Separation and Characterization of Dimethylnitrobiphenyl and Dimethylbiphenylamine Isomers by Chromatographic and Spectrometric Methods

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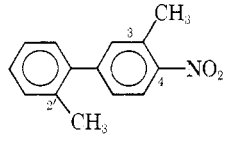
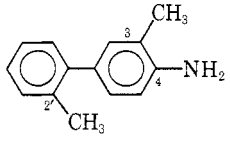
Diazotized 4-nitro-*m*-toluidine when coupled with toluene yields a mixture of dimethyl positional isomers of 2′-, 3′-, or 4′-dimethyl-4-nitrobiphenyl. The 2′,3-dimethyl-4-nitrobiphenyl isomer is of interest as a precursor for the carcinogen 2′,3-dimethyl-4-biphenylamine and the related hyroxamic acid. The 2′,3-dimethyl-4-nitro isomer is separated on a preparative scale from the 3,3′- and 3,4′-dimethyl isomers in a cyclohexane–heptane–nitromethane liquid–liquid partition chromatography system. The chromatographic system resolves 2′,3-dimethyl-4-biphenylamine from the two corresponding dimethylamine isomers and from the nitro compounds. The 2′,3-dimethylnitro or amine isomers may be analyzed by GLC on a 4-mm × 5-foot column of 2% OV-17 on Gas Chrom P. The methyl positional isomers of the nitro and amine compounds and their corresponding hydrocarbons have been characterized and compared to reference hydrocarbons by mass spectrometry. The 2′,3-dimethyl nitro and amine compounds are additionally characterized by physical constants, IR, and UV spectral data.

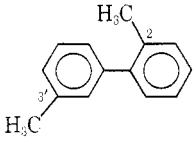
Walpole *et al. (1)* have described the induction of bowel tumors in rats by 2′,3-dimethyl-4-biphenylamine (2′,3-DMBA), a compound which is representative of a class of aromatic amines of occurrence in industry. Subsequent work has demonstrated similarities between the lesions induced in rats by 2′,3-DMBA and the related colonic disease in man *(2)*. Colonic tumors are of relatively infre-



2′,3-dimethyl-4-nitrobiphenyl



2′,3-dimethyl-4-biphenylamine



2,3′-dimethylbiphenyl

quent occurrence in laboratory animals, making their experimental study difficult. The property of 2′,3-DMBA to induce bowel tumors in rats thereby furnishes a useful model for studies on chemically-induced colonic tumors. Investigations have been undertaken in our laboratory on the biochemical mode of action of 2′,3-DMBA in the rat with regard to the *in vivo* activation of the amino group and the metabolism of the carbon skeleton. In the course of the work, it has been determined that all commercial preparations of the aromatic amine available to us, which appeared to be homogeneous by paper and thin-layer chromatography in several solvent systems, are actually various mixtures of 2′,3-, 3,3′-, and 3,4′-DMBA. The need for pure 2′,3-DMBA for biochemical studies on the model has led to the development of chromatographic methods which will separate it from other methyl isomers which are formed during its synthesis. The nitro analog, 2′,3-dimethyl-4-nitrobiphenyl (2′,3-DMNB), is useful as a chemical precursor for the synthesis of related hydroxamic acids with this carbon skeleton. The aromatic amines do not appear to be directly carcinogenic and the related *N*-hydroxy compounds have been suggested as more proximal carcinogens which may be esterified *in vivo* and then are highly reactive in nucleophilic substitution reactions *(3)*.

This communication describes the preparation and characterization of 2′,3-DMBA and its nitro analog, 2′,3-DMNB, free of other methyl isomers. The methyl positional isomers of the nitro, amino, and hydrocarbon compounds have been characterized by mass spectrometry.

EXPERIMENTAL

Synthesis of Compounds. 4-Nitro-*m*-toluidine was prepared most successfully by the procedure of McGookin and Swift *(4)*. *m*-Aceto toluidide was nitrated in acetic acid, sulfuric acid, nitric acid solution. From the nitration products, a single crystallization from ethanol yielded the principal isomer, 4′-nitro-*m*-acetotoluidide, which melted at 115 °C, and after recrystallization from the melt, remelted at 120.5–121.5 °C (all melting points are corrected), literature mp 102 °C *(5)*. Hydrolysis to the free base yielded yellow needles with a mp of 134–137 °C (literature, 134 °C) *(4)* and a molecular weight, determined by mass spectrometry, of 152. The nitroamine was diazotized and coupled with toluene by the procedure of Wilson to yield a crude product of isomeric dimethylnitrobiphenyls *(6)*. The yield of 2′,3-DMNB was 15–20%. The hexane-soluble residue from the reaction was dissolved in benzene and pigments were removed by chromatographing over alumina (Woelm, neutral, activity 1, Alupharm Chemicals, New Orleans, La.). Elution with benzene gave an orange oil from which the nitro isomers 2′,3-, 3,3′-, and 3,4′-DMNB were each resolved by liquid chromatography (*vide infra*). The principal component, 2′,3-dimethyl-4-nitrobiphenyl, was isolated and then crystallized from methanol, mp 59.5–60 °C (literature 56 °C) ethanol λ_{max} 287 nm, ϵ = 8,400; cyclohexane λ_{max} 280 nm, ϵ = 9,600. The infrared spectrum was recorded with a Beckman IR-9 infrared spectrophotometer and is presented in Figure 1. No absorption was observed in the 2800–3700 cm^{-1} region. The absorption bands in the 1300–1700 cm^{-1} region include typical nitro group absorptions.

Individual dimethylnitrobiphenyl isomers were reduced quantitatively to the corresponding amine by reacting with hydrogen for two hours at one atmosphere and at ambient temperature in the presence of 5% palladium on charcoal catalyst. The catalyst was removed, the reduction products were recovered and used directly for gas chromatography–mass spectrometry. For chemical charac-

[1] Author to whom reprint requests should be addressed.

(1) A. L. Walpole, M. H. C. Williams, and D. C. Roberts, *Brit. J. Ind. Med.*, **9**, 255 (1952).
(2) H. J. Spjut and J. S. Spratt, Jr., *Ann. Surg.*, **161**, 309 (1965).
(3) J. A. Miller and E. C. Miller, "Physico-Chemical Mechanisms of Carcinogenesis," The Israel Academy of Sciences and Humanities, Jerusalem, 1969, pp 237–61.
(4) A. McGookin and S. R. Swift, *J. Soc. Chem. Ind.*, **58**, 152 (1939).
(5) J. B. Cohen and H. D. Dakin, *J. Chem. Soc.*, **83**, 331 (1903).
(6) R. H. Wilson, *J. Appl. Chem.*, **3**, 37 (1953).

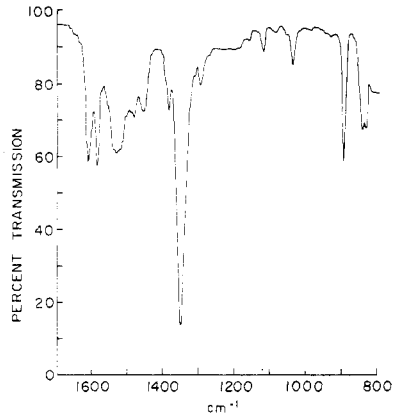


Figure 1. IR spectrum of 2′,3-dimethyl-4-nitrobiphenyl

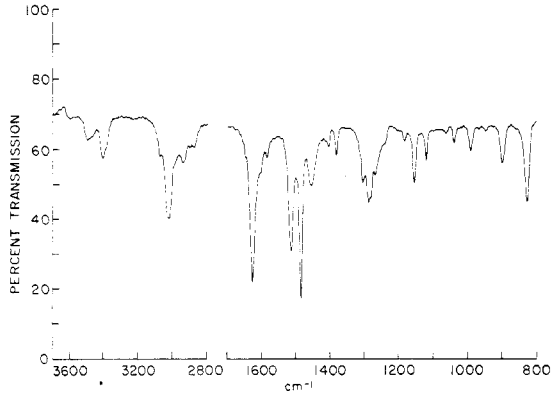


Figure 2. IR spectrum of 2′,3-dimethyl-4-biphenylamine

terization, 2′,3-DMBA was chromatographed in the cyclohexane–heptane–nitromethane system and then distilled in a molecular still to yield a pale yellow oil; ethanol λ_{max} 262 nm, ϵ = 11,800; cyclohexane λ_{max} 259.5 nm, ϵ = 13,300. The IR spectrum of the amine is shown in Figure 2. Typical amine absorptions are seen in the 2800–3700 cm^{-1} and 1200–1700 cm^{-1}.

Submilligram amounts of each amine derived by hydrogenation from the specific DMNB were diazotized with 100 μmol of concentrated hydrochloric acid, 50 μmol of sodium nitrite, and 50 μl of water and then deaminated overnight at ambient temperature by adding 1 ml of 30% solution of hypophosphorous acid *(6)*. Yields of the corresponding dimethylbiphenyl hydrocarbon were about 70%. The hydrocarbon was recovered by hexane extraction of the reaction mixture and used for GC–MS.

Reference Hydrocarbons. The 2,3′-, 3,3′-, and 3,4′-dimethylbiphenyls were synthesized from bromotoluenes and methylcyclohexanones and were donated by G. W. Griffin of Louisiana State University, New Orleans *(7)*.

Liquid Partition Chromatography. Nitromethane and silica gel (Unisil, 100/200 mesh, Clarkson Chemical Co., Inc., Williamsport, Pa.) were thoroughly mixed in the proportion of 0.5 ml of nitromethane per gram of silica gel. Cyclohexane and heptane (mobile phase) (1:1) was saturated with an excess of nitromethane (15 ml per liter of cyclohexane–heptane). The nitromethane–silica gel was packed into a 0.9- × 100-cm column with the mobile solvent. Preparations of the dimethyl-4-nitrobiphenyls or the dimethyl-4-biphenylamines were eluted with the mobile phase and 2-ml fractions were collected. Fractions were analyzed by gas–liquid chromatography.

Mass Spectrometry. Mass spectra were determined with an LKB Model 9000 mass spectrometer equipped with a gas chromatography inlet. The 3-mm × 6-foot column was packed with 1% OV-17 on 100/120 mesh silanized Gas Chrom P. Spectra were determined with an ionizing potential of 70 eV and an accelerating potential of 3.5 kV. It was very important to obtain the spectra with the most uniform conditions possible. Helium was the carrier gas. A given compound (nitro, amine, or hydrocarbon) was determined repeatedly and within a limited time period of several days, thereby minimizing instrumental parameters for comparisons among all isomers.

(7) J. L. Laseter, U. Mende, and G. W. Griffin, *Org. Mass Spectrom.*, **4**, 599 (1970).

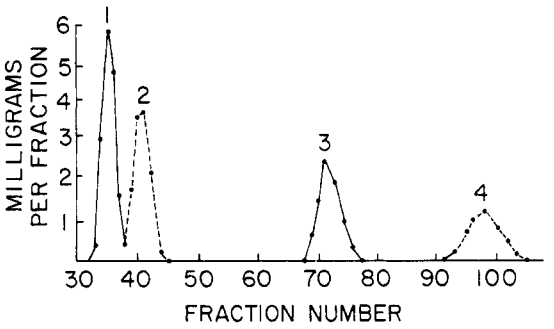


Figure 3. Liquid chromatography of DMNB and DMBA isomers
1. 2′,3-DMNB
2. 3,3′-DMNB (slightly leading) plus 3,4′-DMNB (slightly trailing)
3. 2′,3-DMBA
4. 3,3′- plus 3,4′-DMBA

Gas Chromatography. GLC was conducted by standard techniques utilizing 6-foot U-tube columns in a Barber-Colman oven, a flame ionization detector, and a Cary Model 21 vibrating reed electrometer. Experimental conditions are specified in Figure 4.

RESULTS AND DISCUSSION

The liquid partition chromatography systems which have been described are very useful for the separation of the 2′,3-dimethylnitro and amine compounds from each other and from their respective methyl positional isomers. Typical separations are shown in Figure 3. The three dimethylnitro isomers are obtained from the coupling reaction in the relative amounts of 2′,3- 60%, 3,3′- 24%, and 3,4′- 16%. An efficient system is required to separate the 2′,3-isomer from the other two. The 2′,3-DMNB, peak 1 of Figure 3, can be obtained in crystalline form free of the other dimethylnitro isomers. The overlapping of peak 1 and peak 2 (3,3′-DMNB + 3,4′-DMNB) occurs only in the final two minor fractions of peak 1. The system has a high capacity for preparative work. As much as 230 mg of a mixture of nitro isomers has been chromatographed at one time on the described column. The column was obviously overloaded and thus the peaks were flat-topped and the overlapping was greater. However, a relatively large amount of 2,3′-DMNB was obtained free of the other two isomers. Peak 2, the mixed 3,3′- and 3,4′-DMNB compounds, appeared to be displaced by peak 1 and was eluted in later fractions.

For this study it was desired to separate the 3,3′- and 3,4′-DMNB isomers from each other for identification purposes. Peak 2, Figure 3, is a composite of the two partially resolved isomers. By recycling the composite peak and repeatedly collecting enriched fractions, it was possible to obtain small amounts of each isomer. Each compound was used for further work only after it was determined to be free of the other methyl isomer by gas chromatography (*vide infra*). Submilligram amounts of each nitro isomer were obtained and were converted to the corresponding hydrocarbon by reduction to the amine, followed by deamination. The amines and hydrocarbons were each studied separately by mass spectrometry.

The separation of the amine, 2′,3-DMBA, peak 3, Figure 3, from the other methyl positional isomers, 3,3′- and 3,4′-DMBA, peak 4, was easily achieved. Columns as short as 30 cm gave resolution of peaks 3 and 4 with only minor overlapping. The composition of peak 4, the mixed 3,3′- and 3,4′-DMBA has not been determined. The res-

olution of these two compounds has not been achieved by paper, thin-layer, liquid, or gas chromatography.

The analysis of this series of compounds has been most conveniently accomplished by gas chromatography using a 2% OV-17 column (Figure 4). With this column, the compounds of principal interest, 2′,3-DMNB and 2′,3-DMBA, are completely and rapidly resolved (Figure 4, *a* and *c*). Only semiquantitative indications of 3,3′-DMNB and 3,4′-DMNB are obtained. The corresponding two amines are not resolved. Base-line separation of all three nitro isomers has been achieved with a 4-mm × 12-foot column of 5% OV-25 on Gas Chrom P at 220 °C. Because of the extended elution times with the 5% OV-25 column, the 2% OV-17 column was preferred for routine work. Other phases, such as OV-225 or Poly-I-110, have been less satisfactory.

Preparative gas chromatography with the 12-foot OV-25 column appeared to be an attractive route for purification of small amounts of 3,3′- and 3,4′-DMNB for identification purposes. A 1% split went to the flame ionization detector of the gas stream. The two isomers were each prepared by conventional trapping in a small glass capillary tube at the time of the detection of the peak. Rechromatography of such preparations on the same column always showed the appearance of new peaks in the chromatogram. It seems reasonable that changes in the compound occurred during the collection step perhaps by contact with atmospheric oxygen. In contrast, the more laborious fractionation in the liquid chromatography system yielded preparations of 3,3′- 3,4′-DMNB which were homogeneous by gas chromatography.

Thin-layer chromatography with silica gel G (Merck) has been of little use in the study. The nitro and amine isomers have not been resolved. The amine compound underwent undesirable changes on the plates to unknown products. Chromatography of 2,3′-DMBA has sometimes resulted in the appearance of new spots on the chromatogram. The recovery of amine and hydrocarbon isomers from plates has been low. The use of several different solvents did not improve the recoveries.

The preparative gas chromatography and the thin-layer chromatography results suggest that these compounds should be handled with care to prevent undesirable chemical changes. This conclusion is supported by a study on the photoisomerization of the hydrocarbons (bitolyls) by UV radiation via a benzvalene mechanism *(8)*. That these reactions occur can be rationalized by considering that the most easily separated amine or nitro isomers, 2′,3-DMNB and 2′,3-DMBA, are the compounds with the greatest dihedral angle between the aromatic rings as compared to the corresponding 3,3′- and 3,4′-dimethyl isomers *(9)*. These compounds also have the most distinctive mass spectra in the series. The corresponding hydrocarbon, 2,3′-dimethylbiphenyl, also has greater photolability than the 3,3′- and 3,4′-dimethylbiphenyls *(8)*.

The mass spectra determined in this investigation allow the nitro and amine positional isomers to be distinguished from each other. These compounds are connected through their derived hydrocarbons with structurally defined hydrocarbons synthesized by an independent route *(7)*. The three nitro isomers have base peaks furnished by the molecular ion with an m/e of 227. The three methyl positional isomers, 2′,3-, 3,3′-, and 3,4′-, may be distinguished

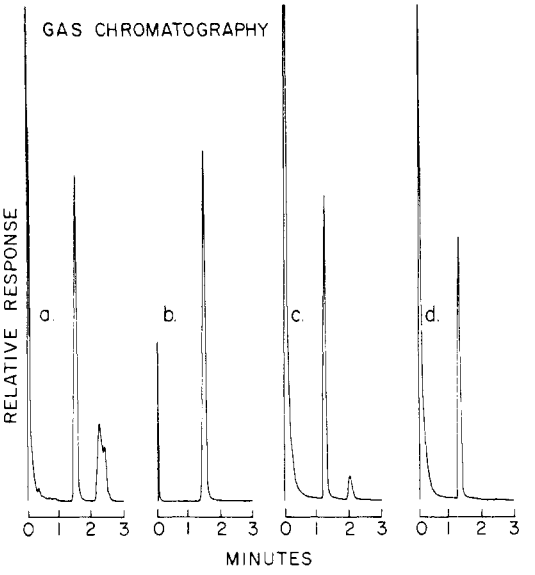


Figure 4. Gas–liquid chromatography on 4-mm × 5-foot OV-17 on Gas Chrom P (silanized) at 178 °C, N_2 flow rate 100 ml per min

a. Dimethylnitrobiphenyl isomers from coupling reaction. Minutes of elution time: 2′,3-DMNB 1.52, 3,3′-DMNB 2.28, 3,4′-DMNB 2.43

b. 2′,3-DNB after liquid chromatography

c. Isomers. 2′,3-DMBA at 1.25 min, 3,3′- plus 3,4′-DMBA at 2.03 minutes

d. 2′,3-DMBA after liquid chromatography

from each other by the [M − 17] peaks with corresponding relative intensities of 68.6, 60.7, and 46.3. The [M − 62] peaks are also useful with intensities of 65.8, 63.5, and 56.3. The corresponding amine methyl isomers similarly have base peaks of the molecular ion with an m/e of 197. The [M − 1] peak distinguishes the three isomers (as above) with relative intensities of 18.2, 9.4, and 24.5. The 3,3′- and 3,4′-hydrocarbons each have a base peak of the molecular with an m/e of 182. However, the 2,3′-dimethyl hydrocarbon (corresponding to the 2′,3-dimethyl amino or nitro compounds) has a base peak given by the [M − 15] ion, with intensities of 35.8 and 38.0 for the 3,3′ and 3,4′ isomers. The [M − 1] peaks gave relative intensities of 18.3 (2,3′ isomer), 20.0, and 26.3 (3,3′ and 3,4′ isomers). The mass spectra of the hydrocarbons synthesized in this study agree with the spectra of the reference hydrocarbons which were determined on the same instrument and under the same conditions, thereby confirming the structures of these methyl positional isomers.

The relative intensities for the hydrocarbons which are reported in this study differ from those previously reported in the literature *(7)*. The prior work was done with a Hitachi instrument while the present studies utilized the LKB-9000 mass spectrometer. The differences cannot be ascribed to differences in preparation as the reference hydrocarbons for this study were obtained from the laboratory which prepared them for the earlier work. The differences in the mass spectra between this work and the earlier study *(7)* are ascribed to possible differences between instruments and operating conditions.

Wilson fractionally distilled the nitro compounds from the coupling reaction and reduced the products with Raney nickel *(6)*. A lower boiling fraction yielded 2′,3-DMBA as shown by deamination and subsequent oxidation to 2,3′-biphenyldicarboxylic acid. A higher boiling fraction of the nitro compounds similarly yielded 3,3′-biphenyldicarboxylic acid, indicating the presence of

(8) U. Mende, J. L. Laseter, and G. W. Griffin, *Tetrahedron Lett.*, **43**, 3747 (1970).

(9) H. Suzuki, "Electronic Absorption Spectra and Geometry of Organic Molecules," Academic Press, New York, N.Y., 1967.

3,3′-DMNB in the starting material. The 3,4′-dimethyl isomer, described in this study, was not reported. A UV characterization of 2′,3-DMAB has been reported from work on the labeling of the amine with tritium by catalytic exchange *(10)*. It is now recognized that the characterizations previously reported utilized a mixture of methyl isomers. The tritiated 2′,3-DMBA from exchange labeling was purified from radioactive impurities by partition chromatography and was shown to be free of other methyl isomers by the methods described here.

The availability of pure 2,3′-DMNB now provides a tool for further investigations with this drug on the mechanism of the chemical induction of colonic tumors. Nitro-substituted aromatic compounds have been suggested as possible precursors for carcinogenic compounds *in vivo (11)*. It has been shown that *N*-hydroxy compounds can be esterified *in vivo* with sulfate or other anions. The resulting ester has an increased carcinogenic activity *(12)*. In this series of compounds, only one brief sentence in the literature states that 3,3′-DMBA has carcinogenic activity, probably similar to that of 2′,3-DMBA *(1)*. The 3,4′-isomer has not been reported. Thus, 2′,3-DMNB and 2′,3-DMAB are compounds of prime interest for biochemical study and are now available free of other methyl positional isomers. The biological role of other positional isomers of this series remains to be determined.

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(10) M. W. Noall, *J. Label. Compounds*, **5**, 192 (1969).
(11) J. H. Weisberger, *Cancer*, **28**, 60 (1971).
(12) J. R. DeBaun, J. Y. R. Smith, E. C. Miller, and J. A. Miller, *Science*, **167**, 184 (1970).

On-the-Fly Gas Chromatography–Infrared Spectrometry Using a Cholesteric Liquid Crystal–Effluent Interface

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On-the-fly infrared spectra of organic vapors in quantities as small as 50 μg have been obtained using a cholesteric liquid crystal–effluent interface. The liquid crystal fractionates sample from carrier gas. The technique yields spectra which are solution rather than gas phase spectra, eliminating problems of rotational structure and vapor–liquid frequency shifts. The system can also be used for on-the-fly spectra of organic vapors in an air stream, with comparable efficiency. A technique for extending the system to accommodate higher boiling materials is described.

A wide variety of techniques for obtaining infrared spectra of gas chromatograph effluents have been described in the literature *(1)*. Each of these may be designated as either a trapping or an on-the-fly technique. While trapping methods potentially offer the maximum sensitivity for infrared analysis (if 100% of the effluent can be transferred to the infrared cell), they have disadvantages which limit their utility. The trapped effluent can be concentrated in the IR cell allowing small quantities (~0.1 μg) to be measured. However, the time required to trap an effluent, transfer it to a suitable IR cell, and record the infrared spectrum can result in considerable delay between exit of the effluent from the GC and availability of a plotted spectrum for analysis and decision making. There are thus many applications where an on-the-fly analysis is required even if this means increasing sample size.

The development of rapid scanning dispersive spectrometers and interferometric Fourier spectrometers led to development of on-the-fly GCIR techniques. These systems offer approximately real time spectral analysis but have their own limitations. Operating on the effluents as they exit from the gas chromatograph, these techniques must record spectra of dilute solutions of sample in a carrier gas. In addition, they must be able to generate correct relative spectral intensities for a sample which varies in concentration with time. The result of these sampling problems is a minimum sensitivity of only 10–50 μg *(2)*.

Additional problems associated with on-the-fly GCIR techniques are the gaseous phase of the sample and the determination of when the sample is in the infrared cell. A spectrum recorded on a gas phase sample will contain rotational structure which distinguishes it from its liquid phase counterpart. The gas phase spectrum often shows frequency shifts for some bands of up to 20 cm^{-1} compared to the liquid spectrum. These differences from liquid spectra can hamper the identification of materials if comparison with a liquid phase reference spectrum is used.

Determination of when a sample enters an on-the-fly GCIR cell is generally made for a particular set of experi-

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(1) "Ancillary Techniques of Gas Chromatography," L. S. Ettre and W. H. McFadden, Ed., Wiley-Interscience, New York, N.Y., 1969.
(2) J. O. Lephardt, Ph.D. Thesis, University of Maryland, 1972.