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Methods for the Isolation and Characterization of Constituents of Natural Products

V. Separation of 2,6-Dinitrophenylhydrazone Pyruvamides into Classes and Resolution of the Individual Members

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INTRODUCTION

In part IV of this series, preparation of a homologous series of 2,6-dinitrophenylhydrazone pyruvamides of *n*-aliphatic primary amines and a partial series of symmetrical secondary aliphatic amines was described (*3*). A brief discussion of the potential utility of pyruvyl chloride 2,6-dinitrophenylhydrazone for derivatizing amines in lipids was presented. This entailed the mild conditions under which the derivatives are formed, and properties which they possess enable them to be isolated directly from lipids in a lipid-free condition. It was also mentioned that the pyruvamides could be separated into classes, that is, into primary and secondary amine derivatives. This paper describes the method for performing the class separation both quantitatively by column adsorption chromatography, and qualitatively by thin-layer adsorption chromatography. The rapid resolution of an homologous series of members of both classes by thin-layer partition chromatography is also described.

APPARATUS AND MATERIALS[1]

Magnesium oxide (catalog no. 2477) suitable for chromatographic use was obtained from the J. T. Baker Co., Phillipsburg, New Jersey. The

[1] Reference to certain products or companies does not imply an endorsement by the Department over others not mentioned.

powder had an adsorption index (Food and Drug yellow no. 4) of 12-13 and was used without further treatment. Celite 545 and Analytical Grade Celite were products of the Johns-Manville Co., Baltimore, Maryland. Chloroform, benzene and methanol were ACS grade; diethylamine (Baker) was redistilled; hexane was the high purity grade obtained from the Phillips Petroleum Co., Bartlesville, Oklahoma; heptane was a product of Eastman Kodak Co., Rochester, New York; silica gel G was obtained from Brinkmann Instruments, Westbury, New York.

The thin-layer chromatographic equipment was the same as previously described (*1*). A borosilicate glass column with a coarse fritted glass disc was employed for the column chromatography. The dimensions were 2.2 cm i.d. by 29 cm measured from the top of the column to the disc.

EXPERIMENTAL

Separation of 2,6-dinitrophenylhydrazone pyruvamides of primary and secondary amines into classes by column adsorption chromatography. One g of MgO and 9 g of Celite 545 are slurried in about 50 ml of $CHCl_3$ and the slurry poured through a long-stemmed funnel into the column. The slurry is packed under moderate air pressure until a few ml of $CHCl_3$ remain above the bed. The sides of the column are carefully washed down with a few ml of $CHCl_3$ and the washings are permitted to drain. The derivatives are quantitatively transferred to the column using a minimum of $CHCl_3$. After the last of the solution has just drained, the sides of the column are washed with a few ml of $CHCl_3$ and when this has percolated into the bed, a small wad of glass wool is placed just above the bed and development of the chromatogram is begun with 75 ml of $CHCl_3$. The secondary amine derivatives move off readily with this solvent as a greenish band. The primary amine derivatives remain near the top of the column as a violet band and are eluted with 10% methanol in $CHCl_3$.

Separation of 2,6-dinitrophenylhydrazone pyruvamides of primary and secondary amines into classes by thin-layer adsorption chromatography. Thirty g of silica gel G are slurried with 60 ml distilled water and spread over five 8 $\times$ 8 inch plates in the usual manner. The plates are left to dry overnight at room temperature and then activated for 1 hour at 100°C prior to use. The derivatives are spotted from benzene solution and the plate is developed for about 30 minutes with diethylamine: heptane (1:1). The secondary amines move as greenish spots, the primary amine derivatives as violet spots. Evaporation of the diethylamine from the finished plate gives spots with the original yellow color.

Separation of homologous series of primary and secondary pyruvamides by normal thin-layer partition chromatography. The methods for preparing the normal thin-layer partition plates is identical to that described for separating homologous series of 2,6-dinitrophenylhydrazone derivatives of pyruvic acid esters (*1*), except that 65 ml of absolute ethanol are used instead of 60 ml when preparing the slurry.

The derivatives are spotted from benzene solution and developed (in an equilibrated tank lined with filter paper) with hexane:benzene (7:3) saturated with polyethylene glycol 400. At the end of the development, the plate is removed, inspected and placed in a tank containing cotton wetted with diethylamine. This procedure produces violet spots which can be detected much more readily than the original yellow spots.

Separation of Primary Amine Derivatives by Reversed-Phase Thin-Layer Partition Chromatography. The reversed-phase thin-layer plates are prepared as previously described (*1*). The derivatives are spotted from

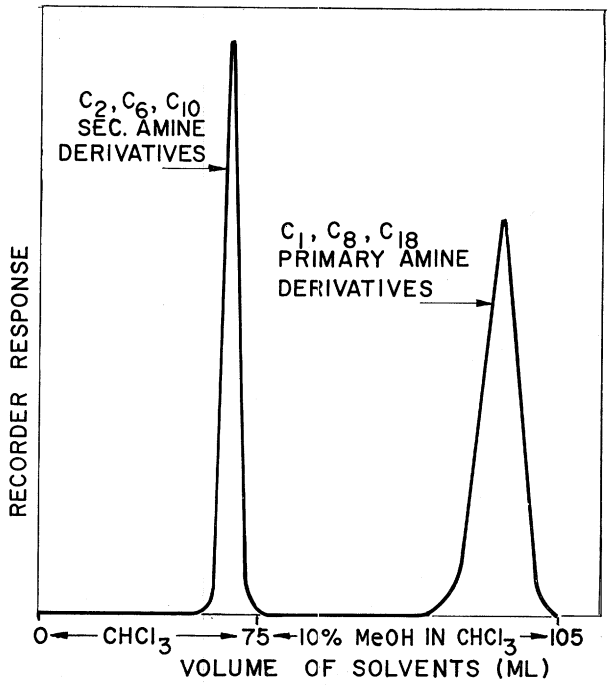

FIG. 1. Class separation of primary from secondary pyruvamide 2,6-dinitrophenylhydrazones by column absorption chromatography on magnesia-celite.

benzene solution at the origin of the plate. The top inch of support is scraped from the plate and the plate is placed scraped-side down in an equilibrated tank lined with filter paper. At the end of this time the plate is inverted and developed for approximately 1 hour in the solvent system acetonitrile:water (3:1) not previously saturated with the stationary phase (Nujol).

RESULTS AND DISCUSSION

Figure 1 shows the separation of primary and secondary amine derivatives achieved by adsorption chromatography on the magnesia-celite column. Approximately 0.2 μmole (each of the C_2, C_6 and C_{10}) secondary amine derivatives and 0.2 μmole (each of the C_1, C_8 and C_{18}) primary amine derivatives were selected for study since preliminary work indicated that the C_{10} secondary and C_{18} primary amine derivatives would be the most difficult pair to separate of the amine derivatives prepared in this laboratory. A 98.2% recovery of the secondary amine class and a 100.6%

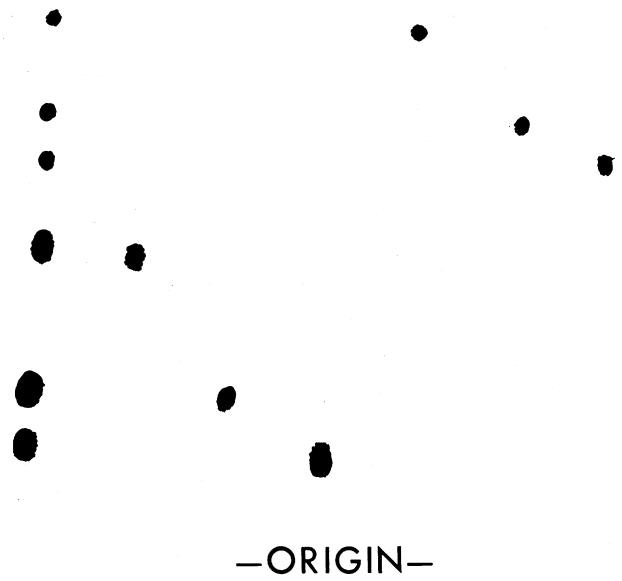

FIG. 2. Class separation of primary from secondary pyruvamide 2,6-dinitrophenylhydrazones by thin-layer adsorption chromatography on Silica Gel. G. Solvent system—diethylamine:heptane (1:1). Left to right: mixture of C_{18}, C_8, C_1 primary amine and C_{10}, C_6, C_2 secondary amine derivatives; then C_{18}, C_8, C_1 primary amine and C_{10}, C_6, C_2 secondary amine derivatives.

recovery of the primary amine class was achieved. The two classes are easily separated and readily distinguished by the differences in color of the derivatives on the adsorbent. The entire chromatographic separation takes approximately 30 minutes to complete. The data in Fig. 1 were obtained by monitoring the effluent from the column continuously using a 0.2 ml flow-through cell in an Hitachi-Perkin Elmer Model 139 spectrophotometer and recording the readings with a Honeywell-Brown 5 mv recorder.

Separation of the primary and secondary amine classes by thin-layer adsorption chromatography on silica Gel G is shown in Fig. 2. As with column adsorption chromatography, the secondary amine derivatives are

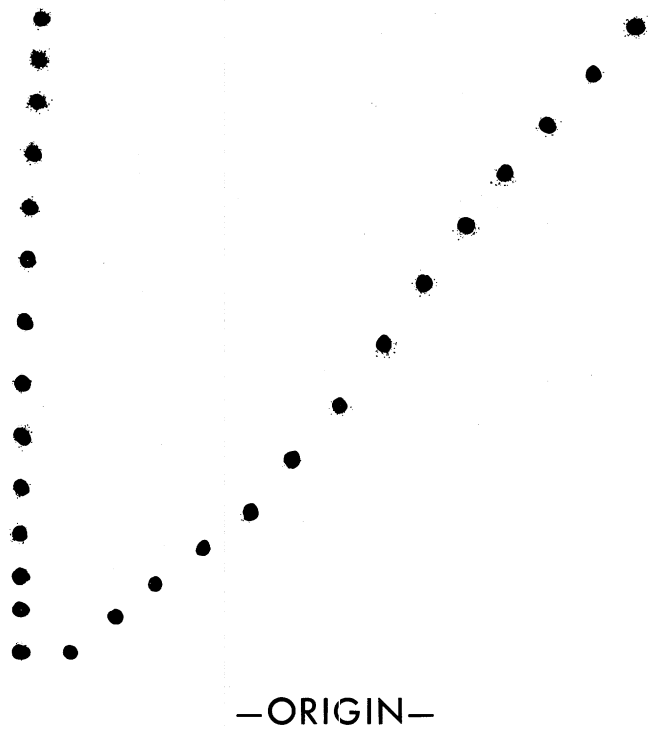

FIG. 3. Separation of primary amine pyruvamide 2,6-dinitrophenylhydrazones by normal partition chromatography. Support-Microcel T-38; stationary phase—polyethylene glycol 400; mobile phase—hexane:benzene (7:3) saturated with stationary phase. Diagonally from top to bottom C_{14} through C_1 derivatives. Column on left represents mixture of all 14 derivatives.

greenish and the primary amine derivatives are violet, even though a different adsorbent and solvent system are employed. The separation shown in Fig. 2 was achieved in about 30 minutes. The primary amine derivatives are detectable in the amount of 1.8×10^{-4} μmoles; the secondary amines in the amount of 5.4×10^{-4} μmoles.

Separation of the C_1 through C_{14} primary amine derivatives by normal thin-layer partition chromatography is depicted in Fig. 3. The C_{15} amine separates from the C_{14} but not from the C_{16} derivative and, therefore, amine derivatives above C_{14} are not included. It should be noted that the solvent system used to separate the amines is more polar than that used (*1*) to separate the alcohol derivatives. Approximately 1.5×10^{-4} μmoles of derivatives can be detected on the plate after exposure of the plate to diethylamine vapor.

Separation of the five secondary amine derivatives prepared in this laboratory is shown in Fig. 4. Although the secondary amine derivatives

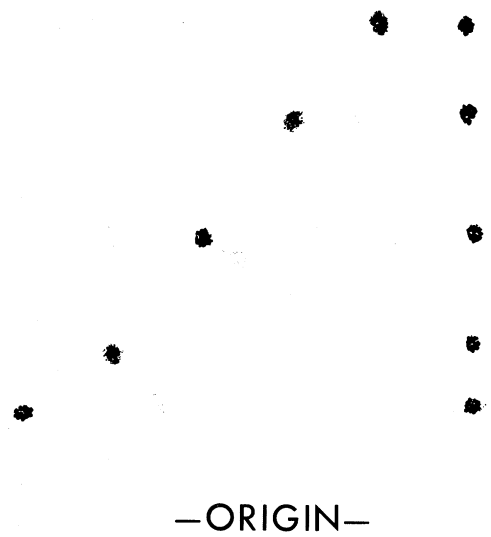

FIG. 4. Separation of secondary amine pyruvamide 2,6-dinitrophenylhydrazones by normal partition chromatography. Support-Microcel T-38; stationary phase—polyethylene glycol 400; mobile phase—hexane:benzene (7:3) saturated with stationary phase. Diagonally from top to bottom C_{10}, C_8, C_6, C_4, C_2 symmetrical secondary amine derivatives. Column on right represents mixture of all five amine derivatives.

higher than C_{10} were not prepared, it is likely that the C_{12} and C_{14} derivatives would also separate well if the primary amine separation is used as an analogy.

Figure 5 shows the separation of the higher primary amine derivatives by reversed-phase thin-layer chromatography. The C_{18} through the C_{11} derivatives are well separated. The C_{10} derivative separates from the C_{11} but does not separate clearly from the C_9 and is, therefore, not shown. Approximately 2×10^{-4} μmoles can be detected after exposure of the plate to diethylamine vapor. No reversed-phase partition chromatography was carried out on the secondary amine derivatives since the C_{10} derivative was the longest chain derivative prepared.

Class separation of the amine derivatives should facilitate the analysis of complex mixtures of amines isolated from biological material. Moreover, since in the class separation of alcohol derivatives (described in part III of this series (*2*) no greenish bands are produced on the magnesia, the

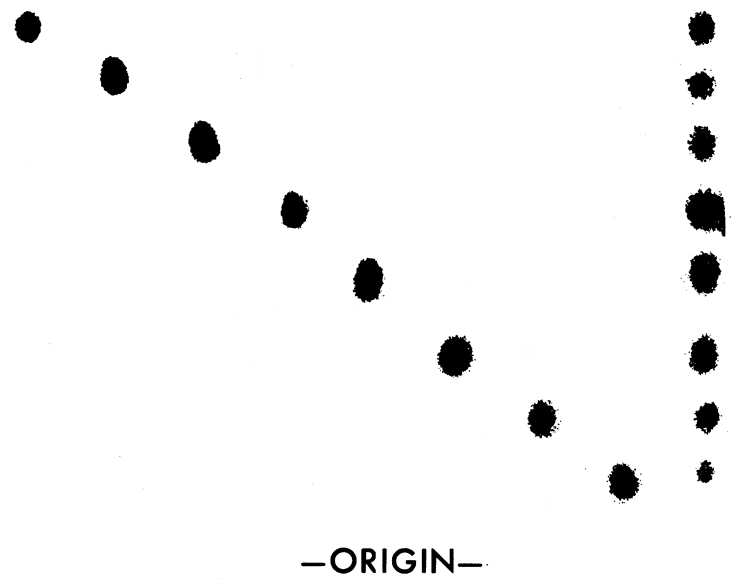

FIG. 5. Separation of primary amine 2,6-dinitrophenylhydrazones by reversed-phase partition chromatography. Support-Microcel T-38; stationary phase—mineral oil; mobile phase—acetonitrile:H_2O (3:1). Diagonally from top to bottom C_{11} through C_{18} primary amine derivatives. Column at right represents mixture of the 8 derivatives.

appearance of a greenish band in a mixture of unknown derivatives may indicate the presence of secondary amine derivatives. Work is in progress to attempt to class separate the alcohol derivatives from the amine derivatives.

SUMMARY

Class separation of the 2,6-dinitrophenylhydrazone derivatives of *n*-aliphatic primary and symmetrical secondary aliphatic amines is described. Both quantitative column adsorption and qualitative thin-layer adsorption chromatographic procedures are presented. In both procedures the classes are cleanly separated and they can also be distinguished readily by color; the secondary amine derivatives are green and the primary amine derivatives are violet during chromatography. Approximately 0.2 μmole of a derivative is easily seen in the column procedure whereas 1.8×10^{-4} μmole of a primary derivative and 5.4×10^{-4} μmole of a secondary amine derivative can be followed in the thin-layer procedure.

Methods for the separation of a homologous series of the primary amine derivatives and a partial series of secondary amine derivatives by thin-layer partition chromatography is also described. Approximately 1.5×10^{-4} μmoles of derivative can be utilized in this procedure. Reversed-phase separation of the higher primary amine derivatives by thin-layer chromatography is also presented.

REFERENCES

1. Schwartz, D. P., and Brewington, C. R., Methods for the isolation and characterization of constituents of natural products. II. Separation of homologous series of esters of pyruvic acid 2,6-dinitrophenylhydrazone by thin-layer chromatography. *Microchem. J.* **12**, 1-6 (1967).
2. Schwartz, D. P. Brewingon, C. R. and Shamey, Jennie. Methods for the isolation and characterization of constituents of natural products. III. Separation of alcohol esters of pyruvic acid 2,6-dinitrophenylhydrazone by column and thin-layer chromatography. *Microchem J.*, **12**, 186-191 (1967).
3. Schwartz, D. P., and Brewington, C. R. Methods for the isolation and characterization of constituents of natural products. IV. Amide derivatives of amines with pyruvyl chloride 2,6-dinitrophenylhydrazone. *Microchem. J.* **12**, 192-195 (1967).