Phytochemistry, 1971, Vol. 10, pp. 1135 to 1140. Pergamon Press. Printed in England

ALKYLPHENOLS AND ARYLNITRILES IN A BIOLOGICALLY ACTIVE NEUTRAL SUBFRACTION OF CIGARETTE SMOKE CONDENSATE*

R. L. MILLER and R. L. STEDMAN

Eastern Utilization Research and Development Division, Agricultural Research Service, United States Department of Agriculture, 600 East Mermaid Lane, Philadelphia, Pennsylvania 19118, U.S.A.

(*Received* 16 *June* 1970)

Abstract—A tumorigenic neutral subfraction of cigarette smoke condensate eluting from silicic acid with benzene and soluble in dimethylsulfoxide was shown to contain the following constituents: 2-butylphenol; 2-isobutylphenol; 2-ethyl-6-methylphenol; 3-ethylbenzonitrile; 2,5-dimethylbenzonitrile; 2-ethylbenzonitrile; 2,6-dimethylbenzonitrile; 4-ethylbenzonitrile and 2,3-dimethylbenzonitrile. Evidence was also obtained for the presence of 2-methylbenzonitrile and 2-ethyl-5,6-dimethylphenol. None of these components has been reported previously in condensate. The partition coefficients in ether/1N NaOH ranged from 1·1 to 44 for the four phenols, indicating that their occurrence in the neutral fraction would be expected. The amounts of constituents that were isolated and identified ranged from 0·6 to 2·2 mg/kg smoke condensate.

INTRODUCTION

THE NEUTRAL fraction of cigarette smoke condensate (CSC) is a major contributor to the tumorigenic activity of CSC in animals.[1] On fractionation of the neutral substances by solvent partitioning,[2] tumorigenic activity is observed in all subfractions, and the nitromethane-soluble subfraction is the most active.[3] Compositional studies on the latter have shown the presence of polynuclear aromatic hydrocarbons,[4,5] quinones,[6] myristicin,[7] secondary arylamines,[8] benzyl esters[9] and cinnamonitrile.[9] In more recent work, the neutral substances of CSC have been separated by silicic acid chromatography and solvent partitioning,[10,11] and tumorigenic activity has been found in several subfractions.[12] The present report concerns the isolation and identification of several components in one of these active subfractions.

* Part XLIII in the series "Composition studies on tobacco".

[1] E. L. WYNDER and D. HOFFMANN, *Tobacco and Tobacco Smoke. Studies in Experimental Carcinogenesis*, p. 225, Academic Press, New York (1967).
[2] A. P. SWAIN, J. E. COOPER and R. L. STEDMAN, *Cancer Res.* **29**, 579 (1969).
[3] F. G. BOCK, A. P. SWAIN and R. L. STEDMAN, *Cancer Res.* **29**, 584 (1969).
[4] D. HOFFMANN and E. L. WYNDER, *Anal. Chem.* **32**, 295 (1960).
[5] G. GRIMMER, *Beitr. Tabakforsch.* **1**, 291 (1962).
[6] W. J. CHAMBERLAIN and R. L. STEDMAN, *Phytochem.* **7**, 1201 (1968).
[7] I. SCHMELTZ, R. L. STEDMAN, J. S. ARD and W. J. CHAMBERLAIN, *Science* **151**, 96 (1966).
[8] R. L. MILLER, L. LAKRITZ, C. J. DOOLEY and R. L. STEDMAN, *Tobacco Sci.* **11**, 35 (1967).
[9] I. SCHMELTZ, C. J. DOOLEY, R. L. STEDMAN and W. J. CHAMBERLAIN, *Phytochem.* **6**, 33 (1967).
[10] R. L. STEDMAN, R. L. MILLER, L. LAKRITZ and W. J. CHAMBERLAIN, *Chem. & Ind.* 394 (1968).
[11] A. P. SWAIN, J. E. COOPER, R. L. STEDMAN and F. G. BOCK, *Beitr. Tabakforsch.* **5**, 109 (1969).
[12] F. G. BOCK, A. P. SWAIN and R. L. STEDMAN, *J. Nat. Cancer Inst.* in press.

RESULTS AND DISCUSSION

Acids, bases and insoluble substances were removed from CSC, and the neutral fraction was chromatographed on activated silicic acid. The light petroleum, 25% benzene in light petroleum (BP) and benzene (B) eluates were each partitioned between cyclohexane (CH) and dimethyl sulfoxide (DMSO) giving six subfractions, of which three (BP–CH, BP–DMSO, B–DMSO) showed tumorigenic activity in mice. By further adsorptive and GLC separations of B–DMSO, several components were isolated having i.r. spectral characteristics of alkylphenols. The occurrence of such compounds in the neutral fraction of CSC has not been previously reported[13] and is of special interest because of the known tumor-promoting properties of some members of this group.[14]

Table 1 summarizes the findings. The i.r. spectrum of the first isolated component showed strong absorption at 2·79 μ (unbonded —OH), a doublet of medium intensity at 3·26–3·31 μ (aromaticity) and strong bands characteristic of the C—H stretching of alkyl groups in the 3·39–3·51 μ region. A single, strong band at 13·35 μ suggested the presence of

TABLE 1. ALKYLPHENOLS AND ARYLNITRILES IN CIGARETTE SMOKE CONDENSATE

Isolate	Identity*			K†	Level‡
	i.r.	MS	GC		
2-Butylphenol	+	+	+	44	2·2
2-Isobutylphenol	+	+	+	11	1·2
2-Ethyl-6-methylphenol	+	+	+	1·1	2·2
2-Ethyl-5,6-dimethylphenol	+	−	±	5·4	1·5
2-Methylbenzonitrile	+	−	±	−	1·2
2,5-Dimethylbenzonitrile	+	+	+	−	1·2
2,3-Dimethylbenzontrile 4-Ethylbenzonitrile	+	+	+	−	1·5
2,6-Dimethylbenzonitrile 2-Ethylbenzonitrile	+	+	+	−	1·3
3-Ethylbenzonitrile	+	+	+	−	0·6

* Based on spectral (i.r. = infrared, MS = mass) and gas chromatographic (GC) comparisons of isolated (I) and synthetic (S) compounds (or pairs of compounds). (+), I and S cochromatograph or have identical spectra. (±), G.C. retention times of I and S are identical but cochromatography not actually done. (−), Not determined. Small amounts of a possible dimethyl isomer accompanied the isolated 3-ethylbenzontrile.

† Partition coefficient (ether–1N NaOH).

‡ mg/kg smoke condensate (isolated amounts).

four adjacent unsubstituted hydrogen atoms in a mononuclear aromatic structure.[15] The mass spectrum of the isolated compound gave a molecular peak of 150 ($C_{10}H_{14}O\cdot^+$), a base peak of 107 ($HOC_6H_4CH_2^+$) and major fragments of 121 (loss of $C_2H_5\cdot$), 108 (loss of C_3H_6) and 77 ($C_6H_5^+$). All of these spectral characteristics were suggestive of an alkylphenol having a C_4 aliphatic sidechain in the 2-position. 2-Butylphenol was synthesized by rearrangement of butyl phenyl ether in the presence of $AlCl_3$ at room temp.[16] A number of products was obtained on separation of the reaction mixture by gas chromatography,

[13] R. L. STEDMAN, *Chem. Rev.* **68**, 153 (1968).
[14] R. K. BOUTWELL and D. K. BOSCH, *Cancer Res.* **19**, 413 (1959).
[15] D. D. SHREWSBURY, *Spectrochim. Acta* **16**, 1294 (1960).
[16] R. A. SMITH, *J. Am. Chem. Soc.* **56**, 1419 (1934).

including 2-butylphenol, 2-*sec*-butylphenol and polyalkylphenols. The synthetic 2-butylphenol and the alkylphenol isolated from CSC were identical with respect to gas chromatographic and spectral characteristics.

The i.r. spectrum of the second component separated from B–DMSO was essentially identical to that of the isolated 2-butylphenol. The mass spectrum of the unknown was also very similar with a molecular peak of 150, a base peak of 107 and major fragments of 108 and 77. However, the peak at 121 present in 2-butylphenol was absent in this isolate. These data indicated that the isolated compound was a probable isomer of 2-butylphenol. 2-Isobutylphenol was synthesized by thermal rearrangement[17] of 2-methylallyl phenyl ether to 2-(2-methylallyl)phenol which was then hydrogenated to the desired product. The i.r. spectrum of the synthetic isobutylphenol indicated substitution in the 2- rather than the 4-position. The gas chromatographic and spectral characteristics of the synthetic 2-isobutylphenol and the compound isolated from CSC were identical.

Two other phenols were isolated from B–DMSO. One of these showed strong i.r. absorption for aromaticity at 12·03, 13·03 and 13·50 μ and medium absorption at 11·33 and 13·27 μ. This pattern suggested the presence of multiple substitution in the ring. The mass spectrum gave a molecular peak at 136 and a base peak at 121 (loss of $CH_3\cdot$). 2-Ethyl-6-methylphenol was synthesized by acetylation of 2-methylphenol followed by $AlCl_3$-catalyzed rearrangement of the resulting 2-methylphenyl acetate to 2-acetyl-6-methylphenol and Clemmensen reduction of the latter. The synthetic and isolated compounds cochromatographed and gave i.r. and mass spectra that were identical.

The other isolated phenol also appeared to have multiple substitutions with strong i.r. absorption at 11·5, 12·4 and 13·3 μ. These characteristics were similar to those of authentic 2-ethyl-5,6-dimethylphenol, and the gas chromatographic retention time of the latter was identical to that of the isolated component.

On cursory examination, the occurrence of alkylphenols in the neutral fraction may seem unexpected. Closely related compounds have been reported[13] as constituents of the weakly acidic fraction of CSC, e.g. 2-ethylphenol and 2,6-dimethylphenol. In monocyclic phenols, the ionization of the hydroxyl group is influenced by inductive, resonant and steric effects of substituents.[18] Alkyl substitution in the 2- and 6-positions may reduce the acidity or produce other effects so that the phenol is insoluble in aqueous alkali, e.g. the commonly used commercial antioxidant, BHT (2,6-di-*tert*-butyl-4-methylphenol). To determine whether the bulk of the isolated alkylphenols would be expected in the neutral or weakly acidic fractions of CSC, partition coefficients of the isolated compounds in ether–aq. NaOH were determined (Table 1). The results indicated that most of the 2-butylphenol, 2-isobutylphenol and possibly 2-ethyl-5,6-dimethylphenol should be in the neutral fraction but substantial amounts of the 2-ethyl-6-methylphenol might be in the weakly acidic fraction, depending on the number of successive partitioning steps employed.

The isolated quantities of the alkylphenols were approximately 1·2–2·2 mg/kg CSC, which is equivalent to about 0·024–0·044 μg/cigarette. These levels should be considered minimum values since uncharacterized alkylphenols were also found in adjacent column chromatographic fractions and may have included further amounts of the identified components.

In addition to the aklylphenols, several substituted benzonitriles were isolated and

[17] Q. R. Bartz, R. F. Miller and R. Adams, *J. Am. Chem. Soc.* **57**. 371 (1935).
[18] E. S. Gould, *Mechanism and Structure in Organic Chemistry*, pp. 206, 214–16, Holt, Rinehart & Winston New York (1959).

identified in B–DMSO. Prior to this work, benzonitrile was the only known arylnitrile in tobacco smoke.[19–21] All of these isolates contained the strong i.r. absorptive band at about 4·5 μ characteristic of nitriles. One of the compounds gave a strong aromatic band at 12·25 μ and moderate bands at 11·28 and 14·09 μ. These features suggested the presence of only one or two adjacent unsubstituted hydrogens in the ring, i.e. multiple substitution, including possibly a *meta* configuration. The mass spectrum of the unknown gave a molecular peak of 131, a base peak of 116 (loss of $CH_3\cdot$), and major fragments at 103 (loss of H· and HCN) and 89 (loss of $CH_3\cdot$ and HCN). This pattern of fragmentation is characteristic of alkylbenzonitriles.[22] All of these data indicated that the isolate was a probable dimethylbenzonitrile, such as the 2,5-derivative. Comparison of the spectral characteristics and gas cochromatography of authentic 2,5-dimethylbenzonitrile and the isolated compound showed the two to be identical.

Another isolate having strong nitrile absorption gave three strong i.r. bands for aromaticity at 11·98, 12·78 and 14·08 μ. Thus, in comparison with 2,5-dimethylbenzonitrile, two of the three aromatic bands showed bathochromic shifts, indicating the presence of a larger number of adjacent unsubstituted ring hydrogens in the structure of this isolate. The mass spectrum gave the same qualitative pattern of fragmentation as 2,5-dimethylbenzonitrile, but some difference was observed in the ratio of the molecular peak (131) to the base peak (116). Comparison of the spectral characteristics of the isolate and authentic 2,3-dimethylbenzonitrile showed strong similarities but some minor differences were observed that suggested the presence of a mixture containing 2,3-dimethylbenzonitrile and an isomer thereof. 4-Ethylbenzonitrile was synthesized by reaction between cuprous cyanide and 4-ethylphenyl bromide.[23] The 4-ethyl and 2,3-dimethyl derivatives gave identical gas chromatographic retention times. A 1 : 1 mixture of the isomers showed i.r. and mass spectra that were identical to the isolate. It was concluded that the isolate was a mixture of 2,3-dimethylbenzonitrile and 4-ethylbenzonitrile.

A similar case was encountered with another isolate having the characteristics of a nitrile. The i.r. spectrum indicated the possible presence of a mixture of components, including structures with *ortho*-substituents and/or 1,2,3-trisubstitution. The mass spectrum was similar to the mixture of isomers described above. 2,6-Dimethylbenzonitrile was synthesized by the reaction of the corresponding dimethylphenyl bromide and cuprous cyanide and was found to have the same gas chromatographic retention time as the unknown. Examination of other possible isomers revealed that 2-ethylbenzonitrile cochromatographed and had spectral similarities with the isolate and 2,6-dimethylbenzonitrile. A 1 : 1 mixture of the synthetic ethyl and dimethyl compounds had i.r. and mass spectra that were identical to the isolate. It was concluded that the latter was a mixture of 2,6-dimethylbenzonitrile and 2-ethylbenzonitrile.

3-Ethylbenzonitrile was also identified among the nitriles. The i.r. spectrum showed probable *meta*- substitution [λ_{max} 12·54 μ(s), 14·50 μ(s)], and the mass spectrum was similar to the other nitriles discussed above. Synthesis of the 3-ethyl derivative was achieved by

[19] E. GLOCK, R. K. ODLAND, N. L. BODENHAMER and R. W. PHILLIPS, JR., *Abstracts 22nd Tobacco Chemists Res. Conf.* Richmond, Va. (1968).
[20] K. GROB and J. A. VÖLLMIN, *Beitr. Tabakforsch.* **5**, 52 (1969).
[21] R. L. PECK, S. F. OSMAN and J. L. BARSON, *Tobacco Sci.* **13**, 38 (1969).
[22] H. BUDZIKIEWICZ, C. DJERASSI and D. H. WILLIAMS, *Mass Spectrometry of Organic Compounds*, pp. 416–8, Holden-Day, San Francisco (1967).
[23] L. FRIEDMAN and H. SHECHTER, *J. Org. Chem.* **26**, 2522 (1961).

Friedel–Crafts condensation of benzonitrile and diethyl ether in a pressure bomb.[24] The synthetic compound and the isolate cochromatographed and had i.r. and mass spectra that were almost identical. However two minor i.r. bands appearing at 12·23 and 14·62 μ in the isolate were absent in the synthetic compound and the ratios of molecular and base peaks in their respective mass spectra differed slightly. It was concluded that the isolated substance contained principally 3-ethylbenzonitrile but that small amounts of another component was present, such as a dimethyl isomer.

Another isolate having absorption for the nitrile group gave a strong i.r. band at 13·20 μ suggestive of *ortho* substitution. Authentic 2-methylbenzonitrile had an i.r. spectrum and a gas chromatographic retention time that was identical with the isolated compound. Based on these similarities of chromatographic and spectral data and the occurrence of isomeric and homologous alkylbenzonitriles in these fractions, it was concluded that 2-methylbenzonitrile was present in this isolate.

From our experience in these isolations, it appears that the mass spectra of dimethyl and ethyl derivatives of benzonitrile may be differentiated by comparison of the ratios of the molecular peaks (P) to the P-15 peaks since the ethyl derivatives tend to give relatively smaller molecular peaks. For a definitive conclusion, however, a more complete series of derivatives should be investigated.

In addition to alkylphenols and alkylbenzonitriles, indoles were found in B–DMSO, including skatole, a well-known smoke constituent. Some of these components may have included alkyl- and arylindoles that were reported recently in CSC.[25–27]

The isolated nitriles and phenols comprise a very small proportion of the total weight in fraction B–DMSO. The contribution of these compounds to the tumorigenicity is obviously unknown at this time. Although 2-butylphenol is tumor-promoting when tested on mouse skin at high concentration,[14] it is questionable whether the level isolated from CSC is contributing substantially to the observed activity therein, if possible synergistic effects with other tumorigenic agents[28,29] are discounted. Although the bulk of the carcinogenic hydrocarbon, benzo[a]-pyrene, in CSC appears[10,11] in fraction BP–DMSO, about 3% of the total hydrocarbon in CSC is also present[10] in B–DMSO. The contributions of all of these components to the biological activity remain to be investigated.

EXPERIMENTAL

Isolation of Phenols and Nitriles

Cigarette smoke condensate (2 kg) obtained by mechanical smoking of 100,000 domestic, 85 mm, nonfilter cigarettes was fractionated as described previously.[10,11] In brief, the acids and bases were removed from the condensate by solvent partitioning, the neutral fraction was chromatographed on activated silicic acid and the column was eluted with light petroleum, 25% benzene in light petroleum and benzene. The residue from the benzene eluate was then partitioned between cyclohexane and dimethylsulfoxide (DMSO) giving 26·3 g of DMSO-soluble material which was rechromatographed on silicic acid (1000 g, 64 mm × 82 cm column). Elution was performed with light petroleum (4 l., fractions 1–2, 2 l. each). 5% benzene in light petroleum (v/v) (4 l., fractions 3–4, 2 l. each), 25% benzene in light petroleum (v/v) (4·4 l., fractions 5–8, 1·1 l. each), benzene (4 l., fractions 9–30, 180 ml each) and other solvents of increasing polarity. Fraction 25 was separated by gas chromatography on SE-30 (20% on Chromosorb W, 0·63 cm × 3·0 m column) under the following conditions: detector, 270°; injector, 270°; flow rate (helium), 60 ml/min; and oven temp., 150°

[24] G. BADDELEY, *J. Chem. Soc.* 5229 (1949).
[25] D. HOFFMANN and J. RUBIN, *Beitr. Tabakforsch.* **3**, 409 (1966).
[26] D. HOFFMANN, G. RATHKAMP and H. WOZIWODZKI, *Beitr. Tabakforsch.* **4**, 253 (1958).
[27] D. HOFFMANN and G. RATHKAMP, *Anal. Chem.* **42**, 366 (1970).
[28] B. L. VAN DUUREN, A. SIVAK, A. SEGAL, L. ORRIS and L. LANGSETH, *J. Nat. Cancer Inst.* **37**, 520 (1966).
[29] E. BINGHAM and H. L. FALK, *Arch. Environ. Health* **19**, 779 (1969).

for 5 min then programmed at 4°/min to 275°. Three groups (A–C) of peaks eluting at 150–182°, 182–206° and 206–220°, respectively, were collected and each group was rechromatographed on Carbowax 20M (20% on Chromosorb W, 0·63 cm × 3·0 m column) under the same conditions as above except that a maximum temp. of 250° was used.

Separation of A yielded 2-methylbenzonitrile (retention time on Carbowax 20M, 13·8 min); 2-ethylbenzonitrile (15·4 min); 2,6-dimethylbenzonitrile (15·4 min); 2,5-dimethylbenzonitrile (16·6 min); 3-ethylbenzonitrile (17·6 min); 4-ethylbenzonitrile (18·8 min); 2,3-dimethylbenzonitrile (18·8 min); and 2-ethyl-6-methylphenol (20·9 min).

Separation of B yielded an unidentified phenol (25·1 min); 2-ethyl-5,6-dimethylphenol (26·5 min); 2-isobutylphenol (27·8 min); 2-butylphenol (30·1 min); and indoles (36·5 min).

Separation of C yielded a number of components that were phenols, nitriles and indoles by i.r. spectral analysis, including skatole (39·8 min).

Synthesis of 2-Butylphenol

Anhydrous $AlCl_3$ (26·6 g) was added incrementally to butyl phenyl ether (30 g) with cooling, and the mixture was kept at room temp. for 24 hr. The mixture was poured into conc. HCl (100 ml) containing ice and Et_2O soluble substances were then removed. The Et_2O extract (500 ml) was dried and evaporated to 25 ml and the products were isolated by gas chromatography on SE-30 and Carbowax 20M.

Synthesis of 2-Isobutylphenol

Phenol (42 g), 2-methylallyl chloride (49·5 g) and anhydrous K_2CO_3 (76 g) were added to acetone (63 ml) and the mixture was heated under reflux on a steam bath for 24 hr. After cooling, water and light petroleum were added and the organic layer containing the phenyl ether was separated and washed with aqueous 10% NaOH. After removal of the solvent, the residue was heated at 230–240° for 30 min to rearrange the phenyl ether. The products were then hydrogenated in EtOH with Pt_2O (7·5 g) at 3 atm for 5 min. 2-Isobutylphenol was isolated by gas chromatography on SE-30 and Carbowax 20M.

Synthesis of 2-Ethyl-6-methylphenol

A mixture of 2-methylphenol (36 g) and acetyl chloride (26·1 g) was heated (steam bath) until HCl evolution was completed. Anhydrous $AlCl_3$ (44·5 g) was added and the mixture heated at 120° for 15 min to effect rearrangement of the ester to the ketophenol. Clemmensen reduction of the latter was accomplished by refluxing in 1:1 (v/v) conc. HCl–H_2O containing Zn amalgam (200 g) to which were added 25 ml conc. HCl hourly for 8 hr. The product was isolated by gas chromatography on Carbowax 20M.

Synthesis of 4-Ethylbenzonitrile and 2,6-Dimethylbenzonitrile

For 4-ethylbenzonitrile, 4-ethylphenyl bromide (18·5 g) and CuCN (10·7 g) were refluxed in N,N-dimethylformamide (75 ml) for 4 hr. The mixture was poured into a mixture of H_2O (300 ml) and conc. HCl (50 ml) containing $FeCl_3$ (200 g). After 20 min at 60–70°, the layers were separated and the aqueous layer was extracted with toluene (2 × 125 ml). All organic layers were combined and washed successively with aq. HCl and aq. NaOH. After drying the organic layer and evaporating the solvent, the residue was distilled and the material boiling at 95–97° (3 mm) was collected. 4-Ethylbenzonitrile was isolated from the distillate by gas chromatography on Carbowax 20M.

2,6-Dimethylbenzonitrile was synthesized by the same general procedure starting with 2,6-dimethylphenyl bromide but the product was isolated by sublimation (3 mm) rather than by distillation.

Synthesis of 3-Ethylbenzonitrile

Benzonitrile (12·9 g), diethyl ether (4·6 g) and anhydrous $AlCl_3$ (35·0 g) were combined in a pressure reactor and heated from 25° to 185° over a 1 hr period. The reaction mixture was poured over ice and subsequently extracted with Et_2O. 3-Ethylbenzonitrile was isolated and purified by gas chromatography on Carbowax 20M.

Acknowledgements—The authors acknowledge the advice of Drs. A. Eisner and E. S. Rothman and the technical assistance of Mr. W. J. Chamberlain, Miss E. D. Strange and Mr. Douglas H. Ragin.