125. 4-Azidoaniline, a Versatile Protein and Peptide Modifying Agent for Photo Affinity Labeling

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Summary

A modified synthesis of 4-azidoaniline and the use of this compound as a protein and peptide modifying agent for photoaffinity labeling is described. 4-Azidoaniline was diazotized to 4-azidophenyldiazonium and coupled to *N*-acetyltyrosine-ethylester, and to a tyrosine containing analog of bradykinin.

It is shown that the 4-azidoaniline reagent offers great advantages over other protein modifying agents for photoaffinity labeling, *i.e.* very high nitrene reactivity, possibility of tritium or iodine labeling, ligand-receptor complex can be cleaved after isolation. The biological activity of the modified bradykinin analog is measured on rabbit aorta strip in the dark and is similar to the unsubstituted analog. Under the influence of light the modified peptide is able to block partially the myotropic action of bradykinin.

Introduction. – In the past years photo affinity labeling has become a widely used technique whenever specific receptor-ligand interactions were investigated. The great advantage of this technique is that a normally competitive specific ligand analog is introduced which, upon activation with light, welds into the receptor. The resulting covalent ligand-receptor complex is much easier to isolate and to study than a purely reversible ligand-receptor complex. Therefore most photolabeling ligands contain an azido group which can be photolyzed into a very reactive nitrene intermediate, capable to insert into C–H bonds [1]. The photolabeling azido compounds are either totally synthetic analogs of a biological ligand molecule [2] or are introduced onto a pre-existing ligand with an azido bearing modifying agent [3]. Especially with large ligands the second approach is favored over a total synthesis and some modifying agents are already commercially available. One commercial modifying agent is 2-nitro-4-azido-fluorobenzene which reacts with amino groups [4]. The disadvantages of this label are the loss of a positively charged side-chain on the ligand and a relatively sluggish nitrene. The

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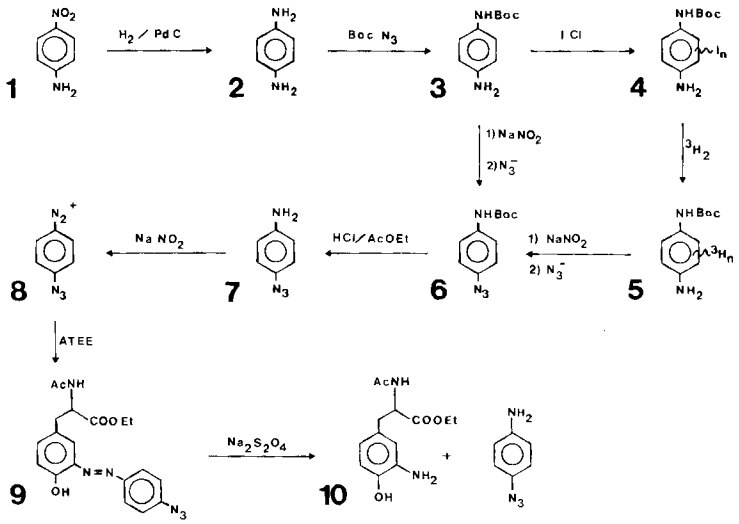
Scheme



nitro group on the aromatic ring has a destabilizing influence on the nitrene which makes it more reactive but the amino function acts inversely and outweighs the influence of the nitro group [5].

A similar compound, 4-azido-2-nitro-phenylsulfenyl chloride has been published for tryptophane [6] residues but suffers from the same disadvantages and does not quickly modify Trp. The alternative 5-azido-2,4-dinitro-phenylsulfenyl chloride seems to incorporate readily but is unstable even in the dark [6].

We propose therefore diazotized 4-azido-aniline (**7**), a historic compound [7], as a modifying agent which combines the following advantages:

- high nitrene reactivity (azo group strongly destabilizes the nitrene) [5];
- specific for Tyr and His and is easily introduced (also amino groups may react at lower pH);
- modified ligand can be photolyzed with visible light, where the precursors are stable;
- the modifying agent can be made highly tritiated or iodinated.

A further property will be very useful, the covalent ligand-receptor complex can be cleaved chemically after isolation with retention of radioactivity on the receptor by simple addition of sodium dithionite [8], similar to the separation of **9** to **10** and **7** *(Scheme)*. This would become very important if the isolated receptor had to be freed of the ligand in order to reconstitute some of its former properties, although the small anilino derivative of the labeling moiety would be retained on the receptor.

4-Nitroaniline (**1**) has been hydrogenated to 4-aminoaniline (**2**) and one amino group protected with *t*-butyloxycarbonyl azide to form 4-Bocaminoaniline (**3**). Iodination with iodine monochloride gave multiple iodination products **4** which upon hydrogenation gave homogenous **3**. (This will later permit catalytic tritiation of **4** in order to obtain radioactive photolabels). Compound **3** was diazotized and

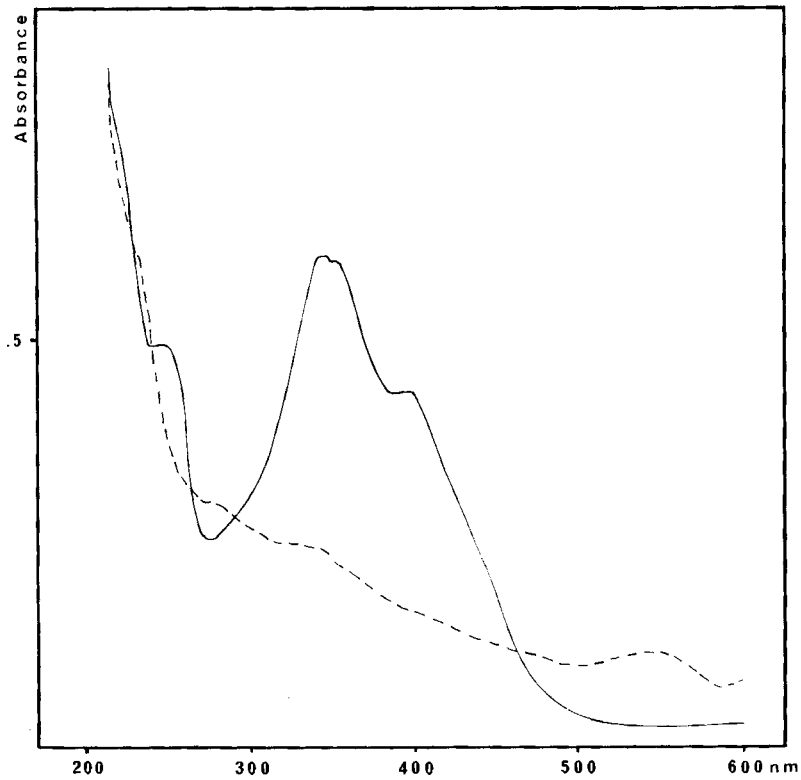


Fig. 1. *UV.-spectrum of* **9**
——— Absorption of **9**; ----- Absorption of photolyzed **9**

treated with sodium azide to yield *N*-Bocaminophenylazide (**6**). The protecting Boc-group was cleaved with hydrogen chloride in ethylacetate to 4-azidoaniline (**7**).

As a first example **7** has been diazotized to **8** which was coupled to N^{α}-acetyltyrosine-ethylester (ATEE) to form the product **9**, N^{α}-acetyl-3′-(4-azidophenylazo)-tyrosine-ethylester. A further sample was the coupling of **8** with [Tyr^5, des-Arg^9]bradykinin **12** [9], which gave the desired product [3′-(4-azidophenylazo)Tyr^5, des-Arg^9]BK (**13**). Both ATEE and **12** were cleaved from their labeling moiety by reduction with sodium dithionite and gave the corresponding [3′-amino]Tyr analogs, identified by TLC. and amino acid analysis. If **9** was photolyzed with long wave length UV., the spectra changed drastically: the infrared band at 2110 cm^{-1} (N_3-stretch) disappeared and in the UV. the maxima at 353 and 394 nm were almost completely reduced *(Fig. 1)*. Compound **13** was tested in the absence of light on rabbit aorta strip, according to [10]. The product showed similar activity as its synthetic precursor with a half maximal dose ED_{50} of 1.9 μg/ml (2 [μM]), an intrinsic activity a^E of 1.0 *(Table)* was completely reversible in the dark *(Table)* although somewhat slower than BK. (Lit. [9] gives 1 [μM] for ED_{50} of **12**).

In order to test the photolabeling capacity **13** has been tested in the same way as angiotensin II analogues recently [10] [11]. The biological activity of **12** is

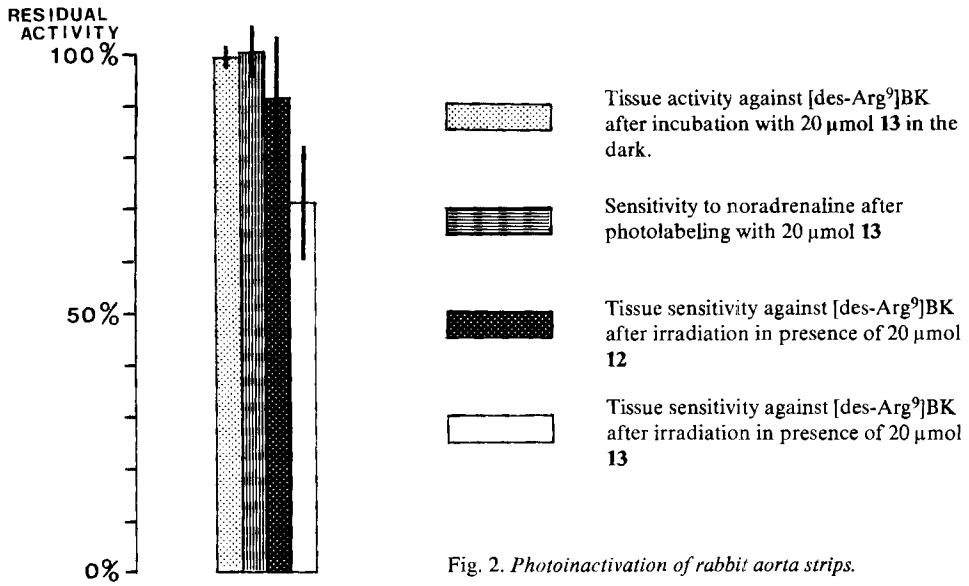


Fig. 2. *Photoinactivation of rabbit aorta strips.*

very low compared to the original [des-Arg^9]BK but almost identical with the product **13**. It was assumed that the substitution does not alter very much the hormonal properties of **12** and is therefore not in a close contact with the receptor of BK. The rather weak but although irreversible and specific inactivation is less dramatic as reported on angiotensin [11] but reasonable with the observed affinities (see *Fig. 2*).

Table. *Biological activities*[a])

	pD_2	a^E
[des-Arg^9]BK [9]	7.20	1.0
[Tyr^5,des-Arg^9]BK, **12**	5.71 ± 0.14	1.0
[3′-(4-azidophenylazo)Tyr^5,des-Arg^9]BK, **13**	5.78 ± 0.21	1.0

[a]) Measurements were made according to [12]; pD_2 is the negative log of the dose which gives half maximal contraction; a^E is the intrinsic activity according to *Ariens* [13].

Experimental Part

General remarks. Thin-layer chromatograms (TLC.) were run on precoated fluorescent *Merck* F-254 silica-gel plates with the following eluents: BAW = 1-butanol/acetic acid/water 5:2:3; BAWP = 1-butanol/acetic acid/water/pyridine 15:3:12:10; CMA = chloroform/methanol/acetic acid 95:5:3; CM = chloroform/methanol 20:1. If not already colored the spots were visualized with UV.-quenching or a modified *Reindel-Hoppe* procedure [14]. Electrophoresis was performed on *Merck* precoated cellulose plates at pH 2.1 and the Rf-values are correlated to picric acid (Rf = 1). ^{1}H-NMR. spectra were obtained with a *Varian* T60 spectrometer using tetramethylsilane (TMS) as internal standard with a chemical shift $\delta = 0$ ppm, s = singlet, d = doublet, t = triplet, qa = quartet, m = multiplet, br. = broad. Infrared spectra (IR.) were recorded on a *Perkin Elmer* 457 instrument and UV./VIS. spectra on a *Beckmann* 25 spectrometer (λ_{max} in nm. logε in parentheses). Mass spectra (MS.) were taken on a HS-30 double beam instrument (*AEI*, England). Optical rotations were determined on a *Zeiss* OLD instrument. Elemental analysis were performed by *Galbraith Laboratories Inc.* Knoxville, Tennessee.

Melting points (m.p.) were taken on a *Thomas Hoover* apparatus in open capillaries and are uncorrected, amino acid analysis on a *Technicon* TSM analyzer equipped with an *Autolab* integrater. Reagents and chemicals were purchased from *G.T. Baker* and used without further purification if not otherwise stated. Biological activities of bradykinin analogues were measured in the same procedure as in [12] except that they were carried out in darkroom condition, and are presented in the *Table*. Abbreviations: RT. = room temperature, i.V. = in vacuo.

4-N-t-Butyloxycarbonylaminoaniline (**3**). To a solution of 6.9 g 4-Nitroaniline (**1**), (mol.-wt. 138.13, 5.0 mmol) in 20 ml of dioxane one spatule tip of Pd/C (10%) moistened with water was added and the reaction mixture hydrogenated at RT. and normal pressure for 24 h. The catalyst was filtered off under nitrogen and the product was directly processed in the next step. TLC. (chloroform): Rf **1** 0.37 (yellow), Rf **2** 0.00 (ninhydrine positive, pink). To the above solution 14 g of *t*-butyloxycarbonyl azide (mol.-wt. 143.15, 9.8 mmol) and 0.6 g diisopropylethylamine were added. The reaction mixture was stirred at 21° for 60 h, evaporated i.V. and chromatographed on 300 g silica-gel (*Merck*, type 40, 70–230 mesh) with CM. A product was collected and recrystallized from ethanol/water yielding 6.63 g of **3** (mol.-wt. 208.26, 63%), m.p. 112–114°. TLC. (CMA) Rf **2** 0.33, Rf **3** 0.60. – ^{1}H-NMR. ($CDCl_3$): 6.5–7.2 (*m*, *AA'BB'*-System, 4 H); 6.3 (br. *s*, 1 H); 3.5 (br. *s*, 2 H); 1.5 (*s*, 9 H).

Iodination and catalytic hydrogenation of 4-N-t-butyloxycarbonylaminoaniline. To a solution of 500 mg of **3** (2.4 mmol) in 15 ml of methanol at 0° 7.2 ml of a 1N ethanolic solution of iodine monochloride *(Alfa Inorganics)* were slowly added under stirring and the 'pH' held between 6.5 and 7.5 with addition of 1N Na_2CO_3. After 1 h the reaction mixture was evaporated i.V. and the residue filtered over 10 g of silica-gel, eluted with CM. The collected product **4** (410 mg) showed in TLC. (CM) several spots, Rf's 0.15, 0.18, 0.22, 0.27, 0.28 (Rf **3** 1.0) due to isomer iodination products. Catalytic hydrogenation of 200 mg of this product mixture with 1 mg Pd/C (10%) for 2 h in methanol gave after filtration and evaporation i.V. 108 mg of homogeneous **3** (identified by TLC. and mixture m.p.), yield 44%.

4-N-t-Butyloxycarbonylamino-phenylazide (**6**). Under continuous stirring 1.0 ml of 1.2M solution of sodium nitrite was slowly added to a solution of 210 mg of **3** (1 mmol) in 20 ml 0.1N HCl in 50% methanol at 0°. After 10 min the reaction mixture was positive to iodine-starch and became negative after addition of 100 mg of sulfamic acid (1 mmol). A solution of 78 mg sodium azide (1.2 mmol) in 100 µl water was added, 15 min later a crystalline precipitate was filtered off and washed with 10 ml of chilled water. After drying 200 mg of **6** (mol.-wt. 234.26, 85%) were obtained, m.p. 98° (dec.). TLC. (CM): Rf **3** 0.21, Rf **6** 0.63, homogenous, ninhydrine positive after acid treatment. – IR. ($CHCl_3$): 2110 cm^{-1} (N_3). – ^{1}H-NMR. ($CDCl_3$): 7.4–6.6 (*m*, *AA'BB'*-System, 4 H); 6.4 (br. *s*, 1 H); 1.45 (*s*, 9 H). – MS.: 234 (M^+).

$C_{11}H_{14}N_4O_2$ (234.26) Calc. C 56.33 H 6.02 N 23.92% Found C 56.40 H 5.82 N 23.80%

4-Azidoaniline (**7**). The suspension of 200 mg of **6** (0.85 mmol) in 1 ml of 6N anhydrous hydrogenchloride in ethylacetate at 0° was warmed up to RT. and left standing for 4 h. The hydrochloride of **7** was precipitated by addition of 10 ml cold diethylether and filtered off. Yield of **7**·HCl was 100 mg (mol.-wt. 170.60, 68%), m.p. 66° (dec.) (ethanol/water) (Lit. [7]: 66°). TLC. (CM) Rf **7** 0.33 (homogenous, ninhydrine positive pink). – IR. (Nuyol) 2110 cm^{-1} (N_3). – ^{1}H-NMR. (d_6-DMSO): 8.8–7.0 (very br., 3 H); 7.5–7.1 (*m*, *AA'BB'*-System, 4 H).

N^{α}-Acetyl-3'-(4-azidophenylazo)tyrosine-ethylester (**9**). Diazotation of 143 mg of **7** (1.0 mmol) with 83 mg sodium nitrite (1.2 mmol) as described above; 200 mg *N*-acetyl-tyrosine-ethylester (mol.-wt. 251.2, 0.8 mmol) were dissolved in 10 ml of ethanol with 1 ml of 2N Na_2CO_3. The first solution was added under absolute darkness to the second and stirred at RT. for 1 h. The following operations have been carried out in a dark room with red pyrex glassware and dimmed red light. To the solution 20 ml of ethylacetate and 20 ml sat. NaCl-solution were added, the upper layer separated, dried over anhydrous sodium sulfate and evaporated i.V. The residue was chromatographed on 12 g silica-gel with chloroform and a product collected. Yield 123 mg of **9** (mol.-wt. 396.41, 39%), m.p. 180° (dec.). – TLC. (chloroform) Rf **9**: 0.18 yellow, Rf ATEE: 0.00, $[\alpha]_D = +18.58°$ ($c = 0.5\%$, ethanol). – UV.: 246 (3.44), 344 (3.53), 394 (sh), 3.37 (see *Fig. 1*). – IR.: (chloroform) 2110 cm^{-1} (N_3). – ^{1}H-NMR. ($CDCl_3$): 10.8 (br. 1 H); 6.8–8.1 (*m*, 7 H); 6.2 (br. 1 H); 5.0 (br. *m*, 1 H); 4.30 (*qa*, $J = 3.5$ Hz, 2 H); 3.20 (*d*, 2 H); 2.0 (*t*, 3 H); 1.3 (*t*, $J = 3.5$, 3 H).

[3'-(4-Azidophenylazo)tyrosine^5,des-arginine^9]bradykinin (**13**). To **7** mg (mol.-wt. 910.22, 10 µmol) of [Tyr^5,des-Arg^9]BK in 200 µl of 0.5N Na_2CO_3 11 mg of **7**·HCl (diazotized as above) have been

slowly added under absolute darkness and stirring at 4°. After 10 min the reaction was stopped by addition of 100 µl 1M phenol in ethanol and the mixture was acidified with 100 µl glacial acetic acid. The mixture has been degassed i.V., applied to a 0.5 cm column *Sephadex* G15, eluted with 0.2N CH_3COOH. The peptide fraction (yellow) has been collected and lyophilized. The product has been resuspended in 100 µl 2N CH_3COOH and further purified with a partition column, G25, 1-butanol/acetic acid/water 5:1:4. The collected fraction contained 2.0 mg (23% yield) of a homogenous yellow product. TLC. (BAWP): Rf **13** 0.52, Rf **12** 0.37; (BAW) Rf **13** 0.14, Rf **12** 0.10; (BIWCl) Rf **13** 0.26, Rf **12** 0.22; electrophoresis Rf **13** −0.64, Rf educt −0.92. - UV.: same as **9**. - IR. (Nuyol): 2110 cm^{-1} (N_3). - Amino acid analysis: Arg-Pro-Pro-Gly-[3'-(4-azidophenylazo)]Tyr-Ser-Pro-Phe; Arg 1.02, Pro 3.0, Gly 1.23, Tyr 0.0, Ser 0.78, Phe 0.91.

Photolysis experiments. Four mercury vapor lamps (*Westinghouse* JC Par 38, 100 W ea) were capped with black light filters (Raymaster 12.6 cm, *G. W. Gates Co.*, Franklin Square, L.I., N.Y., USA) and centered onto a thermostated tissue bath. The bath was surrounded with a tubular shutter. For photolysis experiments a testtube of 2 ml was filled with 1 ml solution and inserted into the tissue bath. After 1 min of irradiation a sample of 0.5 mg of **9** in 50% ethanol has been completely decomposed, TLC. (chloroform) Rf **9**: 0.18. - UV.: 344 and 395, have disappeared *(Fig. 1)* also IR. 2120 cm^{-1} (Nuyol). The same procedure was made for 0.1 mg of **13** in 0.2N CH_3COOH which was photolyzed within 1 min. TLC. (BAWP): Rf **13**: 0.52 disappeared.

Reductive cleavage of N^{α}*-Acetyl-3'-(4-azidophenylazo)-tyrosine-ethylester* (**9**) *and [3'-(4-azidophenylazo)tyrosine*^{5}*,des-arginine*^{9}*]bradykinin* (**13**). A solution of 0.5 mg of **9** (1.4 µmol) in 100 µl dioxane was combined with 3 mg (14 µmol) of sodium dithionite in 200 µl water. The yellow color immediately disappeared and 1 ml of ethylacetate was added. In the organic phase **7** with Rf 0.77 (BAW) and in the aqueous phase **10** with Rf 0.55 were detected and compared with original samples. A solution of 0.1 mg of **13** in 100 µl of 0.2N CH_3COOH and 1 mg of sodium dithionite was added, the yellow color immediately disappeared. The reaction mixture was lyophilized, TLC. (BAWP) and an amino acid analysis performed. Rf product 0.37, Rf **13** 0.52. - Amino acid analysis: Arg-Pro-Pro-Gly-(3'-amino)Tyr-Ser-Pro-Phe: Arg 0.86, Pro 3.0, Gly 1.11, (3'-amino)Tyr [8] 1.02, Ser 0.89, Phe 1.25.

Photolabeling experiments. Rabbit aorta strips have been prepared in the same way as mentioned [12] above and were suspended in the photolysis apparatus. Upon addition of **13** the tissues have been irradiated for 10 min and relaxed afterwards. The residual activity against BK was tested 90 min after this treatment. For specificity tests, the response to noradrenaline has been tested before and after treatment and reference tissues were irradiated in the presence of **12**. The results are presented in *Figure 2.*

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