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DETERMINATION OF HYDROXYBIPHENYLS AS DANSYL DERIVATIVES*

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SUMMARY

The reaction conditions for the fluorigenic labeling of mono- and dihydroxybiphenyls with dansyl chloride were investigated. The hydroxybiphenyls were made to react in an acetone–water solution with a 10-fold excess of dansyl chloride, buffered with sodium carbonate at about pH 11. The reaction was carried out at 45° for 15 min. After hydrolysis of the excess of dansyl chloride and subsequent extraction with *n*-hexane, an aliquot of the reaction mixture was spotted on to a thin-layer plate. The separated derivatives were identified by their R_F values as well as by their mass and fluorescence spectra. The yellow fluorescent derivatives can be analyzed with a relative standard deviation of 4–5 %. Detection limits are in the low nanogram region and linear calibration curves between 5 and 500 ng are observed. As an application of this method, 4-hydroxybiphenyl, which was isolated from the urine of rats treated with biphenyl, was derivatized with dansyl chloride and its identity confirmed by thin-layer chromatography and fluorescence and mass spectrometry.

INTRODUCTION

Biphenyl and *o*-phenylphenol are used as fungicides for the protection of citrus fruits during storage. Some work has been reported on the metabolism of these compounds[1–5]. The isolated metabolites were shown to be mainly 2- and 4-monohydroxybiphenyls as well as 3,4-, 2,5- and 4,4'-dihydroxybiphenyl derivatives.

The need for the development of suitable analytical methods for determining these metabolites became evident and several approaches have been made to solve this problem. Qualitative and semi-quantitative techniques in connection with thin-layer chromatography (TLC) and chromogenic sprays have been suggested by a number of workers [4–7]. Others have used gas chromatography for the investigation of hydroxybiphenyls[8]. UV absorption studies were reported by BERNINGER *et al.*[5] and HARVEY AND PENKETH[9]. The native fluorescence of these compounds has also been investigated[4,10].

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EXPERIMENTAL

Reagents

Analytical-reagent grade dansyl chloride (1-dimethylaminonaphthalene-5-sulfonyl chloride; Aldrich Chemical Co.) was dissolved in acetone to form a 0.1 % solution.

The hydroxybiphenyls tested were 2-hydroxybiphenyl and 4,4′-dihydroxybiphenyl (Eastmann Organic Chemicals) and 4-hydroxybiphenyl and 2,2′-dihydroxybiphenyl (Matheson Coleman and Aldrich Chemical Co.). Solutions of these compounds were prepared in acetone at $1 \cdot 10^{-3}$ molar concentration. The buffer consisted of a 0.1 *M* solution of sodium carbonate in distilled water. The concentration of the sodium hydroxide solution was 1 *N*. A mixture of triethanolamine and 2-propanol in the proportions 20:80 (v/v) was used as a spray solution. The *n*-hexane for extraction and the chromatographic solvents were of reagent grade.

Reaction conditions

Ten microlitres of the hydroxybiphenyl solutions were placed into 2-ml glass-stoppered test-tubes with a 10-μl syringe, then 200 μl of the dansyl chloride solution were added, followed by 30 μl of the buffer. The tubes were sealed and after briefly mixing their contents on a Fisher mini-shaker they were placed in a temperature-controlled water-bath at 43–45°. After a reaction time of 15–20 min, the tubes were removed and cooled to room temperature. Two drops of 1 *N* sodium hydroxide solution were added followed by rapid mixing in order to hydrolyze the excess of dansyl chloride. The dansyl derivative formed was then extracted with 500 μl of redistilled *n*-hexane by whirling the mixture on a mini-shaker. After separation into two layers, 10 μl of the hexane layer were spotted on to a thin-layer plate.

Chromatography

The chromatography was performed by the ascending technique at room temperature in the dark on Merck silica gel plates. The solvent systems were (1) benzene–chloroform (1:1) and (2) *n*-hexane–acetone (7:3). After separation, the plates were dried in a cold stream of air and sprayed with the triethanolamine solution. After spraying, the plates were dried in the dark with cold air for 10 min.

Instrumental analysis

The chromatoplates were evaluated with the Zeiss PMQ II Chromatogram Spectrometer in the fluorescence mode. A mercury lamp, in conjunction with the 365-nm filter, was used as the excitation UV light source. The emission monochromator slit width was set at 0.5 mm for all measurements (except the recording of the spectra). A Honeywell Electronik 194 strip-chart recorder was used. All peaks were measured with a Gelman planimeter.

Mass spectrometric studies

The mass spectra of the dansyl derivatives were obtained with a Du Pont/CEC 21-491 instrument, using the standard probe for direct introduction of the sample into the ion source. The high-resolution measurements were obtained on a Du Pont/CEC 21-110B double-focusing instrument using the peak matching technique.

Synthesis

Dansyl chloride and the hydroxybiphenyls were made to react together in equimolar concentrations in acetone. Solid sodium carbonate was added to the mixture to saturation and the mixture was then stirred at room temperature. After a reaction time of about 2 h, excess 0.1 *N* sodium hydroxide was added. The solution was then extracted twice with an equal volume of ethyl acetate and the combined extracts were washed and then dried with sodium sulfate. After evaporation to dryness *in vacuo*, the green–yellow residue was dissolved in warm aqueous ethanol (95 %) and crystallized from the solution. The synthesized derivatives were run on preparative TLC plates and the silica carrying the pure derivative was removed and eluted with ethyl acetate. Derivatives were recrystallized. Mass spectra of all of the derivatives were recorded and compared with the spectra of products obtained under analytical reaction conditions.

RESULTS AND DISCUSSION

Reaction conditions

The dansylation reaction is usually performed in a mixture of water and acetone at a pH of 9–11. This method proved to be favourable with regard to the competitive kinetic rates of the labeling reaction and the hydrolysis of the dansyl chloride[11–13]. The actual ratio of water (buffer) to acetone in the reaction mixture was found to be not critical and could be chosen according to convenience (see Fig. 1). Similar results were reported by SEILER AND WIECHMANN[13]. It was therefore decided to use 200 μl of acetone (dansyl chloride solution) and 30 μl of water (buffer solution) for further work. A pH of the reaction mixture of about 11 was obtained by using 0.1 *M* sodium bicarbonate solution as buffer. The concentration of the labeling reagent was also found to be not critical. Under the conditions used, a 7–10-fold excess seemed to be satisfactory. The addition of the reagent in stoichiometric amounts, however, was not sufficient, owing to partial hydrolysis by the buffer. Some of the reagent is also consumed as a result of the formation of side products[13]. The reaction proceeds faster at elevated temperature; at 45° the reaction was completed within 15–20 min with no

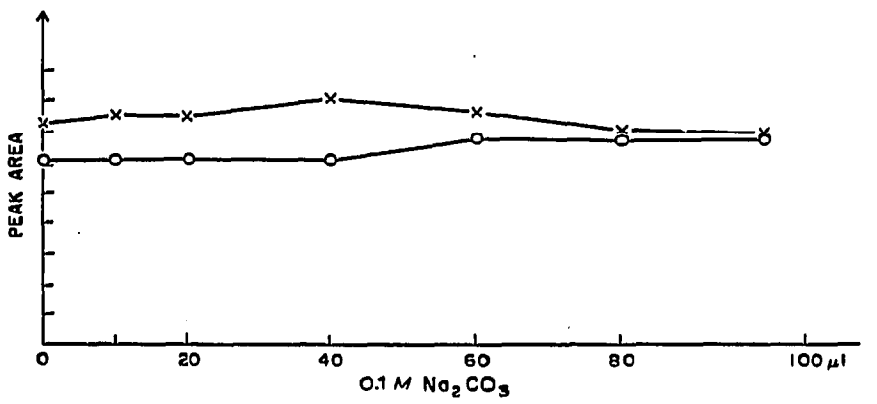

Fig. 1. Influence of reaction conditions on fluorescence intensity of: O—O, 4-hydroxybiphenyl-dansyl derivative; ×—×, 2-hydroxybiphenyl-dansyl derivative.

further derivative formation.This temperature was also optimal with regard to evaporation of the solvent, uniformity of reaction conditions in the different tubes and analysis time. The reproducibility of the reaction at 45° was also found to be best with a reaction time of approx. 15 min (relative standard deviation: 15 min, 5.4 %; 30 min, 8.9 %; 60 min, 8.5 %). After the dansylation reaction is complete, excess of dansyl chloride needs to be hydrolyzed to dansylsulfonic acid so as to prevent hydrolysis on the chromatoplate, which results in highly fluorescent blue streaks. The addition of two drops of 1 *N* sodium hydroxide to the 230 μl of reaction mixture following the 15-min reaction time did not affect the dansyl derivative. The hydrolysis of the dansyl chloride occurred immediately and was visible from the decoloration of the reaction mixture. The resulting dansylsulfonic acid did not interfere in the extraction with *n*-hexane.

Chromatography

For the development of a good fluorigenic labeling technique, an efficient chromatographic separation procedure is essential[14]. Some preliminary chromatographic data are reported in Table I. A good separation between the two monohydroxy isomer derivatives and also the dihydroxy isomer derivatives was obtained with solvent system I (benzene–chloroform, 1:1). With this solvent system, the R_F values are relatively poorly reproducible, but the separation is always satisfactory. The separation time is approx. 30 min. Solvent system II (acetone–hexane, 3:7), while giving improved reproducibility and a shorter development time, separates neither the two monohydroxy nor the two dihydroxy isomers adequately.

TABLE I

CHROMATOGRAPHY OF HYDROXYBIPHENYL-DANSYL DERIVATIVES

Dansyl derivative	R_F *values*[a] *in solvent*[b]	
	I	*II*
4-Hydroxybiphenyl	0.58	0.62
2-Hydroxybiphenyl	0.47	0.62
4,4'-Dihydroxybiphenyl	0.20	0.48
2,2'-Dihydroxybiphenyl	0.10	0.45

[a] Mean values from 4 plates.
[b] I = Benzene–chloroform (1:1); II = acetone–hexane (3:7).

As mentioned previously, some side products may occasionally appear on the plate. They can be formed under the recommended reaction conditions and are yellow fluorescent. It is advisable to run a blank for comparison. The developed and dried chromatoplates were sprayed with triethanolamine in isopropanol, as recommended by SEILER AND WIECHMANN[11,13]. The effect of spray reagents on the fluorescence characteristics as well as intensity of the phenolic dansyl derivatives has been discussed by LAWRENCE AND FREI[15] and was found to be analogous to this study. Triethanolamine proved to be the most useful spray reagent because of an increase in and stabilization of fluorescence.

Fluorescence of the derivatives

The fluorescence characteristics of the hydroxybiphenyl-dansyl derivatives are shown in Table II and Figs. 2 and 3.

When sprayed with the triethanolamine–isopropanol spray reagent, a hypsochromic shift was observed for the emission band on the Zeiss Chromatogram Spectrophotometer (Fig. 2). This shift was not reported for spectra recorded with the Farrand or Aminco-Bowman instrument when monochromatic excitation radiation was used[15]. This shift can be attributed to the broad excitation band (filter with band pass 320–400 mm) used in the Zeiss instrument and to the longer exposure time during the measuring process. A decomposition and corresponding shift in maxima upon prolonged irradiation with UV light from the Zeiss instrument has also been reported for some phenol derivatives by LAWRENCE AND FREI[15]. Prolonged UV irradiation also results in a decrease in fluorescence intensity for the derivatives, as confirmed by LAWRENCE AND FREI for most of their phenolic dansyl derivatives. As a consequence,

TABLE II

FLUORESCENCE CHARACTERISTICS OF SOME HYDROXYBIPHENYL-DANSYL DERIVATIVES

Dansyl derivative	*Farrand*[a]		*Zeiss*[b]	
	Before spraying (nm)	*After spraying (nm)*	*Before spraying (nm)*	*After spraying (nm)*
2-Hydroxybiphenyl	350/520	360/520	530	500
2,2'-Dihydroxybiphenyl	350/520	360/520	535	510
4-Hydroxybiphenyl			520	490
4,4'-Dihydroxybiphenyl			530	480

[a] Excitation filter 7-54, aperture reducer 625, emission filter 3-73.
[b] Excitation filter 365 nm (band pass 320–400 nm).

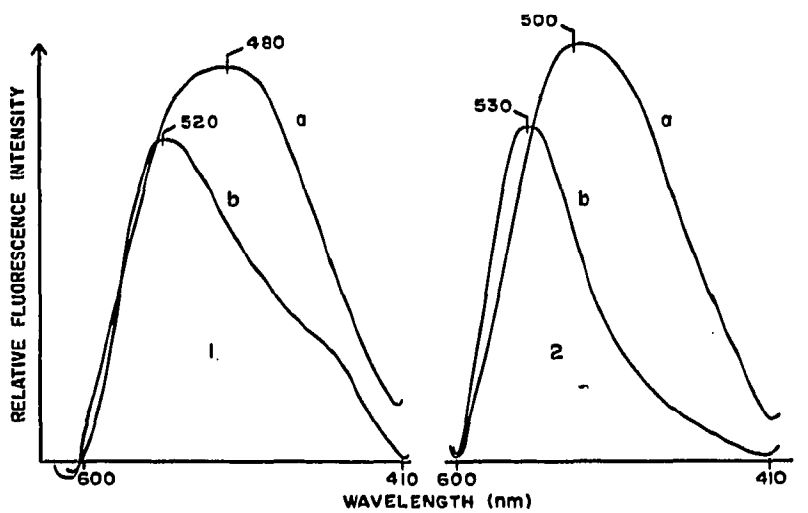

Fig. 2. Emission spectra (Zeiss instrument) of the dansyl derivatives of (1) 4-hydroxybiphenyl and (2) 2-hydroxybiphenyl: (a) after spraying in each instance; (b) before spraying.

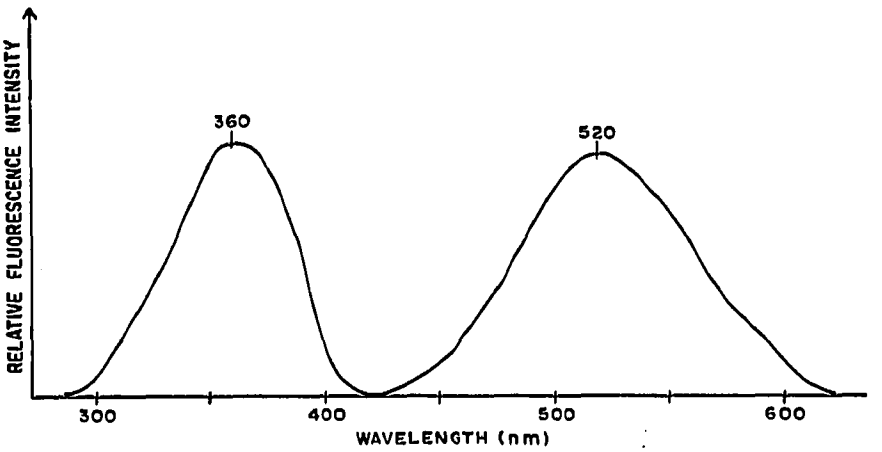

Fig. 3. Fluorescence excitation–emission spectrum of the dansyl derivative of 2,2'-dihydroxybiphenyl (Farrand instrument).

the plates should be handled in the dark and scanning times kept short. Quantitative analysis was possible, however, when these precautions were taken.

In general, treatment of the spots with the spray prior to measurement resulted in enhanced (Fig. 2) and stabilized fluorescence. A typical excitation–emission spectrum of a sprayed dihydroxy derivative recorded with the Farrand UV–visible chromatogram scanner is shown in Fig. 3. The dansyl derivatives of the hydroxybiphenyls were found to be stable when heated at 100° for 20 min after chromatography and before spraying. The fluorescence spectra were not affected by heating.

Identification of the derivatives

The investigation of the chemical nature of the derivatives was carried out by mass spectrometry. Mass spectrometric identification of the dansyl derivatives of biogenic amines has been reported by several workers[16–18]. Recently, mass spectrometry of dansyl amino acids was investigated by SEILER *et al.*[19]. The mass spectra of dansyl derivatives show characteristic major fragments at *m/e* 170 and 171. The occurrence of these major fragments indicates the ionization of a dansyl derivative[16]. In this study (Figs. 4 and 5), those peaks were also found to be the most intensive. In addition, peaks at *m/e* 154, 155, 141, 127, 128 and 115 demonstrate the further

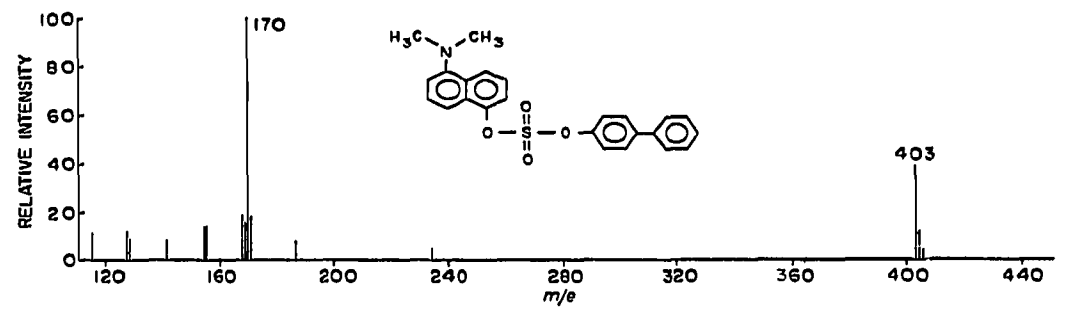

Fig. 4. Mass spectrum (70 eV) of the dansyl derivative of 4-hydroxybiphenyl.

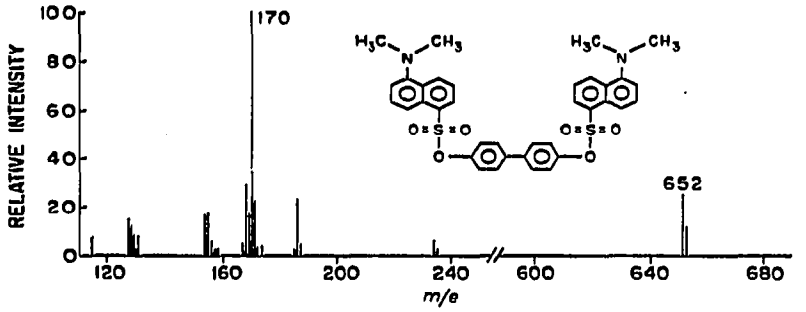

Fig. 5. Mass spectrum (70 eV) of the bis-dansyl derivative of 4,4'-dihydroxybiphenyl.

fragmentation of the dansyl moiety. The molecular ions occur in all spectra, with masses corresponding to their calculated molecular weights. No significant difference was to be found in the fragmentation patterns of the two isomer groups. Final confirmation was obtained by comparing the mass spectra, fluorescence spectra and R_F values for the chromatographed analytical spots with those for the synthetic dansyl derivatives of the same hydroxybiphenyls (see also EXPERIMENTAL).

Reproducibility, detection limits and calibration curves

The reproducibility of the method was determined by simultaneously performing the labeling reaction on six hydroxybiphenyl samples of equal concentration. Aliquots of 10 μl of each sample were then spotted for separation and analysis. The best results were obtained after a reaction time of 15 min. The relative standard deviations did not vary much with concentrations of the derivative and agreed well with the values (4–5 %) reported by FREI AND LAWRENCE[20]. The linearity of the calibration plots (5–500 ng) and the detection limits (*ca.* 2 ng per spot) were very close to those obtained by previously published methods[20].

When the "hydroxybiphenyl fraction" of urine from rats treated with biphenyl (*cf.*, ref. 21) was allowed to react with dansyl chloride as described above, spots were obtained on thin-layer chromatograms which had R_F values and fluorescence and mass spectra identical with those of the authentic dansyl derivative of 4-hydroxybiphenyl. The molecular composition of the isolated dansyl derivative was further confirmed by high-resolution mass measurements (calculated for $C_{24}H_{21}NO_3S$, 403.1242; found, 403.1253).

CONCLUSION

Mono- and dihydroxybiphenyls have been shown to react easily under suitably chosen conditions with 1-dimethylaminonaphthalene-5-sulfonyl chloride. They form highly fluorescent and reasonably stable derivatives that are separated chromatographically from the reaction mixture. Differentiation between the two mono- and between the two dihydroxy isomers is possible. *In situ* fluorimetry of these derivatives permits the determination of hydroxybiphenyls at the nanogram level. The method should be particularly useful for the determination of these compounds in biological samples, as the labeling process can serve as a purification step.

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