# Chemical Reduction of Zoalene to ANOT for Use in Zoalene Residue Studies

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A novel chemical synthesis and purification scheme to produce ANOT (3-amino-5-nitro-*o*-toluamide) was developed to yield an analytical standard grade material for zoalene residue investigations. An original conversion of zoalene (3,5-dinitro-*o*-toluamide) to ANOT was developed. Several literature references were available for the partial reduction of dinitro aryl compounds, but none detailed the conversion of zoalene to ANOT. A chemical synthesis was chosen over a biological synthesis due to the latter's complex reaction matrix, difficult recovery, and low batch yields. Using general partial reduction of dinitro aryl schemes, it was found that 10% palladium on carbon coupled to a cyclohexene reducing agent provided an optimum ANOT yield. Chromatographic techniques were utilized to isolate ANOT from the product mixture and to purify it to a standard grade material (>99% pure). The chemically synthesized ANOT product was characterized using colorimetric, TLC, FTIR, NMR, and LC/MS techniques.

**Keywords:** *ANOT; zoalene; zoaline residue; chemical reduction*

## INTRODUCTION

3-Amino-5-nitro-*o*-toluamide (ANOT) is the primary metabolite of zoalene (3,5-dinitro-*o*-toluamide), a poultry feed additive used as a coccidiostat (Hymas et al., 1960; Runnels et al., 1958). A sufficient quantity of ANOT was desired for use as an analytical standard. Because a current source of ANOT could not be obtained, an attempt was made to synthesize ANOT.

A biological synthesis of ANOT from zoalene in chicken liver tissue (Smith et al., 1963) resulted primarily in the formation of ANOT. The other isomer formed was 5-amino-3-nitro-*o*-toluamide (5-ANOT). The biological synthesis of Smith et al. was a selective reduction of zoalene to ANOT; however, only 30 mg of ANOT could be produced per batch, and it would be difficult to scale up for a higher yield. Chemical means of zoalene reduction were not found to be selective for the synthesis of ANOT; rather, the diamino and 5-amino isomers of ANOT are more prevalent. Aromatic dinitro reduction synthesis schemes were available from the literature, but this search did not provide specific details as to the synthesis of ANOT (Entwistle et al., 1977; Johnstone et al., 1985; Ayyangar et al., 1981, 1983).

The combination of the optimum reduction scheme coupled to chromatographic techniques allowed for an effective recovery of a standard grade of ANOT (Hartman and Silloway, 1955; Loev, 1970; Thiegs et al., 1961). The synthesis scheme of choice consisted of reducing zoalene under a nitrogen blanket using cyclohexene (a reducing agent) in 3A ethanol with a reaction temperature of 51 °C and a catalyst of 10% palladium on carbon (Figure 1). Zoalene was dissolved in the reaction mixture while cyclohexene was slowly added via a peristaltic pump. The reaction progress was monitored by the LC analysis of small aliquots taken over regular intervals. When zoalene could no longer be detected in the LC chromatogram of the reaction mixture, the reaction was stopped. The product mixture was stable at room temperature for several days as long as the headspace was kept purged with nitrogen.

The purification of the reaction product mixture to ANOT was accomplished using multiple liquid chromatographic schemes: (1) the side reaction products were removed using dry column chromatography; (2) the 3-amino and 5-amino isomers of the reaction product were separated on a low-pressure LC system utilizing an alumina column; and (3) the isolated 3-ANOT product was purified using ion exchange chromatography.

The presence of ANOT and the 5-amino isomer in the reaction product was verified by chromatographic (TLC, LC) and colorimetric investigations. The successful synthesis of ANOT in the reaction product was confirmed using mass spectrometry, nuclear magnetic resonance spectroscopy, and infrared spectroscopy. The melting point range of the final purified product was also taken and compared to the reference range.

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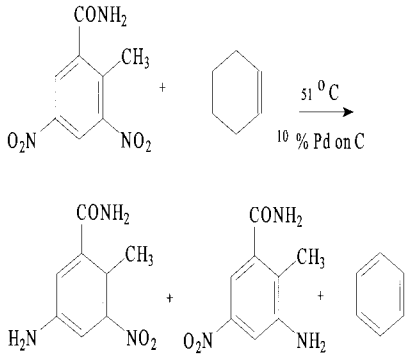


**Figure 1.** Reduction of zoalene to ANOT.

## EXPERIMENTAL PROCEDURES

**Synthesis of ANOT Mixture.** ANOT was synthesized from the chemical reduction of zoalene (Alpharma, Chicago Heights, IL). The starting material (1.41 g, 6.25 mM) was weighed into a 500 mL four-neck flask equipped with an efficient overhead stirrer (furnished with a water-cooled stirrer gland), thermometer, and Hopkins condenser. After the apparatus had been thoroughly purged with nitrogen, 190 mL of anhydrous 3A ethanol was added to the flask. The mixture was stirred at room temperature until total dissolution occurred. A small aliquot was removed to monitor the progress of the reaction. After 3.33 g of 10% Pd on carbon (Aldrich) was added to the flask, the apparatus was again purged with nitrogen.

The reaction was sampled using a 50 mL pear-shaped separatory funnel that was connected to a peristaltic pump by means of C-Flex tubing. Initially, 50.0 mL of a 3.88% (v/v) cyclohexene solution in 3A ethanol was pipetted into the separatory funnel. The temperature of this apparatus was maintained at 51 ± 1 °C.

The reduction of zoalene started with the addition of 50.0 mL of the cyclohexene solution at a nominal rate of 0.05 mL/min. This initial cyclohexene addition is time zero for the reaction. The progress of the reaction was monitored at designated time intervals by removing six drops of sample. This aliquot was diluted with 500 μL of LC mobile phase and filtered prior to injection with a 0.2 μL Teflon syringe filter. The reaction appeared to follow first-order kinetics; samples after 13 h show the absence of zoalene. To determine the actual rate of cyclohexene addition, the addition of the cyclohexene solution was continued until all of the 50.0 mL of solution was added. This addition was completed in 13.5 h. Therefore, the actual rate of cyclohexene solution addition was 0.062 mL/min (equivalent to the addition of 2.4 μL of cyclohexene/min).

At the point of completion, the reaction mixture was filtered through a fine glass fritted funnel and the 10% Pd on carbon was washed thoroughly with anhydrous 3A ethanol. The reaction mixture was evaporated to a moist residue using a Rotovapor under reduced pressure and a temperature setting of 50 °C. The reaction mixture was quantitatively transferred to a recovery flask using methanol. The residue was further evaporated to dryness with the aid of a Rotovapor under the final reduced pressure of 3 mmHg and at 50 °C to give a crude residue weighing 1.37g.

**Isolation of ANOT.** Isolation of ANOT from the crude synthesis product was a three-step process: (1) removal of side reaction products; (2) isolation of crude ANOT; and (3) final purification of crude ANOT.

*Removal of Side Reaction Products Using Dry Column Chromatography.* Dry column tubing (32 mm diameter, Kontes Chromaflex) was sealed at one end by stapling and tightly packing with dry untreated silica gel (J. T. Baker 40 μm flash chromatography packing) to a height of ~25 in. To the top of the column was applied 1 in. of diatomaceous earth (Fisher Scientific Celite 545) and 1 in. of chromatography sand. A 1.8 g mass of crude dry product in 10 mL of methanol was filtered through a 0.2 μm PTFE membrane (Gelman Acrodisc, 25 mm diameter) and applied to the top of the column. When the Celite became saturated, the mobile phase (chloroform/ethyl acetate/methanol 5:5:1) was pumped onto the column at 8 mL/min and gradually slowed to 2 mL/min over a 4 h period. The dry column tube was then laid on a glass plate and examined under a 254 nm light.

The yellow band was removed from the column and extracted with 700 mL of methanol. The extract was filtered through a 12.5 cm Büchner funnel using Whatman No. 40 filter paper. Evaporation of the filtrate was performed with a tared 2 L round-bottom flask at 50 °C. The dry residue was diluted with methanol to produce a 3% (w/v) solution.

*Separation of 3-Amino and 5-Amino ANOT Isomers Using Low-Pressure Chromatography.* Isolation of crude ANOT was carried out with a 48 mm i.d. × 30 cm glass column (Kontes Chromaflex) filled with 11 cm of dry alumina (Acros, neutral, activated, 50-200 μm). The column was part of a low-pressure liquid chromatography system that consisted of a Waters M6000-A isocratic pump, a Gilson model 115 UV detector, and a Gilson FC 203B fraction collector.

The 3% solution of crude ANOT in methanol was filtered prior to application to the column. A Rheodyne type 50 valve equipped with a 1.0 mL Teflon sample loop was used. The column was washed with ~850 mL of the dry column mobile phase prior to use. The ANOT component eluted between 45 and 85 min. After the elution of ANOT, the mobile phase was changed to methanol to elute the 5-ANOT isomer. This isomer eluted between 100 and 150 min. The multiple ANOT fractions collected were evaporated to a dry residue under a stream of compressed air while standing on a heating pad under a hot lamp. The collected residues at this point typically exhibited a relative ANOT peak area of >98% and were combined with other residues of equal or greater purity for further processing.

*Final Purification of ANOT Using Ion Exchange Chromatography.* On the basis of a purification scheme found in the literature (Thiegs et al., 1961), a 113.5 mg sample of ANOT isolated from the low-pressure chromatography procedure was dissolved in 4.00 mL of 80% aqueous ethanol (v/v) and quantitatively transferred to a 10 mm i.d. × 30.5 cm length chromatographic column containing a 6 cm length of wet Dowex 50W-X8 $H^+$ resin. The Dowex resin bed was covered at the top by a 1 cm layer of purified sand. Slight air pressure was needed to elute the components at this desired flow rate (1 drop/5 s). The remainder of the 30.5 cm column was carefully filled with 80% ethanol without disturbing the top of the resin bed.

Fraction I (11 mL) was very light yellow in color and did not contain ANOT. Fraction II (49 mL) was a considerably deeper yellow than either fraction I or the very light yellow fraction III (141 mL). Fractions II and III contained ANOT and were combined. LC analysis of samples from continued elution with 80% ethanol confirmed the absence of ANOT. The recovered ANOT was found to be 99% pure, based on peak area.

The ANOT-containing fractions were evaporated under the house vacuum (19 in Hg gauge pressure) using a temperature of 50 °C. The resulting residue was recrystallized from 1.9 mL of 190 proof ethanol. The wet crystals were washed with cold 190 proof ethanol. The recovered ANOT crystals were dried using an Abderholden drying pistol at the boiling point of methyl ethyl ketone and at a reduced pressure of 3.5 mmHg. The crystals were dried for 1 h at 3.5 mmHg. Using this procedure, 37.8 mg of chromatographically pure ANOT was recovered.

**LC Analysis of ANOT.** The progress of the reaction was monitored using an LC method for the separation of ANOT, 5-ANOT, and zoalene (Morawski and Kyle, 1984; Parks, 1985). The system consisted of a Waters model 6000A isocratic pump and model 490E UV detector, a Wescan model 728 autoinjector configured with a 20 μL fixed volume injection loop, and a Hewlett-Packard Chromjet integrator. Using a Kromasil $C_8$, 5 μm column (Phenomenex, Torrance, CA), the separation was optimized using a 15% (v/v) acetonitrile in water mobile phase

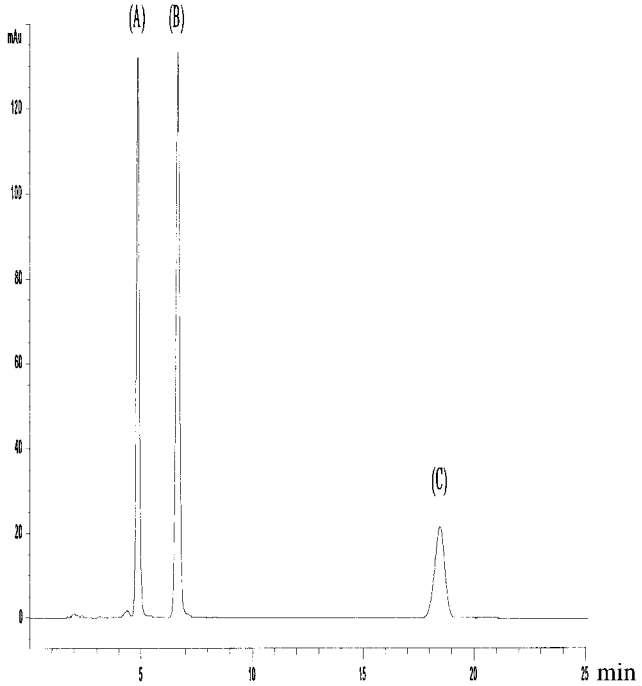


**Figure 2.** LC profile of reaction mixture: (A) ANOT; (B) 5-ANOT; (C) zoalene.

flowing at 1 mL/min. The column dimensions were 4.6 × 150 mm. A wavelength of 254 nm was used for detection. ANOT was found to elute with a 5 min retention time, whereas the 5-ANOT isomer had a retention time of 7 min. Zoalene eluted in 19 min. The peaks were symmetrical and base-line resolved. (Figure 2).

**Preparative Scale LC Analysis of ANOT.** The LC separation was scaled up to collect the separation peaks for identity confirmation. The preparative scale system consisted of two Shimadzu LC-8A pumps configured for binary gradient controlled by a Shimadzu SCL-6B system controller and a 9A autoinjector. A Shimadzu 6A UV detector was configured with a preparative flow cell and used at 254 nm. The profile of Figure 2 retained its integrity using a 25 × 250 mm Kromasil $C_8$, 5 $\mu$m column (Phenomenex) flowing at 18 mL/min using the same 15% acetonitrile mobile phase used with the smaller sized column. Using a 1 mL injection volume, the profile peaks were collected using a Gilson model 201 fraction collector. The ANOT fractions collected were evaporated off using a vacuum rotavap. This system allowed the collection of sufficient quantities of ANOT and 5-ANOT for TLC and colorimetric investigations. The system proved to be too inefficient for large scale fraction collections; thus, a low-pressure LC scheme was developed.

**TLC Analysis of Preparative Chromatography ANOT Fractions.** TLC analyses were performed using the procedure from Owens (1985) on fractions obtained from the preparative LC system. Each fraction was evaporated to a dry residue and dissolved in sufficient methanol to make a 0.3 mg/mL solution. The fraction solutions were applied to Whatman linear HPTLC 10 × 10 cm silica gel (5 $\mu$m) plates using 10 $\mu$L microcaps (Drummond). The plate was developed in a 20 × 20 cm chromatographic developing chamber using a developing solvent of chloroform/ethyl acetate/methanol in the proportions 5:5:1 (v/v), respectively. The fully developed plate was photographed under ultraviolet light using a wavelength of 366 nm. The plate was exposed for 5 s to nitrous acid vapors generated by the addition of sodium nitrite to 8% phosphoric acid. Finally, the plate was sprayed with a Bratton−Marshall solution (0.4% naphthylethylenediamine dihydrochloride in methanol) for spot detection and photographed.

**Liquid Chromatography/Mass Spectrometric Analysis.** For molecular weight confirmation, the reaction product mixture was analyzed using LC/MS. Because the LC procedure used to monitor the reaction did not use a buffer in the mobile phase, no adjustment was necessary for the LC/MS system. The system used was a Waters liquid chromatography system consisting of a model 745 data module, a model 440 UV detector, a model 510 LC pump gradient controller, and a Vestec thermospray interface coupled to a Finnagan TSQ 70 mass spectrometer. The mass spectrometer was tuned to the positive ion (EI) and negative ion (CI) modes with FC43. Thermospray performance was checked using the buffer ions and a 1 mg/mL injection (10 $\mu$L) of a caffeine standard. A 1.18 mg sample from the initial reaction product residue was placed into a 10 mL volumetric flask. Acetonitrile (1.5 mL) was added, and the solution was brought to volume with 18 MΩ water. Dissolution was effected by heating on a steam table. Again, a Kromasil $C_8$, 5 $\mu$m, 4.6 mm × 15 cm column (Phenomenex) was used for the separation. The injection volume was 100 $\mu$L, and the flow rate was 1.0 mL/min. The full scale of the absorbance was set to 0.5, and a 254 nm wavelength was used. The mobile phase was mixed using the gradient controller in an isocratic mode.

**Colorimetric Analysis of Preparative LC Fractions.** Colormetric analysis of preparative chromatography ANOT fractions was performed using a Beckman DU-64 spectrophotometer according to the method of Smith et al. (1963). The analysis solution was prepared by adding 50 $\mu$L of a 9 $\mu$g/$\mu$L ANOT fraction solution in methanol to 2.5 mL of 0.25 N HCl and 125 $\mu$L of 0.1% $NaNO_2$ solution. Upon standing for 5 min, 125 $\mu$L of a 0.5% ammonium sulfamate solution was added. After an additional 3 min, 125 $\mu$L of a 0.1% *N*-1-(naphthyl)-ethylenediamine dihydrochloride solution was added. A colored solution developed immediately, which was scanned from 450 to 650 nm.

**Infrared Spectroscopic Analysis.** FTIR scans of the dry ANOT preparative chromatography fractions were performed using a Matteson Galaxy Series FTIR 5000 spectrometer. A diluted mixture of each ANOT dry fraction in anhydrous KBr was prepared and ground to a fine powder in a small marble mortar (~1% w/w). Each dilute mixture was scanned using the technique of diffuse reflectance. The FTIR was used in transmittance mode with a resolution of 4 $cm^{-1}$ and a scan range of 400 to 4000 $cm^{-1}$. The Fourier transform was set to operate on 64 scans of each ANOT fraction.

**Nuclear Magnetic Resonance Spectroscopic Analysis.** For NMR analysis, each sample (30.6 mg of ANOT, 30.5 mg of 5-ANOT) was dissolved in 750 $\mu$L of DMSO-$d_6$ (Cambridge Labs, DLM-10, CAS Registry No. 2206-27-11). After dissolution, 2.3 $\mu$L of TMS (Cambridge Labs, CAS Registry No. 75-76-3) was added to each solution. NMR spectra of the final solutions in the $^{13}C$ and proton NMR mode were recorded on a GE QE Plus 300 NMR. (CAS Registry No. were supplied by the author.)

**Melting Point Determination.** Melting ranges of the preparative LC ANOT fractions were obtained using an Electrothermal model 9200 melting point apparatus.

## RESULTS AND DISCUSSION

**Synthesis and Isolation of ANOT.** The Zinnin reaction (a reduction using sodium sulfide), Raney nickel, and palladium on carbon catalysts have all been successfully used in the partial reduction of dinitro aromatic compounds. However, no references were found with specific details for the synthesis of ANOT. Other potential routes of synthesis of ANOT were considered in our laboratory. The high-pressure homogeneous catalyzed reduction of zoalene with tris(triphenylphosphene)ruthenium chloride [$RuCl_2(PPh_3)_3$] looked promising due to its successes with other aromatic compounds (Knifton, 1975a,b) but was not tried in our laboratory due to instrumental constraints. Nitration of 3-amino-*o*-toluamide was investigated, but preliminary experiments yielded nitration in all positions and this scheme was abandoned. Dow Chemical,

**Table 1. Reverse Phase LC Retention Times of ANOT and Related Components**

| | Parks and Doerr | | this work | |
|---|---|---|---|---|
| component | reten,[a] min | rel reten | reten,[b] min | rel reten |
| ANOT | 11.9 | 0.74 | 4.9 | 0.72 |
| 5-ANOT | 16.1 | 1.0 | 6.8 | 1.0 |
| zoalene | 13.2 | n/a | 18.4 | NA |

[a] ANOT and 5-ANOT separated using acetonitrile/water (10 + 90). Zoalene separated using methenol/water (30% + 70%). [b] All three components separated using acetonitrile/water (15% + 85%).

previously the sole origin of ANOT compounds, used a biological synthesis. As ANOT is no longer available from this source and was needed as an analytical standard for metabolism studies, its in-house synthesis was required. The synthesis of ANOT from zoalene is a hydrogenation reaction, and it seemed logical that the use of the Zinnin reaction using either of the two mentioned catalysts could be satisfactorily used for the production of ANOT.

The Zinnin reaction was first attempted for the production of ANOT. This reaction, when applied to the zoalene, was found to be vigorous and resulted primarily in the formation of the 5-ANOT isomer (~90% yield). The formation of ANOT with the Zinnin reaction appeared to be sterically hindered by the methyl group adjacent to the amide.

Raney nickel catalytic hydrogenation was then evaluated for the synthesis of ANOT. Because of the significant number of side reaction products that resulted from the use of Raney nickel, the use of this scheme was discontinued. The yield of ANOT was found to be unacceptably low. Finally, a synthesis scheme found in the literature to reduce aromatic amines utilizing 10% palladium on carbon with cyclohexene as a reducing agent was investigated. When this reaction scheme was used, the number of side reaction products was greatly reduced. In addition, the yield of ANOT had increased to ~44% ANOT on the basis of isomer LC peak areas. This reaction scheme still produced uncharacterized side products. A method of isolating ANOT from the product mixture was needed to use the 10% palladium on carbon reaction procedure.

The procedure developed using 10% palladium on carbon catalysis was optimized to the conditions reported. Optimization experiments showed that use of cyclohexene in the reaction mixture at the start did not produce a significant amount of ANOT. The principal product produced was 3,5-diamino-*o*-toluamide. A slow addition of cyclohexene produced ANOT and 5-ANOT, but in an unfavorable ratio for our purposes. The synthesis of ANOT was much more favorable using a cyclohexene solution in 3A ethanol. For this scheme, a 3.88% (v/v) solution of cyclohexene was used. Another important factor for increasing the ratio of ANOT to 5-ANOT may have been the activity of the catalyst. Different batches of 10% Pd on carbon can produce varying isomer ratios based on the activity of the catalyst.

The LC method made it possible to monitor the progress of the ANOT synthesis and to verify the relative amount of ANOT in the product mixture. Retention times of the ANOT fractions obtained from the LC analysis of the ANOT reaction mixture were found to be consistent with the literature values (Table 1).

**Table 2. TLC Data of ANOT Fractions from Preparative LC**

| preparative LC fraction | Parks and Doerr: $R_f$ value | this work: $R_f$ value |
|---|---|---|
| ANOT | 0.56 | 0.53 |
| 5-ANOT | 0.45 | 0.44 |
| sulfadimethoxide[a] | 0.74 | 0.73 |

[a] Sulfadimethoxide used as a control.

Dry column chromatography was applied to this isolation strategy after it was discovered that fluorescent impurities were still in samples purified solely using the low-pressure chromatographic system. This technique was very simple and a highly effective way to remove side reaction products from the ANOT isomers. The chromatography was performed in a flexible nylon tube using silica gel as the stationary phase packing. Elution continued until the mobile phase had reached the bottom of the 25 in. column. Side reaction products were found to adhere to the top of the column, with the ANOT compounds eluting further down. At least two different side reaction products were retained at the top of the column: a dark brown band (visible in white light) at the apex and a strongly fluorescent green band immediately following. The column was cut at a region between the fluorescent green band and a further eluting yellow band which contained the ANOT isomers. This fluorescence was used to determine the region of separation between the side reaction products and the ANOT isomers. Whereas there was no appreciable separation between the ANOT isomers, there was a satisfactory separation between the side reaction products and the ANOT isomers. The portion containing ANOT isomers was extracted with methanol, filtered on a Büchner funnel, and washed with additional methanol. The filtrate (containing isolated ANOT isomers) was transferred to a round-bottom flask and evaporated to a dry residue. This residue was diluted with methanol to obtain a 3% solution.

Low-pressure preparative chromatography allowed for the separation and collection of ANOT and 5-ANOT. Though the 3-amino and 5-amino isomers of ANOT could be resolved and collected on the preparative scale LC system, the system proved to be too inefficient for collecting large amounts of ANOT. Transferring the TLC procedure that had already proved to be effective in resolving the ANOT isomers to a low-pressure column chromatography system allowed the use of more reaction product on the column while maintaining the effective resolution of isomers.

ANOT was the first major band to elute as the 5-ANOT isomer was strongly retained by the stationary phase. A change of mobile phase was required to effectively elute the 5-ANOT isomer. Each injection of a 3% solution prepared at the end of the dry column step onto a 480 mm × 11 cm alumina column yielded ~12 mg of pure product (~98% pure).

Final purification of the synthesized ANOT was performed using ion exchange chromatography. This scheme was incorporated because it was used not only to purify the original ANOT standards (Thiegs et al., 1961) but also to up the baseline of the LC chromatogram of the recovered ANOT fractions. This final step yielded an analytical grade material suitable for metabolism and residue studies.

**Characterization of the Chemically Synthesized ANOT.** The $R_f$ values from the TLC analysis of frac-

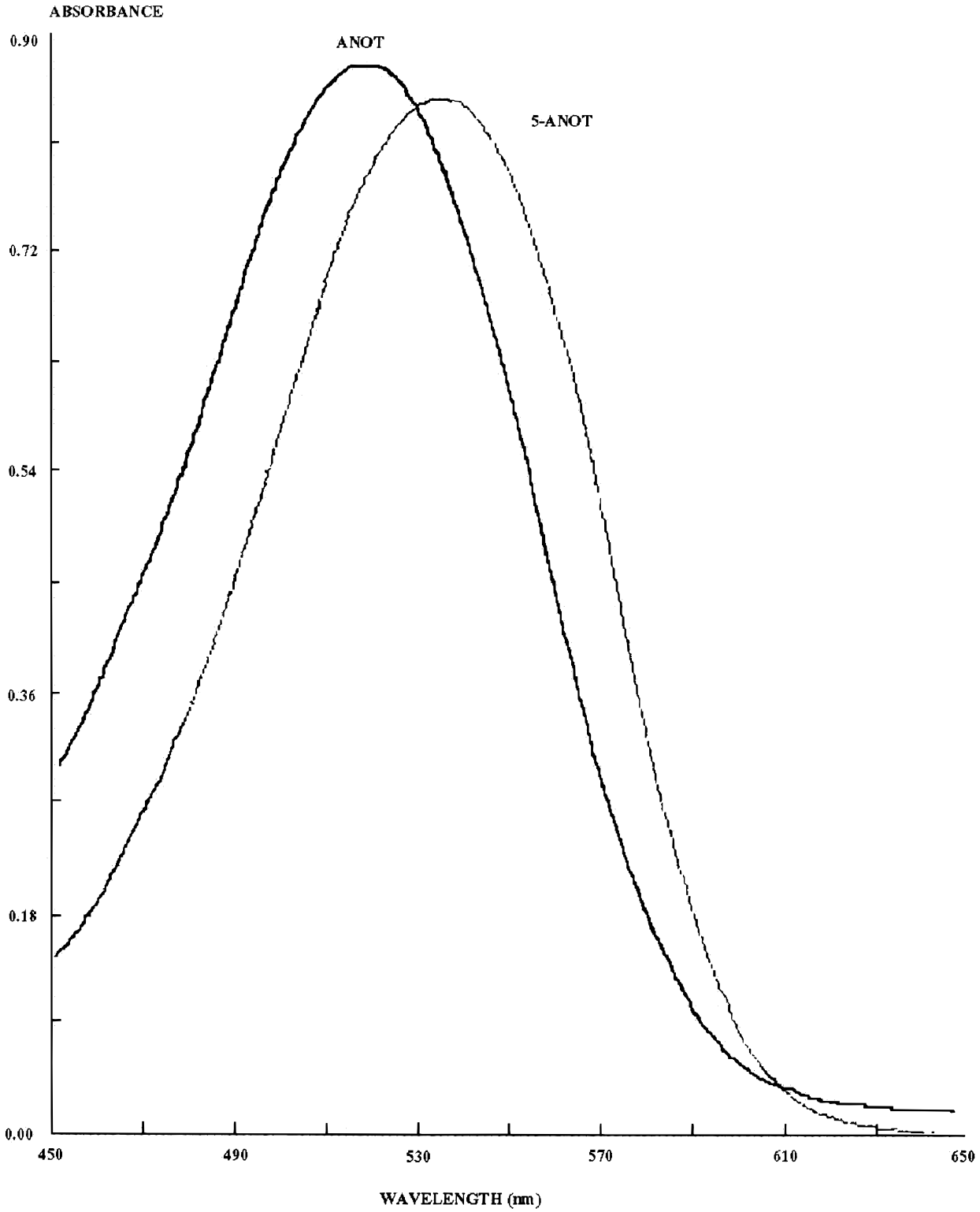


**Figure 3.** Colorimetric analysis of ANOT fractions.

tions collected from the preparative scale LC system were calculated and compared to the Dow Chemical reference values (Table 2). The relative $R_f$ values were found to match the literature values. The identity of the fractions as ANOT and 5-ANOT was supported by this analysis.

Colorimetric analysis of preparative LC fractions of the prepared ANOT fraction solutions showed maximum absorbencies that were consistent with ANOT absorbance values from the literature (Figure 3) according to Smith et al. (1963). Slight shifting of the absorbance maxima from the literature values was noted (Table 3) and attributed to normal instrumental error. The colorimetric spectra correlated for the positive identification of the preparative LC fractions as ANOT and 5-ANOT.

Liquid chromatography/mass spectrometry (LC/MS) analysis of the synthesis reaction product yielded results that are consistent with the molecular weight of ANOT. Positive ion thermospray mass spectra for the ANOT

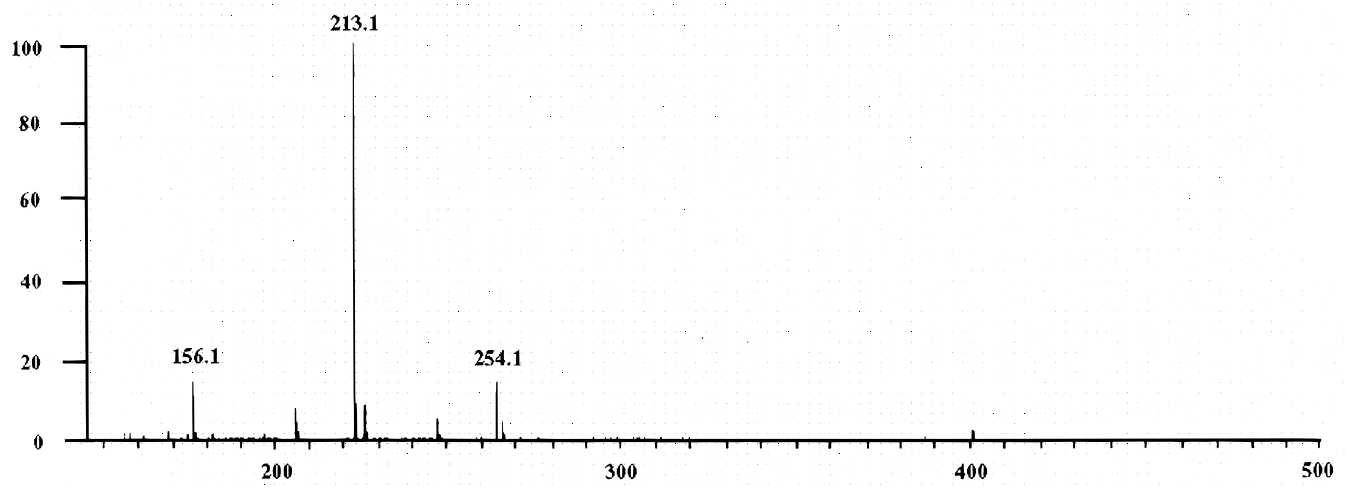


**Figure 4.** Mass spectrum of ANOT from the LC/MS analysis of the reaction product.

**Table 3. Colorimetric Absorbance Maxima of ANOT Fractions from Preparative LC**

| fraction | Smith et al: absorbance max, nm | this work: absorbance max, nm |
|---|---|---|
| ANOT | 525 | 520 |
| 5-ANOT | 540 | 535 |

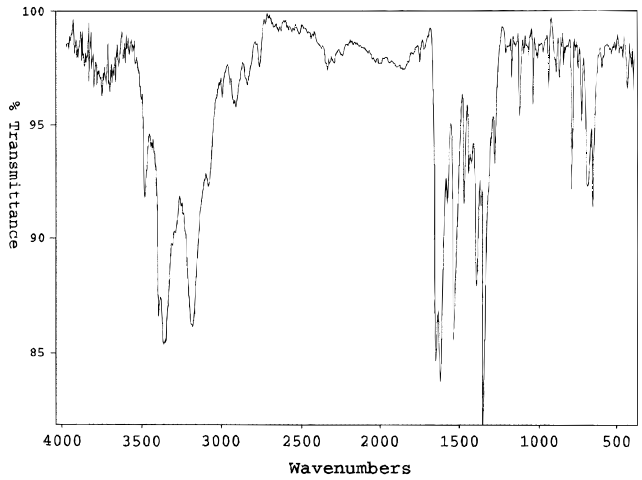


**Figure 5.** FTIR analysis of isolated ANOT.

peak was consistent with the structure of ANOT (Figure 4). The analysis produced the characteristic M $H^+$ (196 amu) and M $NH_4^+$ (213 amu) ions expected for ANOT. The negative ion thermospray analysis produced a single ion representing M $CH_3COO^-$ (254 amu), which is also consistent with the ANOT structure.

The FTIR spectra of the preparative LC fraction produced the characteristic IR stretches associated with the functional groups of ANOT (Figure 5) according to Smith et al. (1963). The figure illustrates the important response of the ANOT functional groups (Shriner et al., 1980; Silverstein et al., 1991): the N—H stretch of the primary amine coupled doublet (asymmetric 3373 $cm^{-1}$; symmetric 3290 $cm^{-1}$), the overlap C═O stretch of the amide I band (1695−1640 $cm^{-1}$), the C—N stretch of a primary aromatic amine (1425 $cm^{-1}$, 1340−1250 $cm^{-1}$), the asymmetric $ArNO_2$ $(N═O)_2$ stretch (1520 $cm^{-1}$), the symmetric $N(═O)_2$ stretch (1347 $cm^{-1}$), and the aromatic C—N stretch for $ArNO_2$ (850 $cm^{-1}$). The IR spectrum confirms the expected functional groups of ANOT are present.

**Table 4. NMR Response for ANOT and 5-ANOT**

| ANOT | | | 5-ANOT | | |
|---|---|---|---|---|---|
| position/ proton | calcd (ppm) | found (ppm) | position/ proton | calcd (ppm) | found (ppm) |
| | | $^{13}C$ NMR | | | |
| C3 | 148.4 | 148.5 | C3 | 150.0 | 150.8 |
| C5 | 146.3 | 146.0 | C5 | 169.7 | 170.0 |
| | | $^{1}H$ NMR | | | |
| $-CH_3$ | 2.2 | 2.2 | | 2.6 | 2.2 |
| $-NH_2$ | 6.6 | 5.8 | | 5.9 | 5.9 |
| $-CONH_2$ | 7.6 | 7.6, 7.9 | | 7.6 | 7.6. 7.9 |
| C4 | 7.1 | 7.3 | | 7.0 | 6.8 |
| C6 | 7.9 | 7.5 | | 7.4 | 7.0 |

The $^{13}C$ NMR spectra for the C3 and C5 carbon agree fairly well with the calculated values for the 3- and 5-ANOT isomers found in Table 4. The proton NMR spectra for the two isomers are consistent with their respective structures. Chemical shifts for the protons (Table 4) other than those on the aromatic ring are very similar and agree closely with the calculated spectra for the two compounds. The two amido hydrogens show different chemical shifts (0.35 ppm shift for ANOT and 0.33 ppm shift for 5-ANOT). Integration of the proton signals for both compounds is consistent with their structures. The signals from protons C4 and C6 on the aromatic ring are shifted downfield (7.32 and 7.51 ppm, respectively) in the ANOT because of their proximity to the 5-nitro group compared to that in the 5-ANOT. This is expected due to the larger contribution of shielding from the nitro group at this position. In the 5-ANOT structure, the C4 and C6 protons resonate downfield at 6.81 and 7.05 ppm, respectively, because the shielding effect is not as large. As a final confirmation of the identity and purity of the synthesized ANOT, the melting point of the recovered product was determined. The light yellow crystals melted quickly at 198 °C, well within a 1 °C range. The melting point reported in the literature is within the range of 198.5−199.5 °C (Thiegs, 1994; *The Merck Index*, 1996).

**Conclusion.** Our results demonstrate that the cyclohexene reduction of zoalene using 10% palladium on carbon coupled with chromatographic isolation methods is a reliable and efficient means of ANOT synthesis. The synthesis is a simple one-step process, although an extensive cleanup was necessary to isolate the ANOT. The ANOT produced was isolated from the synthesis products using three chromatographic techniques: dry

column, low-pressure liquid, and ion chromatography. The dry column chromatographic technique isolated the ANOT isomers from the reaction matrix. Because the dry column technique did not resolve ANOT from its 5-ANOT isomer, a low-pressure column chromatography scheme developed from a TLC method effectively isolated ANOT from the 5-ANOT isomer. ANOT was further purified to an analytical grade material of >99% purity after an ion exchange chromatographic cleanup procedure. The synthesis and purification methodology presented was successful in producing an analytical grade standard of ANOT.

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