitate was filtered, washed with cold water, and dried in vacuo. VI was crystallized from ethyl acetate: yield, 3.2 g (87%); mp 140–143 °C; homogeneous on TLC on silica gel G, R_f 0.80 in benzene; IR (Nujol) 2090 cm^{-1}, N_3 asymmetric stretch. Anal. Calcd for $C_{13}H_{10}N_4O_2S$ (286.2): C, 54.53; H, 3.52; N, 19.57. Found: C, 54.57; H, 3.64; N, 19.61.

2-Nitro-5-azidophenylsulfenyl Chloride (2,5-NAPS-Cl). VI was converted to 2,5-NAPS-Cl by reaction with sulfuryl chloride according to Kharasch & Langford (1973). VI (1.08 g, 4.2 mmol) was suspended in 3 mL of dichloromethane. Sulfuryl chloride (0.4 mL) was added, and the mixture was left at room temperature for 10 min. The resulting solution was concentrated in vacuo on a water bath (60 °C) to an oil. Petroleum ether (40 mL) was added, the oil was dissolved by warming, and the solution was then left overnight at −20 °C. 2,5-NAPS-Cl was obtained as yellow crystals: yield, 0.75 g (77%); mp 84–86 °C (recrystallization from petroleum ether yielded a product with mp 85–86 °C); homogeneous on TLC on silica gel G, R_f 0.64 in benzene; IR (Nujol) 2110–2090 cm^{-1}, N_3 asymmetric stretch. Anal. Calcd for $C_6H_3N_4O_2SCl$ (230.6): C, 31.25; H, 1.31; N, 24.29. Found: C, 31.35; H, 1.42; N, 24.07.

Preparation of the 2,5-NAPS Derivative of N-Acetyltryptophanamide (VII). *N*-Acetyltryptophanamide was converted to VII by reaction with 2,5-NAPS-Cl as described for III. The product, VII, was crystallized from ethyl acetate–benzene twice and obtained in 42% yield: mp 173–175 °C dec; TLC [silica gel G, $CHCl_3$–methanol (6:1 v/v)]: R_f 0.40; IR (Nujol) 2110–2100 cm^{-1}, N_3 asymmetric stretch. Anal. Calcd for $C_{19}H_{17}N_7O_4S$ (439.3): C, 51.93: H, 3.90; N, 22.31.

Found: C, 52.06; H, 4.05; N, 22.35.

Preparation of NAPS Derivatives of ACTH. ACTH (8.45 mg) was dissolved in 0.2 mL of distilled water. Methionine (50 mg) and glacial acetic acid (2.8 mL) were added. 2,4-NAPS-Cl (20 mg) was added, and the reaction mixture was left in the dark at room temperature for 4 h. Distilled water (30 mL) was then added, and the excess reagent was extracted with ethyl acetate. The organic phase was extracted with 0.1% acetic acid, and the combined aqueous phase was lyophilized. The product was subjected to gel filtration on a Sephadex G-25 column (1 × 38 cm) equilibrated with 0.1 N formic acid. The modified ACTH was obtained in a yield of 6.4 mg (73%). This material was further purified by partition chromatography (Yamashiro, 1964) on Sephadex G-50 (1.5 × 58 cm) in the solvent 1-butanol–pyridine–0.1% acetic acid (5:3:11 v/v) as previously described (Canova-Davis & Ramachandran, 1976). 2,4-NAPS-ACTH was isolated in the overall yield of 62% (5.4 mg) after partition chromatography. TLC on silica gel G in 1-butanol–pyridine–acetic acid–water (5:5:1:4 by volume) (BPAW) revealed a single yellow, ninhydrin positive spot, R_f 0.41; ACTH, R_f 0.36.

2,5-NAPS-ACTH was prepared in a similar manner by reaction of 2,5-NAPS-Cl with ACTH and purified by gel filtration and partition chromatography. 2,5-NAPS-ACTH was obtained in 52% yield after partition chromatography: homogeneous on TLC on silica gel G in 1-butanol–pyridine–acetic acid–water (5:5:1:4 by volume), R_f 0.41.

Preparation of Tritiated 2,5-NAPS-ACTH. Synthetic human [3,5-^{3}H-Tyr^{2,23}]ACTH with a specific radioactivity of 90 Ci/mmol was prepared as described (Ramachandran & Behrens, 1977). To 9 μg (2 nmol) of the tritiated hormone in 0.05 mL of 90% acetic acid, 1.25 mg of methionine and 0.5 mg of 2,5-NAPS-Cl were added. The reaction mixture was kept in the dark for 4 h with occasional shaking. The tritiated 2,5-NAPS-ACTH was isolated by gel filtration on a Sephadex G-25 column (0.8 × 26 cm) equilibrated with 90% acetic acid and pretreated with 1 mg of polylysine to decrease losses due to adsorption. 2,5-NAPS-[^{3}H]ACTH was further purified by partition chromatography on Sephadex G-50 as described above.

Photolysis. Irradiation was conducted in Pyrex glass containers using a Blak-Ray UV lamp emitting principal radiation at 366 nm. Solutions were kept at 0 °C by means of an ice–water bath at a distance of 10–15 cm from the lamp. Enzymatic digestion of peptides and peptide maps was performed as described in the preceding article (Canova-Davis & Ramachandran, 1980).

Results

Synthesis of 2,4-NAPS-Cl. The major difficulty encountered in our initial studies (Ramachandran & Canova-Davis, 1977) was in the preparation of bis(2-nitro-4-aminophenyl) disulfide (I) from 4-chloro-3-nitroaniline. The reaction was sluggish and the yields were extremely low (<5%). The yield of I was significantly improved (45%) by using 4-fluoro-3-nitroaniline. I was converted to the azido derivative II in high yield (92.5%). Chlorinolysis of II with sulfuryl chloride resulted in the formation of 2,4-NAPS-Cl in 36% yield.

Synthesis of 2,5-NAPS-Cl. The isomer 2,5-NAPS-Cl was prepared by a procedure different from that used commonly for preparing arylsulfenyl chlorides. We adopted the procedure described by Kharasch & Langford (1973) for the preparation of 2,4-dinitrophenylsulfenyl chloride. The scheme for the synthesis of 2,5-NAPS-Cl is shown in Figure 1. 3-Fluoro-4-nitroaniline (IV) was synthesized and converted to 2-nitro-5-aminophenylbenzyl sulfide (V) by reaction with benzyl

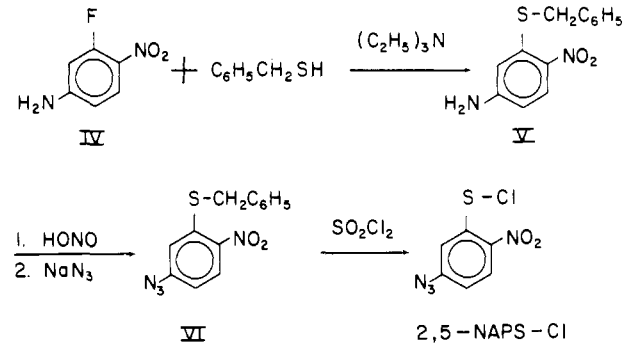

FIGURE 1: Scheme for the synthesis of 2-nitro-5-azidophenylsulfenyl chloride (2,5-NAPS-Cl).

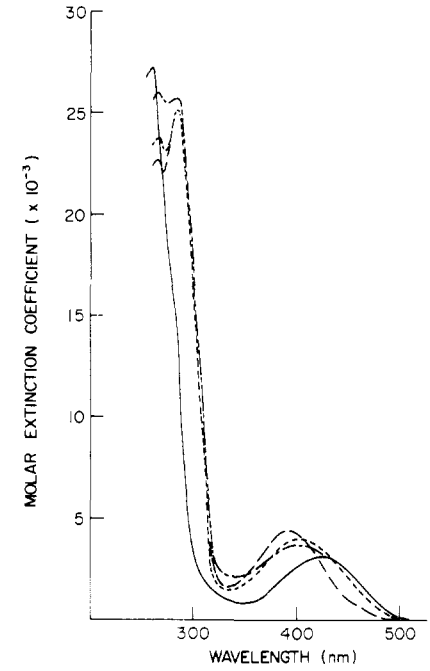

FIGURE 2: Absorption spectra of 2,4-NAPS-Cl and derivatives: (—) 2,4-NAPS-Cl in glacial HOAc; (– – –) III in glacial HOAc; (- - -) III in 2 N HOAc; (–·–) 2,4-NAPS-ACTH in 2 N HOAc.

mercaptan. It was necessary to use a stronger base (triethylamine) than pyridine which was used by Kharasch & Langford (1973). No conversion of IV to V could be observed in the presence of pyridine. V was converted to the azido derivative VI in high yield. Chlorinolysis of VI with sulfuryl chloride proceeded smoothly and rapidly (10 min). 2,5-NAPS-Cl was obtained in 77% yield.

Both 2,4-NAPS-Cl and the isomer 2,5-NAPS-Cl as well as all intermediates were obtained in crystalline form. The microanalysis of all compounds was highly satisfactory. All compounds containing an azido group showed strong absorption in the infrared spectrum around 2100 cm^{-1}, which is due to the characteristic asymmetric stretch vibration of the azido group. The two new arylsulfenyl chlorides were found to be quite stable when stored in the dark under dry conditions.

Absorption Spectra. The absorption spectra of the NAPS chlorides and the NAPS derivatives of Ac-Trp-NH_2 and ACTH are shown in Figures 2 and 3. Both NAPS chlorides exhibit absorption bands near 400 and 280 nm. The major difference is the broad absorption band at 300–350 nm observed in the spectrum of 2,5-NAPS-Cl, which is not present in the spectrum of 2,4-NAPS-Cl. The long-wavelength absorption band is intensified and shifted to shorter wavelengths upon reaction with Trp residues. The absorption spectral

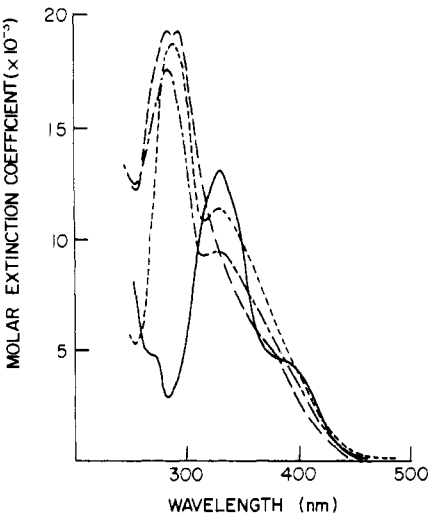

FIGURE 3: Absorption spectra of 2,5-NAPS-Cl and derivatives: (—) 2,5-NAPS-Cl in glacial HOAc; (– – –) VII in glacial HOAc; (- - -) VII in 2 N HOAc; (- - -) 2,5-NAPS-ACTH in 2 N HOAc.

Table I: Absorption Spectral Characteristics of NAPS Chlorides and Derivatives

compound	solvent	λ_{max} (nm)	ϵ
2,4-NAPS-Cl	HOAc	425	3 100
		261	27 200
N-Ac-(2,4-NAPS)-Trp-NH_2 (III)	HOAc	390	4 300
		286	25 200
		263	22 600
	2 N HOAc	400	3 900
		286	24 900
		263	23 700
2,4-NAPS-ACTH	2 N HOAc	400	3 700
		283	24 900
		265	26 000
2,5-NAPS-Cl	HOAc	330	13 200
N-Ac-(2,5-NAPS)-Trp-NH_2 (VII)	HOAc	293	19 300
		285	19 300
	2 N HOAc	327	11 400
		288	18 700
2,5-NAPS-ACTH	2 N HOAc	325	9 400
		283	17 600

maxima and the molar extinction coefficients of the two NAPS chlorides and the NAPS derivatives of *N*-acetyltryptophanamide and ACTH are listed in Table I. The long-wavelength bands of the 2,5-NAPS derivatives are twice as intense as the long-wave-length absorption of the 2,4-NAPS derivatives. However, the opposite is true for the short-wavelength absorption. The extinction coefficient of Ac-(2,4-NAPS)-Trp-NH_2 at 285 nm in 2 N HOAc is 9300, compared to a value of 6300 for this absorption of the 2,5-NAPS derivative in the same solvent. The molar extinction coefficients of 2,4-NAPS-ACTH and 2,5-NAPS-ACTH in 2 N HOAc for the long-wavelength band were very similar to those of the model compounds III and VII, respectively. This result strongly suggests that the NAPS derivatives of ACTH are selectively modified at a single site in the hormone, namely, the tryptophan residue.

Kinetics of Modification of Tryptophan. The kinetics of the reaction of 2,4-NAPS-Cl with *N*-Ac-Trp-NH_2 was followed spectrophotometrically by monitoring the increase in absorbance at 300 nm (Figure 4). The rate of reaction of 2,5-NAPS-Cl with the model compound was monitored at 295 nm and is also shown in Figure 3. It is evident that modification of the Trp residue by the photoreactive arylsulfenyl chlorides is very rapid and is complete within 30 min at room

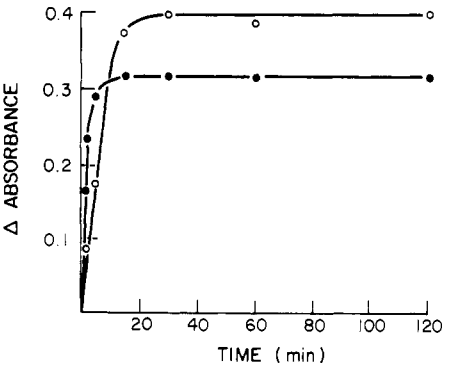

FIGURE 4: Kinetics of reaction of NAPS chlorides with *N*-Ac-Trp-NH_2. Equal volumes (2.5 mL) of a 20 mM solution of *N*-Ac-Trp-NH_2 in glacial HOAc and 40 mM 2,4-NAPS-Cl or 2,5-NAPS-Cl in glacial HOAc were mixed at room temperature. At various times 0.1 mL of the reaction mixture was removed and diluted to 25 mL with glacial HOAc. The increase in absorbance at 300 nm for 2,4-NAPS-Cl (●) and that at 295 nm for 2,5-NAPS-Cl (○) were measured. The reagents alone showed no change in absorbance over several days in glacial HOAc.

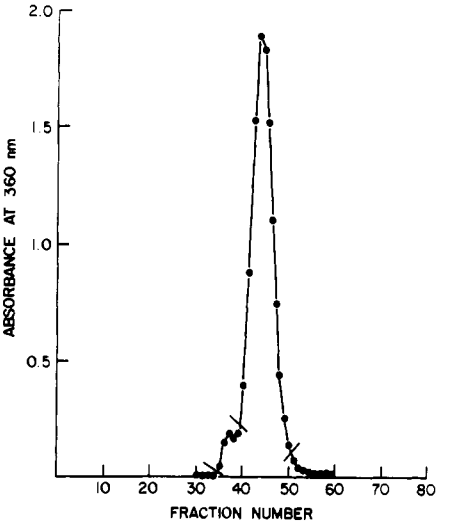

FIGURE 5: Partition chromatography of 2,5-NAPS-ACTH on Sephadex G-50 (1.5 × 58 cm); 0.9 mL/fraction; flow rate, 20 mL/h.

temperature. 2,4-NAPS-Cl appears to react significantly faster than 2,5-NAPS-Cl. The times required for half-maximal reaction are 1 min for 2,4-NAPS-Cl and 6 min for 2,5-NAPS-Cl.

Preparation and Characterization of NAPS Derivatives of ACTH. ACTH was reacted with the NAPS chlorides in 90% acetic acid as described under Materials and Methods for 4 h and purified by gel filtration and partition chromatography. The purification of 2,5-NAPS-ACTH by partition chromatography is shown in Figure 5. The R_f value of 0.64 is in agreement with the increased hydrophobic character of 2,5-NAPS-ACTH compared to porcine ACTH (R_f 0.53). 2,4-NAPS-ACTH has a R_f of 0.65 in the same solvent system. The NAPS derivatives of ACTH were homogeneous by TLC on silica gel G. The amino acid compositions of enzymatic digests of ACTH and the two NAPS derivatives are given in Table II. It is apparent that the Trp residue is completely modified in both 2,4-NAPS-ACTH and 2,5-NAPS-ACTH. The results in Table II also demonstrate that the tyrosine, methionine, and histidine residues in the modified hormones are intact. The lower values for lysine, proline, glycine, valine, and arginine in the digests of the NAPS derivatives compared to unmodified ACTH suggest that the segment Trp-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro is difficult to digest

Table II: Amino Acid Composition of ACTH and NAPS Derivatives[a]

amino acid	ACTH	2,4-NAPS-ACTH	2,5-NAPS-ACTH
Trp	1.07	-	-
His	1.06	1.13	1.03
Lys	2.33	1.23	0.98
Arg	2.01	1.71	1.23
Asp	0.72	0.50	0.49
Ser ± Asn	2.70	2.58	2.55
Glu	5.00	5.00	5.00
Pro	2.96	2.84	2.73
Gly	2.08	0.93	1.09
Ala	2.81	2.92	2.93
Val	2.19	1.65	1.45
Met	0.97	0.99	0.99
Leu	2.15	2.34	2.28
Tyr	1.85	1.92	1.74
Phe	2.87	3.00	2.98

[a] The peptides were digested with acid protease followed by leucine aminopeptidase as previously described (Canova-Davis & Ramachandran, 1976).

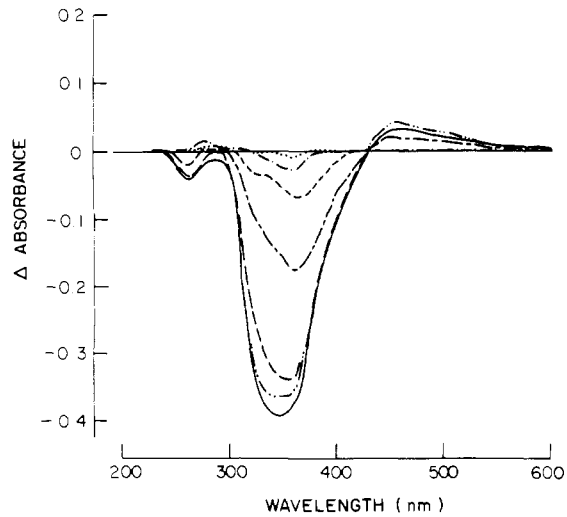

FIGURE 6: Difference spectra of photolyzed Ac-(2,5-NAPS)-Trp-NH_2. A solution of Ac-(2,5-NAPS)-Trp-NH_2 (1.52×10^{-4} M) in 2 N HOAc (optical density at 366 nm, 0.67) was photolyzed at 0 °C as described under Materials and Methods, and the difference spectrum of the photolyzed sample was measured against an unphotolyzed sample at various times: (···) 5 min; (-·-) 15 min; (- - -) 30 min; (-·-·-) 60 min; (— — —) 120 min; (—) 180 min; (-·-·-) 240 min.

after modification of the Trp residue. Peptide maps of tryptic digests of the NAPS derivatives of ACTH were identical with that of ACTH except for the position of the Trp peptide [see Figure 1 of Canova-Davis & Ramachandran (1980)]. In the map of the modified hormones the Trp peptide was missing and a new, yellow, ninhydrin positive spot was observed. The modified Trp peptide was eluted with 0.1 N ammonia, hydrolyzed with acid, and analyzed. The amino acid composition was found to be $Lys_{1.98}Pro_{1.02}Gly_{1.95}Val_{1.05}$, thus confirming that the new yellow spot represented the modified Trp peptide.

Photolysis. The photoreactivities of the NAPS derivatives of Ac-Trp-NH_2 and ACTH were investigated in the solvent 2 N acetic acid by irradiating with 366-nm radiation from a Blak-Ray lamp. The spectral changes produced upon photolysis of Ac-(2,5-NAPS)-Trp-NH_2 are shown in Figure 6. A small increase in absorption at 450 nm and a large decrease in the absorption at 350 nm are evident in the difference spectrum. The kinetics of photolysis of VII in 2 N HOAc are also shown in Figure 6. The spectral changes are nearly complete after 2 h of irradiation. The spectral changes produced in 2,5-NAPS-ACTH and the kinetics of photolysis of

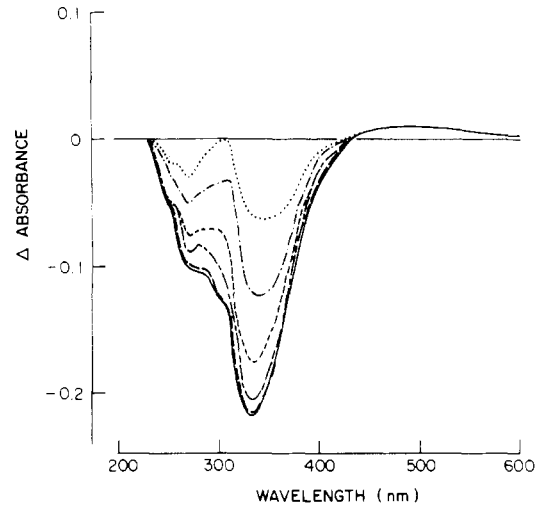

FIGURE 7: Difference spectra of photolyzed 2,5-NAPS-ACTH. A solution of 5.68×10^{-5} M 2,5-NAPS-ACTH in 2 N HOAc (optical density at 366 nm, 0.24) was photolyzed as described under Figure 6. Symbols are the same as in Figure 6.

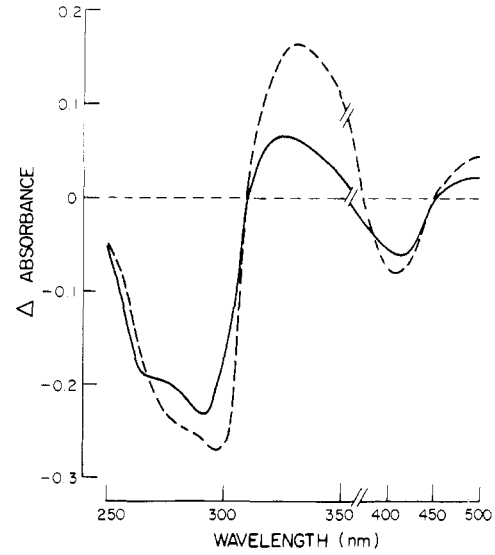

FIGURE 8: Difference spectra of photolyzed Ac-(2,4-NAPS)-Trp-NH_2 and 2,4-NAPS-ACTH. Ac-(2,4-NAPS)-Trp-NH_2 (1×10^{-4} M) (- - -) and 2,4-NAPS-ACTH (5.2×10^{-5} M) (—) in 2 N HOAc were photolyzed for 2 h as described under Materials and Methods, and the difference spectrum was measured against the corresponding unphotolyzed samples.

2,5-NAPS-ACTH are shown in Figure 7. There is a large decrease in absorption at 335 nm and a smaller decrease around 280 nm. 2,5-NAPS-ACTH appears to be more readily photolyzed than the model compound VII. The spectral changes are nearly complete by 60 min, whereas irradiation for 180 min was required for complete photolysis of VII. The difference spectra of photolyzed vs. unphotolyzed 2,4-NAPS-ACTH and the model compound III are presented in Figure 8. In addition to the minima around 425 and 295 nm, there is a maximum at 325–330 nm in the difference spectra. Both 2,4-NAPS-ACTH and III are photolyzed more rapidly than the corresponding 2,5-NAPS derivatives (Figure 9). Photolysis of 2,4-NAPS-ACTH is almost complete within 15 min. The model compound III is photolyzed more slowly but faster than VII. The difference spectra of the photolyzed NAPS derivatives of ACTH are very similar to the difference spectra of the corresponding photolyzed model compounds.

Covalent Attachment of 2,5-NAPS-ACTH to Pituitary Protein. To test the utility of NAPS-ACTH derivatives for

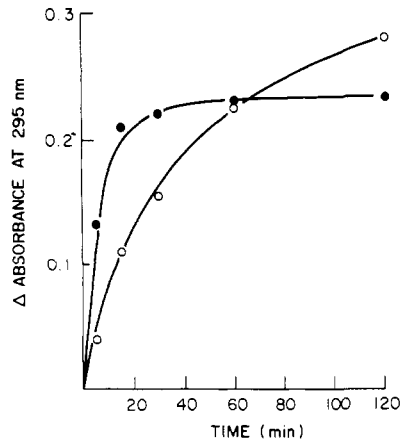

FIGURE 9: Kinetics of photolysis of 2,4-NAPS derivatives. Difference spectra were measured at various times as described under Figure 8 for III (optical density at 366 nm, 0.18) (○) and 2,4-NAPS-ACTH (optical density at 366 nm, 0.1) (●).

photolabeling purposes, the interaction of ACTH with the pituitary protein fraction FI was examined. Tritiated 2,5-NAPS-ACTH was incubated with the crude ACTH binding protein fraction FI isolated from ovine pituitary glands as described previously (Canova-Davis & Ramachandran, 1976). The mixture was then photolyzed at 0 °C for 1 h and subjected to gel filtration on Sephadex G-75 in the presence of 5 M guanidine hydrochloride. Figure 10 shows that FI which is excluded on Sephadex G-75 is linked covalently to the tritiated 2,5-NAPS-ACTH by this procedure. A small amount of radioactivity remains associated with FI in the control which was not photolyzed. When the photolysis was conducted in the presence of a 33-fold excess of unlabeled ACTH, an 80% inhibition of labeling of FI was observed. In addition to FI (the peak at the void volume) and tritiated 2,5-NAPS-ACTH (the major peak), an additional peak of radioactivity was observed emerging between FI and tritiated 2,5-NAPS-ACTH when the photolyzed samples were subjected to gel filtration. This peak of radioactivity was also seen when tritiated 2,5-NAPS-ACTH was photolyzed in the absence of FI (data not shown) and corresponds to the dimer of the ACTH derivative.

Discussion

The synthesis of the two new arylsulfenyl chlorides described here was undertaken because of the instability of 2,4-dinitro-5-azidophenylsulfenyl chloride and its derivatives. The presence of a nitro group ortho to the azido group is not desirable since these compounds tend to form benzofluoroxan derivatives readily (Bailey & Case, 1958). Therefore, the location of the azido group in *o*-nitrophenylsulfenyl chloride is limited to the 4, 5, or 6 positions on the phenyl ring. Since the 6 position is ortho to the –SCl group and may lead to steric hindrance as well as reaction with the indole during photolysis of the NAPS derivative of tryptophan compounds, we decided to prepare nitrophenylsulfenyl chlorides containing the azido group in the 4 or 5 positions.

Our studies on the synthesis of 2,4-NAPS-Cl show that this reagent may be prepared more readily starting with 4-fluoro-3-nitroaniline rather than 4-chloro-3-nitroaniline. The synthesis of arylsulfenyl chlorides by the original procedure of Hubacher (1935) is not very satisfactory since the disulfide intermediates such as I are difficult to dissolve and chlorinolysis of the disulfides proceeds in low yields. Even with sulfuryl chloride, prolonged refluxing was necessary to convert II to 2,4-NAPS-Cl, and the yield was low (36%). The alternative method described by Kharasch & Langford (1973) for the

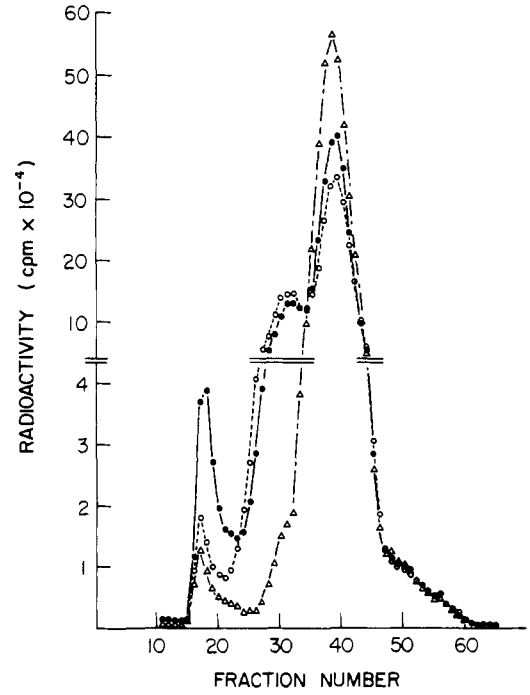

FIGURE 10: Gel filtration of tritiated 2,5-NAPS-ACTH–FI complex. FI protein (0.1 mg) dissolved in 0.1 mL of 0.1 M sodium acetate buffer, pH 5.0, was mixed with 0.3 ng of tritiated 2,5-NAPS-ACTH (3.6×10^6 cpm) and 30 μg of unlabeled 2,5-NAPS-ACTH in 0.1 mL of the same buffer. The mixture was incubated at 4 °C for 12 h in the dark and photolyzed at 0 °C for 1 h. After lyophilization the photolyzed mixture was dissolved in 0.2 mL of 5 M guanidine·HCl in water and chromatographed on a Sephadex G-75 column (0.8 × 29 cm) equilibrated with 5 M guanidine·HCl: flow rate, 2.4 mL/h; fractions, 0.2 mL/tube; (●–●) photolyzed tritiated 2,5-NAPS-ACTH–FI protein complex; (○---○) 2,5-NAPS-ACTH–FI protein complex photolyzed in the presence of 1 mg of ACTH; (Δ–·–·–Δ) 2,5-NAPS-ACTH–FI complex not subjected to photolysis.

preparation of 2,4-dinitrophenylsulfenyl chloride was, therefore, adopted for the synthesis of 2,5-NAPS-Cl. As shown in Figure 1, 3-fluoro-4-nitroaniline was readily converted to 2-nitro-5-aminophenylbenzyl sulfide (V). Diazotization of V and reaction with NaN_3 yielded VI, which was converted to 2,5-NAPS-Cl in good yield (77%) rapidly by chlorinolysis with sulfuryl chloride at room temperature. It is evident that this scheme can be adopted also for the synthesis of 2,4-NAPS-Cl by starting with 4-fluoro-3-nitroaniline. In general, the method of Kharasch & Langford (1973) is preferable for the synthesis of arylsulfenyl chlorides.

Both 2,4-NAPS-Cl and the isomer 2,5-NAPS-Cl reacted rapidly with the indole group of tryptophan residues (Figure 4). The faster rate of reaction observed with 2,4-NAPS-Cl suggests that this reagent may be more useful when short reaction times are necessary. The nucleophilic reactivity of the sulfenyl chloride is increased by the presence of the azido group in the para position in 2,4-NAPS-Cl since the azido group is electron withdrawing with a Hammet constant of $\sigma_p^- = 0.116$ (Miller, 1968). The absorption spectra of the 2,4-NAPS-Trp derivatives are also considerably different from the spectra of the 2,5-NAPS-Trp compounds due to the differences in the electronic interaction of the azido and nitro groups in the two isomeric compounds. The red shift of the long-wavelength band in III compared to VII suggests that the presence of the electron-withdrawing azido group para to the thioether group in III augments the interaction of the nitrophenyl group with the indole moiety.

Selective, quantitative modification of the single Trp residue in ACTH was accomplished using either reagent. The two

NAPS derivatives of ACTH were obtained in a homogeneous state after partition chromatography. Selective modification at the Trp residue was demonstrated by analysis of the spectra, TLC, amino acid analysis of an enzymatic digest, and peptide maps of tryptic digests.

The photoreactivities of the NAPS derivatives of ACTH and the two model compounds III and VII were readily seen upon irradiation. The NAPS derivatives of ACTH appeared to undergo photolysis more readily than the corresponding model compounds even though the optical densities of the model compounds at the wavelength of irradiation were higher. III was found to undergo photolytic decomposition at the same rate in 95% ethanol (data not shown). The reason for the faster rate of photolysis of the NAPS derivatives of ACTH is not known. However, these results suggest that NAPS-ACTH may be more efficient in labeling specific receptors than indicated by the photolysis of the model compounds.

A significant difference was also observed between the rates of photolysis of the 2,4-NAPS compounds and the 2,5-NAPS compounds. The 2,4-NAPS derivatives were photolyzed more rapidly at comparable optical densities. These differences may be related to the substituent effects of the azido group discussed earlier. Which NAPS-ACTH should be used in photoaffinity labeling studies depends not only on the photochemical properties but also on the affinity of the modified ACTH for the specific receptor. Preliminary studies indicate that 2,5-NAPS-ACTH is a better inhibitor of ACTH-induced cyclic AMP production in isolated rat adrenocortical cells than 2,4-NAPS-ACTH. Studies of the biological properties of the two NAPS-ACTH derivatives and their use in photoaffinity labeling of ACTH receptors on adrenocortical cells are in progress.

The results presented in Figure 10 clearly show that 2,5-NAPS-ACTH can be attached covalently to proteins with an affinity for ACTH. The incorporation of radioactivity into the pituitary binding protein fraction FI following photolysis in the presence of tritiated 2,5-NAPS-ACTH was specifically blocked by the presence of excess ACTH. These results indicate that the NAPS-ACTH derivatives can be used for selectively labeling biological macromoles that bind specifically to ACTH. Preliminary studies with isolated rat adrenocortical cells have shown that photolysis with tritiated 2,5-NAPS-ACTH results in selective labeling of ACTH binding sites.

The results presented in this article demonstrate that 2,4-NAPS-Cl and 2,5-NAPS-Cl are highly useful reagents for the selective introduction of photoreactive groups at Trp residues of polypeptides. Such photoreactive groups are useful not only for covalent attachment of a ligand to its receptor but can also be used for probing the three-dimensional structure of peptides and proteins in solution. NAPS-ACTH can be used to investigate whether there is any preferred structure in ACTH by analyzing the products of photolysis. These studies as well as studies of the reaction of these reagents with sulfhydryl groups and amino groups are in progress.

Acknowledgments

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