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PRE-CHROMATOGRAPHIC DERIVATIZATION OF PRIMARY AND SECONDARY AMINES WITH A POLYMERIC ANHYDRIDE FOR IMPROVED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETECTION

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SUMMARY

A polymeric anhydride containing *o*-acetylsalicyl as the labelling moiety was utilized as a derivatization reagent in conjunction with high-performance liquid chromatography for primary and secondary amines. The derivatization reactions were performed off-line, before the chromatographic separation. Standards were prepared, characterized by melting point, UV, IR, NMR, mass spectrometry, and elemental analysis, and these were then used as external standards to determine the percent reaction. The derivatives are easily chromatographed on a reversed-phase high-performance liquid chromatographic system and can be monitored by a UV detector at 196 nm, or an electrochemical detector in the oxidative mode, with or without post-column photolysis. No pH suppression of the eluent was needed. The minimum detection limits of underivatized and derivatized amines were determined. There was a decrease of 3 to 4 orders of magnitude in minimum detection limits as a result of these off-line derivatizations. The minimum amount of amine that can be detected through derivatization is also reported.

INTRODUCTION

The determination of small amounts of aliphatic amines is a commonly confronted problem in organic analysis. Gas chromatographic determination of these amines at low concentrations is limited by adsorption and decomposition in the column, ghosting phenomena, tailed elution peaks, and low detector sensitivity. A common method of overcoming these limitations is to convert amines into a derivative that has a selective sensitivity increase using electron-capture or flame ionization detection. Several derivatization reagents, such as flophemesyl chloride[1], pentafluorobenzoyl chloride[2], benzenesulfonyl chloride[3], and dimethylthiophosphinic chloride[4], have been used for this purpose.

Amines have also proved difficult to handle in liquid chromatography (LC)

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for several reasons: 1) low UV detection sensitivities; 2) variable ionization during separation requiring pH suppression which is detrimental to silica based stationary phases; and 3) strong interaction with many LC supports[5]. However, these compounds are easily derivatized to products that are strongly UV absorbing[6–8], fluorescent[9], or electrochemically active[10,11]. The resulting derivatives are usually chromatographed with minimal difficulty and have better detectabilities. Today, most of the derivatizations performed for high-performance liquid chromatography (HPLC) involve the use of homogeneous reactions in which the reagent is present in solution and is mixed with a solution of the substrate of interest[9–11]. Recently, there has been some work involving the use of solid phase reagents for derivatizations to be used in conjunction with HPLC[12–16]. Several advantages of using solid phase reagents for derivatization have been discussed[12–14].

Anhydrides are commonly used as acylating reagents for amines, and the highly reactive anhydride function can be immobilized on insoluble polystyrene. This results in a polymer capable of acylating simple aliphatic amines. Digenis reported the preparation and reactivity of polymeric activated anhydrides containing incorporated labels[17–19]. All of this work dealt with the use of such polymeric reagents for synthetic organic purposes, such as a multi-step preparation of penicillin derivatives and analogs. However, none of this work involved any HPLC approaches, and there was no suggestion at that time that such polymeric reagents might prove useful for either off-line or on-line derivatizations related to HPLC applications. In our preliminary studies, we have chosen the polymeric anhydride containing *o*-acetylsalicyl as the labelling moiety, since this group provides high UV absorptivity, electrochemical activity, and activates the nucleophilic substitution reaction compared to other labels, such as phenyl, benzyl, and thienyl[18].

We have pursued these reactions off-line, using this polymeric anhydride as the derivatization reagent. We have investigated the reactivities of various primary and secondary amines with this polymeric anhydride, and have been able to show that with mild, off-line conditions (60°C, 20 min) it is possible to obtain up to 96% of the expected derivative. This polymeric anhydride has advantages similar to other supported reagents[20] including: 1) enhanced stability of anhydrides after immobilization on the polystyrene; 2) simplicity of operation; derivatizations are performed on the polymeric anhydride in reaction pipettes under mild conditions; no preparation of the reagent solution was needed; 3) reactions on the polymeric support often are more selective and give fewer side products; and 4) reactions not possible in solution because of lack of solubility of one or more of the reagents can be carried out in high effective reagent concentrations on a polymeric support. The percent reactions have been optimized with regard to time, temperature and solvent. Minimum detection limits (MDLs) of the derivatives and underivatized amines have been determined by LC–UV, liquid chromatography with oxidative electrochemical detection (LC–ED), and oxidative ED after post-column photolysis (LC–*hv*–ED). There was a decrease of 3 to 4 orders of magnitude in MDLs as a result of these off-line derivatizations.

EXPERIMENTAL

Reagents

Chloromethylated polystyrene (4.2 meq/g) used for the preparation of the po-

lymeric anhydride was obtained from Bio-Rad Labs (Richmond, CA, U.S.A.). The chemicals used throughout this study were obtained from a variety of commercial suppliers including: Aldrich (Milwaukee, WI, U.S.A.), Alfa Products (Danvers, MA, U.S.A.), J. T. Baker (Phillipsburg, NJ, U.S.A.), MCB Manufacturing Chemists (Gibbstown, NJ, U.S.A.), Fisher Scientific (Fair Lawn, NJ, U.S.A.), VWR Scientific (San Francisco, CA, U.S.A.), Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). These chemicals were of the highest purity commercially available when necessary, and were used as received without further purification.

HPLC solvents were obtained from MCB Manufacturing Chemists, as their Omnisolv brand HPLC solvent. All solvents were used as received after filtered through a 0.45-μm solvent filtration kit/filter (Millipore, Bedford, MA, U.S.A.) and degassed with stirring under vacuum.

Apparatus

The HPLC system consisted of a Waters 6000A solvent delivery system/pump, a Waters U6K syringe loading injection valve (Waters Chromatography Division, Millipore, Milford, MA, U.S.A.) and an SE 120 dual pen recorder (Brown, Boveri, Metrawatt/Goerz Division, Vienna, Austria). Chromatographic columns used consisted of a Waters μBondapak (TM) C_{18} reversed-phase column, 30 cm × 7.8 mm I.D. (semipreparative) and an 8 mm or 5 mm I.D. Radial-Pak Resolve(TM) C_{18} column used in a RCM-100 Radial Compression Module. The detectors used consisted of a Waters Model 480 variable-wavelength UV detector (Waters), a Bioanalytical Systems (BAS) (West Lafayette, IN, U.S.A.) Model LC-4A amperometric electrochemical detector, glassy carbon working electrode, Ag/AgCl reference electrode, stainless-steel counter electrode. At times the BAS electrochemical detector was used in conjunction with a post-column photolysis unit. The photolysis apparatus was a Photronix Model 816 UV batch irradiator (Medway, MA, U.S.A.). The configuration of a photolytic LC–ED system has been described[21].

The instrumentation used to characterize the *o*-acetylsalicylamide derivatives consisted of a Varian T-60 NMR spectrometer (Palo Alto, CA, U.S.A.), a Perkin-Elmer 599B Infrared Spectrophotometer (Perkin-Elmer, Norwalk, CT, U.S.A.), a Thomas Hoover capillary melting point apparatus (Arthur H. Thomas, Philadelphia, PA, U.S.A.), a Perkin-Elmer Lambda 3B UV/VIS Spectrophotometer equipped with the PE data station, and a Finnigan 4000 mass spectrometer (Sunnyvale, CA, U.S.A.) or a Nuclide magnetic sector mass spectrometer (State College, PA, U.S.A.).

Preparation and characterization of the polymeric anhydride containing o-acetylsalicyl as the labelling moiety

The polymeric anhydride I (Fig. 1a) was prepared as previously described by Digenis[18], with an exception that trichloromethylchloroformate was substituted for phosgene, and the loading of the starting polymer was 4.2 mequiv. Cl/g instead of 1.8 mequiv. Cl/g. The loading was determined by saponification of the polymeric reagent and quantitation of the amount of salicylic acid formed by this hydrolysis reaction. The quantitation was performed using an external standard of salicylic acid by HPLC–UV. We were able to obtain reagents having upwards of 1.28 mequiv./g polymer which corresponds to *ca.* 30.4% of maximum loading based on the loading of Cl originally present in the chloromethylated polystyrene.

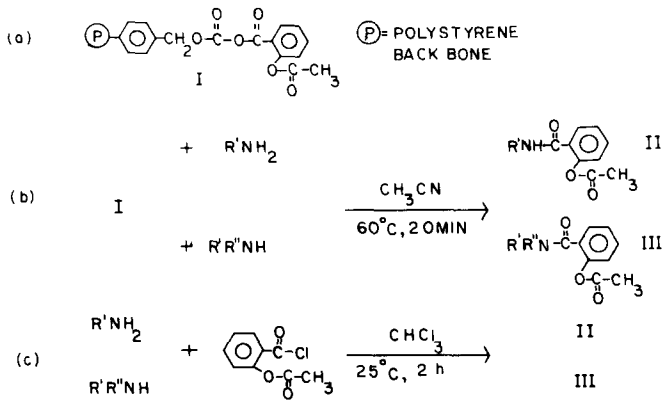

Fig. 1. (a) Structure of polymeric anhydride containing *o*-acetylsalicyl as the labelled moiety; (b) derivatization reactions of primary and secondary amines with polymeric anhydride; (c) preparation of authentic standards of *o*-acetylsalicylamides.

Preparation, isolation and characterization of o-acetylsalicylamides

Standards of I and II (Fig. 1b) have been prepared as described below and shown in Fig. 1c. These were characterized by UV, IR, NMR, mass spectrometry (MS), and elemental analysis, and were then used as external standards to determine percent reaction.

A sample of 30 mmol of *o*-acetylsalicyloyl chloride was dissolved in 10 ml of chloroform in a round-bottomed flask. A 50% molar excess of the amine (*n*-propylamine, *n*-butylamine, nonylamine, diethylamine, or morpholine) was added dropwise, with stirring, and this reacted for 2 h at room temperature. The reaction mixture was washed twice with 10 ml of 15% hydrochloric acid. The chloroform layer was removed by roto-evaporation. The reaction mixture was dissolved in acetonitrile, filtered and subjected to semipreparative HPLC using a μBondapak C_{18} column (30 cm × 7.8 mm I.D.). The *o*-acetylsalicylamides were collected and rotoevaporated to dryness leaving the pure compound.

Reaction procedure

Reactions were carried out in laboratory-made reaction pipettes. These were made by cutting a disposable pipette to a total length of *ca.* 5 cm and plugging the narrow end with a small amount of glass wool. The narrow end was then plugged with a plastic column cap, and the polymeric anhydride was added. For reactions using this polymeric reagent, the exact amount need not be weighed each time, but after the solvent containing the substrate is added, there should be some dry reagent still present (all of the liquid should be absorbed by the reaction bed; the amount used was 0.1–0.2 g). The top of the pipette was then sealed with Parafilm, and the tube was then heated by a water bath at the optimized temperature for a optimized time (60°C, 20 min). The reaction mixture was washed into a volumetric flask with acetonitrile to be used for the HPLC analysis, the mixture was filtered, and injected into the HPLC for quantitation of the derivatization.

Off-line derivatizations of primary and secondary amines

To determine percent derivatizations of the amines with this reagent, 100 μl of amine solutions (0.0066 *M*) were added to 0.1 g of the polymeric anhydride contained in a reaction pipette. Here, and throughout this paper, the percent derivatization (or percent reaction) will be referred to as the percentage of the original substrate converted into the labelled derivative, not the percentage of the original compound that is absent at the completion of the reaction time. After the amine was added, the pipettes were heated by a water bath at 60°C for 20 min. The reaction mixtures were washed into a 2-ml or 1-ml volumetric flask with acetonitrile, and 25 μl of this solution were injected into the chromatograph. Derivatization reactions for each amine were repeated three times. At least three injections were made for each sample solution. The derivatization reaction is shown in Fig. 1b.

To illustrate the increase in detectability that occured following derivatization, a 200-μl solution of butylamine (482 ppm) was derivatized under the above conditions (Figs. 2 and 3).

To show the separation of the derivatives of an amine mixture, 100 μl of a mixture of four amines (0.132 μmol each) were added to the polymeric anhydride in a reaction pipette and heated at 60°C for 20 min. The reaction mixture was washed into a 1-ml volumetric flask with acetonitrile, and 10 μl of the reaction mixture were injected into the chromatograph (Fig. 4).

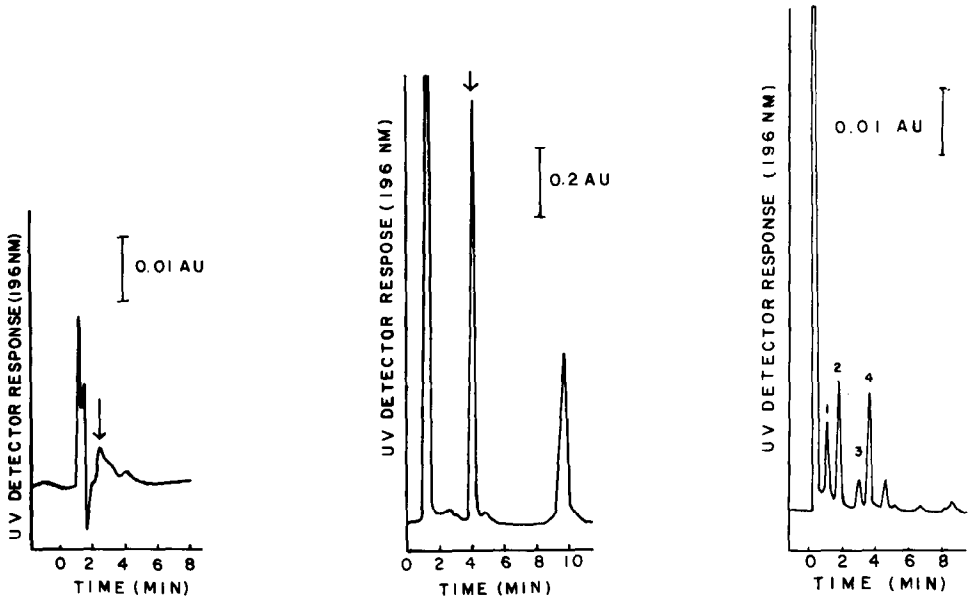

Fig. 2. The arrow shows 200-μl injection of 482 ppm butylamine without derivatization. Radial-Pak C_{18} column, 10 cm × 8 mm I.D., 5 μm; mobile phase, acetonitrile–water (50:50) flow-rate, 1.5 ml/min.

Fig. 3. The arrow shows the derivative peak of 482 ppm butylamine. The chromatographic conditions are as in Fig. 2.

Fig. 4. Liquid chromatogram of a mixture of four amines after derivatization at 60°C for 20 min with polymeric anhydride. Each amine is equimolar (0.132 μmol). Radial-Pak C_{18} column, 10 cm × 5 mm I.D., 5 μm; mobile phase acetonitrile–water (30:70) flow-rate, 1.5 ml/mins. Peaks: 1 = morpholine; 2 = propylamine; 3 = diethylamine; 4 = butylamine.

To experimentally determine the minimum amount of amine that can be derivatized, a 200-μl solution of butylamine (100 ppb*) was derivatized; the reaction mixture was washed to 200 μl with acetonitrile, and 25 μl of this solution were injected into the chromatograph.

Determination of minimum detection limits

Comparison of MDLs using different instruments can be difficult to present in a fair way. In this report, all MDLs were normalized to a signal-to-noise (S/N) ratio of 2:1 for a 200-μl injection.

RESULTS AND DISCUSSION

Molar absorptivity of the amines and their labelled derivatives

All the UV spectra show maximum absorbance at *ca.* 196 nm using acetonitrile as solvent. The molar absorptivities of the derivatives at 196 nm are in the range of 24 500–28 400 M^{-1} cm^{-1} which are *ca.* ten times higher than that at 254 nm, and therefore the UV detector was set to 196 nm throughout these studies. The molar absorptivity of the labelled derivatives at 196 nm are *ca.* 3 orders of magnitude higher than that of the underivatized amines (10–41 M^{-1} cm^{-1}). For complex matrices, there is a possibility that interferents may elute at a similar k' to the derivative at 196 nm. In this case, better selectivity can be achieved at 254 nm.

Optimization of solvent, temperature and time

One of the initial experiments was to determine which solvent would be the best for this type of derivatization. Optimal reaction conditions for the formation of the amine derivative were determined by comparison of peak heights of the derivative as a function of solvent. All other reaction conditions were held constant. Solvents tested included water, methanol, acetonitrile, ethyl acetate, dioxane and hexane. Of all the solvents, acetonitrile showed the best reactivity followed by dioxane, hexane, water, ethyl acetate and methanol. In addition to the better reactivity in acetonitrile, there also were less interference peaks in the acetonitrile blank.

After acetonitrile was chosen as the best solvent, the temperature was optimized, followed by time. Temperatures were varied from 30°C to 80°C holding time constant at 20 min. The optimum temperature of 60°C was then held constant as time was varied from 5 to 40 min. After 20 min, the derivative's peak height increased very slowly with increasing time, and further derivatizations were then performed at 60°C, for 20 min.

Reactivity of amines with the polymeric anhydride

All further reactions of the amines were performed under the optimized time, temperature and solvent conditions. The volume of sample derivatized was 100 μl (Experimental section). An external standard of the labelled derivative was used to determine the percent derivatization. Table I shows the percent derivatization of five amines. The reactivity of a secondary amine toward the polymeric anhydride was

* Throughout this article the American billion (10^9) is meant.

TABLE I

PERCENT DERIVATIZATIONS OF AMINES

*Compound**,**	*Percent derivatizations****
(1) Propylamine	70 (5)§
(2) Butylamine	96 (6)
(3) Nonylamine	78 (5)
(4) Diethylamine	32 (3)
(5) Morpholine	28 (2)

* All amine solutions were equimolar (0.66 μmol).

** Mobile phase: 1, 2 and 4, 50% acetonitrile–water (50:50), 3, acetonitrile–water (75:25); 5, acetonitrile–water (30:70). Flow-rate: 1, 2, 4 and 5, 1.5 ml/min; 3, 2.5 ml/min. Column: Waters Radial-Pak C_{18} column, 10 cm × 5 mm I.D., 5 μm.

*** Reaction conditions: 60°C, 20 min.

§ The numbers in parentheses represent standard deviation. Each data point represents an average of three different reactions, and each reaction mixtures was injected three times ($N = 9$).

2–3 times lower than that of a primary amine. The reactions were performed in an aprotic solvent (acetonitrile) and proceeded by the tetrahedral mechanism[22]. Steric effects play the major role in these reactions. This makes primary amines a better nucleophile than secondary amines, even though a secondary amine is more basic than primary amines. The percent derivatization of morpholine is slightly lower than diethylamine owing to the electron-withdrawing effect of the oxygen, which reduced its nucleophilicity.

Detectability as a result of derivatization

Table II lists the MDLs of the amines and the labelled derivatives monitored at 254 nm and 196 nm. The MDLs using a UV detector were experimentally determined by normalizing the S/N ratio to 2:1. This data was consistent with the molar absorptivity data. To illustrate the increase in detectability that occured following derivatization, a 200-μl solution of butylamine (482 ppm) was derivatized under the optimized conditions. The reaction mixture was monitored by LC–UV at 196 nm. Fig. 2 shows a 200-μl injection of 482 ppm butylamine. The tailed peak is not uncommon for an underivatized amine. Fig. 3 shows the improvement in UV response after derivatization. In addition to the amine derivatives, there also is a peak eluting at 10 min, which was seen in blanks and corresponded to an impurity released during the reaction procedure. This peak was well separated from the amine derivatives and doesn't interfere with their detection. The detector setting in Fig. 3 is 20 times less sensitive than that in Fig. 2, and the reaction mixture was diluted with 2000 μl of acetonitrile.

The derivatives were also amenable to ED, with or without post-column photolysis. Since the amines studied were not electrochemically active, considerable improvements in LC–ED detectability were achieved through derivatization. Table III shows the MDLs of *o*-acetylsalicylamides with oxidative and photolytic LC–ED detection. The UV detector and both types of electrochemical detectors are suitable for chromatographic detection of the amine derivatives. UV detection at 196 nm gave

TABLE II

COMPARISON OF MDLs AT 196 nm AND 254 nm FOR AMINES AND DERIVATIVES*

Compound	*MDLs at 196 nm*	*MDLs at 254 nm*
Propylamine**	55 ppm	–***
Butylamine	104 ppm	–
Nonylamine	37 ppm	–
Diethylamine	50 ppm	–
Morpholine	–	–
Propyl-*o*-acetyl salicylamide§	11 ppb	220 ppb
Butyl-*o*-acetyl salicylamide	34 ppb	273 ppb
Nonyl-*o*-acetyl salicylamide	113 ppb	1067 ppb
Diethyl-*o*-acetyl salicylamide	23 ppb	267 ppb
Morpholino-*o*-acetyl salicylamide§§	1.5 ppb	1453 ppb

* Radial-Pak C_{18} column, 10 cm × 8 mm I.D., 5 μm; mobile phase, acetonitrile–water (50:50); flow-rate, 1.5 ml/min.

** Mobile phase, acetonitrile–water (30:70).

*** Not determined.

§ Flow-rate, 2.5 ml/min.

§§ Mobile phase, acetonitrile–water (20:80).

lower MDLs than by the electrochemical approaches. Table IV lists the MDLs of the amines after derivatization with the three detector approaches. All MDLs after derivatization were calculated assuming: 1) the same chromatographic conditions used to determine the MDLs of the *o*-acetylsalicylamides listed in Tables II and III; 2) reaction conditions of 60°C, 20 min; 3) 200-μl injections; and 4) percent derivati-

TABLE III

MDLs OF *o*-ACETYLSALICYLAMIDES WITH LC–ED AND LC–*hv*–ED*

Compound	*MDL with LC–ED*	*MDL with LC–hv–ED*
Nonyl-*o*-acetyl salicylamide**	135 ppb	47 ppb
Butyl-*o*-acetyl salicylamide	171 ppb	121 ppb
Morpholino-*o*-acetyl salicylamide	29 ppb	19 ppb
Diethyl-*o*-acetyl salicylamide	357 ppb	57 ppb
Propyl-*o*-acetyl salicylamide	175 ppb	119 ppb

* Mobile phase, water–methanol (65:35), 0.2 *M* sodium chloride in water; flow-rates, 1.5 ml/min; injection volume, 200 μl; working potential, +1.0 V *vs.* Ag/AgCl; column, Radial-Pak C_{18} (10 cm × 5 mm I.D., 5 μm); working electrode, glassy carbon electrode.

** Mobile phase, methanol–water (80:20); flow-rate, 2.5 ml/min.

TABLE IV

MDLs OF THE AMINES AFTER DERIVATIZATION WITH UV DETECTION AT 196 nm, OXIDATIVE LC–ED AND LC–*hv*–ED

MDLs after derivatization were calculated assuming: 1) the same chromatographic conditions used to determine the MDLs of the *o*-acetylsalicylamides listed in Tables II and III; 2) reaction conditions of 60°C, 20 min; 3) 200-μl injection; and 4) percent derivatizations as listed in Table I. The formula is:

$$\text{MDL of standard derivative} \times \frac{\text{MW of amine}}{\text{MW of derivative}} \times \frac{1}{\text{percent reaction}}$$

Compound	*UV at 196 nm*	*LC–ED*	*LC-hv–ED*
Butylamine	11 ppb	55 ppb	39 ppb
Propylamine	4 ppb	67 ppb	45 ppb
Diethylamine	23 ppb	346 ppb	55 ppb
Nonylamine	68 ppb	81 ppb	28 ppb
Morpholine	2 ppb	36 ppb	24 ppb

zations as listed in Table I. By comparison of Table II and IV, the amount of amine that can be detected is at least 3 orders of magnitude lower as a direct result of this off-line derivatization.

The MDLs listed in Table IV were calculated values assuming that the percent derivatization is independent of amine concentration. These values were confirmed by experimentally derivatizing 200 μl of 100 ppb butylamine in acetonitrile. Experimentally, the MDL by UV was 27 ppb which was close to the calculated MDL of 11 ppb. The variation in MDL may have been due to small changes in percent derivatization as a function of concentration or from sample lost during the washing procedure.

Separation of the mixture of amines after derivatization with the polymeric anhydride

In order to demonstrate that a variety of amines could simultaneously be derivatized and have their derivatives separated chromatographically, four different amines were reacted with the polymeric reagent. A chromatogram of the reaction mixture is shown in Fig. 4. Both primary and secondary amines can be monitored with the same derivatization and chromatographic conditions.

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