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RESEARCH ARTICLE

Using Gas Phase Reactions of Hexamethylene Triperoxide Diamine (HMTD) to Improve Detection in Mass Spectrometry

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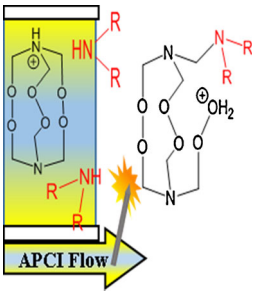

Abstract. Our efforts to lower the detection limits of hexamethylene triperoxide diamine (HMTD) have uncovered previously unreported gas-phase reactions of primary and secondary amines with one of the six methylene carbons. The reaction occurs primarily in the atmospheric pressure chemical ionization (APCI) source and is similar to the behavior of alcohols with HMTD [1]. However, unlike alcohols, the amine reaction conserves the hydrogen peroxide on the intact product. Furthermore, with or without amines, HMTD is oxidized to tetramethylene diperoxide diamine dialdehyde (TMDDD) in a temperature-dependent fashion in the APCI source. Synthesized TMDDD forms very strong adducts (not products) to ammonium and amine ions in the electrospray ionization (ESI) source. Attempts to improve HMTD detection by generating TMDDD in the APCI source with post-column addition of amines were not successful. Signal intensity of the solvent related HMTD product in methanol, $[HMTD+MeOH_2–H_2O_2]^+$ (*m/z* 207.0975), was understandably related to the amount of methanol in the HMTD environment as it elutes into the source. With conditions optimized for this product, the detection of 100 pg on column was accomplished with a robust analysis of 300 pg (1.44 pmol) routinely performed on the Orbitrap mass spectrometers.

Keywords: HMTD, Hexamethylene triperoxide diamine, TMDDD, Tetramethylene diperoxide diamine dialdehyde, Gas-phase reactions, Smines, APCI

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Introduction

Among organic peroxides, hexamethylene triperoxide diamine (HMTD) is an unusual nitrogen-containing molecule with three peroxide functionalities (Figure 1). Combined with its ease of formation, the native sensitivity of this peroxide has facilitated use in illicit explosive devices [2–5]. As part of our research in counterterrorism programs, the quantification of minor amounts of this material by liquid chromatography coupled to mass spectrometric detectors (LC/MS) has become necessary. These systems offer fast and sensitive detection of the intact molecules for unequivocal identification. Many labs have published methods to effectively and robustly detect low levels of HMTD using LC/MS techniques [9–11].

Improving the detection limits for any molecule can be a challenge since there are many variables to consider. Some parameters are greatly dependent on the properties of the analyte itself, with very limited control by the analyst. For instance, on Thermo MS systems, many compounds are rather insensitive to the tube lens voltage, but it is extremely important for peroxides, with a narrow window of optimization. This is not very surprising since the tube lens can be changed to decluster adducts or cause fragmentation to fragile ions. Autotuning algorithms available on most modern MS systems can generally identify these parameters more quickly and efficiently than most users. Parameters within the direct control of the analyst, however, may not be easily identified as the combination of these can become quite extensive. Flow rates, solvents, chromatography columns, and modifiers are among the most practical LC features that can affect MS detection. Ionization type, temperatures, and gas flow rates are the common variables at the ionization source. The process of ionization may be further complicated by variable solvent interactions with compounds of nearly identical structures. Hydrogen/deuterium exchange experiments may be employed in an

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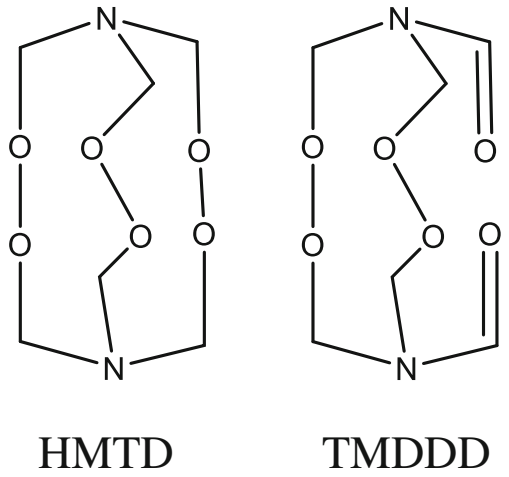


Figure 1. Structure of HMTD and TMDDD

attempt to comprehend these complex gas-phase reactions [12–15]. Understanding these reactions may be crucial for proper identification of an analyte or to make informed decisions about improving detection.

We have already found that choice of mobile phase and ionization source are essential. In both electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI), use of acetonitrile (ACN) in the mobile phase extensively reduces the ionization of most peroxides [6]. However, the use of ACN as a storage solvent is not a problem since this solvent can be easily separated from the analytes prior to ionization [7]. Using APCI and a mobile phase of ammonium acetate/methanol, detection limits of 1 ng on-column were achieved for the $[M+H]^+$ (*m/z* 209.0768) ion. Use of methanol or any alcohol requires attention as HMTD and other cyclic peroxides create gas-phase products with solvent alcohols [1, 7]. HMTD reacts with MeOH in the APCI source to produce the alcohol incorporated product $[HMTD+MeOH_2\text{-}H_2O_2]^+$ with *m/z* 207.0975 ($C_7H_{15}N_2O_5^+$) [1]. This does not appear to negatively affect the HMTD signal intensity. In fact, this product can be used as confirmation of the presence of HMTD along with other in-source fragments frequently observed, including 191.0662, 179.0662, 145.0608, and 88.0393, depending on source conditions [8]. Our efforts to identify the best parameter combination to reduce detection limits have uncovered novel gas-phase reactions that occur with HMTD. Probing these novel reactions has shown the need for high resolution instruments with proper chromatographic separation to assure detection of the correct compound.

Materials and Methods

Chemicals and Reagents

Caution: HMTD is a powerful explosive, and organic peroxides, in general, are sensitive compounds. Take necessary precautions when working with these compounds.

Water, acetonitrile, methanol, (all Optima HPLC grade), ammonium acetate (NH_4OAc, HPLC grade), triethylamine, and aniline were purchased from Fisher Chemical (Fair Lawn, NJ, USA). Methylamine, ethylamine, dimethylamine, 2-nitroaniline, (2-aminoethyl)trimethylammonium were purchased from Sigma-Aldrich (St. Louis, MO, USA). Deuterated formaldehyde (D2), water (D2), and methanol (D4) were purchased from Cambridge Isotope Labs (Cambridge, MA, USA). Isopropylamine, cyclohexlyamine, choline, and hexamethylenetetramine (hexamine) were purchased from Acros Organics (Morris Plains, NJ, USA. Hydrogen peroxide (HP, 50%) was obtained from Univar (Redmond, WA, USA). Unless otherwise stated, the mobile phase used for chromatography consisted of aqueous 10 mM NH_4OAc prepared at neutral pH with methanol (MeOH) as the organic modifier.

HMTD and TMDDD Synthesis

To synthesize hexamethylene triperoxide diamine (HMTD), hexamine (2.43 g, 17.3 mmol) was placed in a round-bottom flask, immersed in an ice bath, and dissolved in 50% hydrogen peroxide (9.88 g, 145 mmol). Anhydrous citric acid (3.61 g, 18.9 mmol) was added in small portions so that the temperature did not exceed 10 °C. The reaction mixture was left in the ice bath and allowed to warm with stirring over 15–18 h. Product was collected by vacuum filtration, washed with deionized water and room temperature methanol, and allowed to dry. A similar procedure was used to produce the d_{12}-HMTD by using d_2-formaldehyde. HMTD was used to produce tetramethylene diperoxide diamine dialdehyde (TMDDD), which was synthesized according to Wierzbicki et al. [16]. This crude product (mp: 156–157 °C) was used for all TMDDD testing.

Instrumentation

Using a ThermoElectron (San Jose, CA, USA) LTQ Orbitrap XL or Exactive mass spectrometer equipped with an atmospheric pressure chemical ionization (APCI) interface, ions were generated and introduced into the ion transfer tube set between 180 and 275 °C (depending on the experimental conditions being tested). All work was performed using positive ion mode. Tune conditions for APCI infusion experiments were varied depending on the parameters being tested: discharge current, 2.5–6.0 μA; N_2 sheath gas, 8–50 arbitrary units (AU); N_2 auxiliary gas, 5–40 AU; vaporizer temperature 180–350 °C; ion transfer tube, 14 V; tube lens, 35–70 V; and skimmer offset (Exactive), 0 V. Minor voltage changes were made at times to improve signal intensity for compounds. For electrospray ionization experiments, spray voltages ranged from 3500 to 5000 V. Other focusing voltages were similar to those used for APCI, with the Tube Lens having the most significant effect on ion abundance. For the LTQ Orbitrap, fragmentation was performed with an isolation width of 1.8 *m/z*, activation time 30 ms, Q_z value of 0.25, and mass resolution was between 7500 to 100,000. For the Exactive MS, mass resolution was 50,000 and the AGC setting was balanced. Solvent delivery was performed using either Thermo infusion syringe pumps or ThermoElectron Accela quaternary pumps. A CTC Analytics HTS PAL autosampler injected directly from either amber glass LC vials with PTFE septa (Agilent

Technologies) or polypropylene, 1 mL 96-well plates with pre-slit silicone plate covers (Analytical Sales and Service). Data collection and analysis were performed with Thermo Xcalibur software ver. 2.2, SP 1.48. Most data reported herein employed full scan MS unless specifically stated. Chromatographic traces are extracted ion chromatograms (XIC) with a mass window of 15 ppm of the expected exact mass. *Note: All masses reported below are exact mass values within ±15 ppm for species less than 130 m/z and ±7.5 ppm for species over m/z 130.*

HMTD/TMDDD Analysis

Direct infusion of HMTD or TMDDD (10 μg/mL or ~48 μM in MeOH) into either the ESI or APCI source was used to optimize voltages for both compounds. Optimized HMTD values were chosen for all analyses since this is the primary compound of interest. The MS gas flows and temperature were initially optimized using mobile phase (MP) infusion with a constant flow of 50% 10 mM NH_4OAc in pump channel B and 50% MeOH in channel A at 230 μL/min flow and directly infusing 20 μL/min HMTD standard (10 μg/mL/48.1 μM in MeOH) into the flow. Monitoring the $[M+H]^+$ ion at *m/z* 209.0768, the vaporizer temperature was set to 250 °C, with the sheath gas at 35 AU and auxiliary gas at 20 AU. Using this optimized system, 40 μL sample volumes of HMTD in 50/50 ACN/water were injected into a LC flow of 250 μL/min with 5% MeOH (channel A) and 95% aqueous 10 mM NH_4OAc (channel B) for introduction onto either a Thermo Syncronis C18 column or an Analytical Sales and Service Advantage Polyfluorinated Phenyl (PFP) column (both 2.1 × 50 mm, 5 μm). Initial conditions were held for 1.5 min before a linear ramp to 35% A/65% B over 1.5 min followed immediately by a linear ramp to 95%A/5% B over the next min. This concentration was held for 2 min before a 30 s transition to initial conditions with a hold of 1.5 min. Fully deuterated HMTD (d_{12}-HMTD) was produced to be used as an internal standard (IS). Extracted ion chromatograms (XIC) were integrated using the Genesis peak detection algorithm in Thermo Xcalibur Quan Browser. Linear dynamic range comparing concentration to peak area response ratio, relative to the IS, extended from 25 ng/mL to 20,000 ng/mL using 10 points and a weighted calibration curve. Solution stability in ACN was previously determined to be up to 40 d at –20 °C [7]. The analysis of TMDDD $[M+NH_4]^+$ ion of *m/z* 224.0877 or $[M+H]^+$ ion of *m/z* 207.0612 were performed by the same method used for HMTD. Separation of TMDDD and HMTD was significant on either the C18 or PFP column.

Solvent Incorporation Studies

To examine the origin of certain products/fragments observed in the LC/MS experiments, HMTD or TMDDD (10 μg/mL) were prepared in solutions containing 1 mM of various amine compounds (see Table 1). Solutions were directly infused into the ESI or APCI source. Due to the strongly basic nature of the amines, most of the compounds were diluted to neutral pH using a small amount of formic acid. To avoid exposing LC columns to the amines, additional studies were performed with 50 mM solutions of amine infused post-column at 5 μL/min into 250 μL/min of mobile phase (final MP concentration ~1 mM). These studies were performed while altering vaporization temperatures and N_2 gas flow. Since alcohol incorporation into HMTD is a known phenomenon, additional studies were performed using MP infusion to determine the gas and temperature conditions in APCI to optimize the methanol incorporated product (*m/z* 207.0975) [1]. Also, studies were performed by altering vaporization temperatures and N_2 gas flow while injecting HMTD onto the system using the PFP or the C18 column. Peak area counts were compared to determine optimal, stable analytical conditions.

Hydrogen/Deuterium Exchange

Hydrogen/deuterium exchange (HDX) studies were performed in 2 ways. To determine the exchange of solvent hydrogen for deuterium on the parent molecule, the d_{12}-HMTD was placed in a solution of 50% MeOH/50% 10 mM NH_4OAc at a concentration of 10 μg/mL. Isopropyl amine was added so the final concentration was 1 mM. This solution was infused into the APCI source at a rate of 20 μL/min. The dominant ion at *m/z* 280.2258, corresponding to the proposed structure and only solvent hydrogen, was trapped and fragmented. To determine the exchange of solvent deuterium for parent hydrogen, 10 μg/mL of HMTD was placed in 50/50 D_2O/CD_3OD with 1 mM

Table 1. Expected and Observed Masses for HMTD in APCI with Fully Incorporated Amine

Organic amine	Expected mass[b]	Observed mass[b]	ΔPPM	pKa
Methylamine	240.1190	240.1185	–2.1	10.62
Ethylamine	254.1347	254.1342	–2.0	10.87
Dimethylamine	254.1347	254.1341	–2.4	10.73
Triethylamine[a]	310.1973	310.1963	–3.2	10.78
Isopropylamine	268.1503	268.1489	–5.2	10.63
Cyclohexlyamine	308.1816	308.1813	–1.0	10.63
Ammonia	226.1034	NR		9.25
Aniline	302.1347	302.1338	–3.0	4.6
2-Nitroaniline	347.1197	NR		–0.28
(2-Aminoethyl)trimethylammonium	311.1925	NR		na
Choline	?	NR		–3.2

[a]Triethylamine forms a HMTD adduct with no observable chemical reaction; NR-no reaction.
[b]TMDDD in ESI presented as 2 H reduction of mass for all (ammonium = 224.0877); na-not available

non-deuterated isopropyl amine. Upon infusion onto the APCI source at 20 μL/min, the dominant ion at *m/z* 271.1691, corresponding to the proposed structure with deuterium exchange of available hydrogen, was trapped and fragmented.

Results and Discussion

Early efforts to identify all species related to HMTD in the APCI source led to the frequently encountered *m/z* 224.0877, associated with $C_6H_{14}N_3O_6^+$. When chromatographically separated, two peaks with this same *m/z* 224 were observed. The first peak eluted early with a major signal of *m/z* 224 and a minor signal of *m/z* 207.0611 $[HMTD–2H+H]^+$. We believed this compound to be TMDDD (Figure 1), with *m/z* 224 being the ammonium adduct. The second peak eluted at the same retention time as HMTD and exhibited all other masses associated with HMTD ionization. Although this second peak showing *m/z* 224 was produced in varying degrees from one analysis to another, we believed this to be TMDDD formed from HMTD in the gas phase under APCI conditions. Marr and Groves had reported that in the gas phase a small amount of HMTD is converted to the dialdehyde product (TMDDD) [17]. When an authentic sample of TMDDD was prepared and analyzed, it eluted with the same retention time (t_R) and peak shape as the early eluting *m/z* 224, indicating that our HMTD standard was contaminated with a small amount of TMDDD. Quantification of the TMDDD and HMTD samples showed that HMTD was contaminated with about 1% TMDDD and TMDDD contained about 1.5% of HMTD (Online Resource Table S-1). Collision induced dissociation (CID) of *m/z* 224 for TMDDD and the *m/z* 224 under the HMTD peak confirmed that HMTD was being oxidized to TMDDD in the APCI source. Very little, if any, conversion of HMTD to TMDDD is observed using ESI.

TMDDD was found to have a significantly better signal in ESI than APCI and a very high affinity for ammonium or amine adducts. In fact, using our standard mobile phase of ammonium acetate/methanol, the minor contamination of TMDDD in HMTD (~1% depending on the batch) produced a signal for the ammoniated TMDDD in ESI that was nearly comparable to the HMTD signal in APCI. Krawczyk discovered that TMDDD also has a high affinity for metal ions in the ESI source and suggested that purposeful oxidation of HMTD be exploited to improve detection levels. This process involved off-line oxidation of the HMTD samples prior to analysis by ESI in the presence of metal ions [18]. In contrast, the APCI source produced a significantly better signal for HMTD than ESI with variable amounts of conversion from HMTD to TMDDD. We then began to consider ways of intentionally increasing this in-source conversion to improve detection limits with the addition of an adducting agent. Since the production of TMDDD from HMTD required heat in the presence of O_2, several experimental parameters were examined in both APCI and heated ESI (HESI) to cause in-source conversion. Attempts to convert HMTD to TMDDD using the HESI source did not succeed even at temperatures exceeding 300 °C. Experiments using APCI show that TMDDD formation increases with increasing temperatures, up to the point (~350 °C) where both HMTD and TMDDD begin to decompose. Figure 2 shows the XIC of products related to HMTD and TMDDD for the same sample analyzed at two different vaporization temperatures in the APCI source (other conditions identical). The signal for ion *m/z* 224 at the t_R ~4.8 min, where HMTD elutes, was nearly 20 times more intense with the vaporizer temperature at 300 °C rather than at 210 °C. Note that the signal area for TMDDD (t_R ~2.1 min) was unaffected by temperature. These results can be observed in Tables S-2 and S-3 of the Online Resource. While *m/z* 224 was increased from 2% to 32% of the total signal by increasing the temperature, the peak at *m/z* 207.0975 was still 46% of the total signal. By summing the peak areas of all HMTD products over all temperatures, it was evident that the largest response for any product was *m/z* 207.0975 at 250 °C, corresponding to methanol incorporation $[HMTD+MeOH_2–H_2O_2]^+$.

Since TMDDD formed very strong ammonium adducts, we attempted to enhance the MS response by use of organic amines. A variety of amines at 1 mM concentration were infused with HMTD into either an APCI or ESI source. Methylamine with HMTD produced abundant signal at *m/z* 238.1034 in ESI and a large signal at *m/z* 240.1190 in APCI; ethylamine produced similar results at *m/z* 252.1190 and *m/z* 254.1347, respectively (Figure 3). It should be noted that each source produced both the low and high *m/z* ions to some degree, but the lower *m/z*, e.g., 238 or 252, clearly dominated in ESI, and the higher *m/z*, in APCI (Figure 3). To clarify these results, the same HMTD sample was injected onto the LC-MS system with post-column addition of the same amines using both ESI and APCI (Figure 4). In ESI, the intense signal at *m/z* 252.1190 eluting early with no detectable *m/z* 254.1347 was the TMDDD contaminant in our HMTD sample. The second peak being HMTD had almost no *m/z* 252, but a reasonable signal for *m/z* 254. Results using APCI showed very low intensity for the TMDDD *m/z* 252 signal (no *m/z* 254 at all) and a very intense signal of *m/z* 254 for HMTD with a small amount of *m/z* 252 present (from TMDDD formed in-source). These results confirm our observations above that TMDDD contaminates HMTD samples. Even HMTD, chromatographically separated from TMDDD, forms TMDDD in the APCI source. Furthermore, it was observed that HMTD produced the strongest signal in APCI, whereas TMDDD was best observed by ESI. Additionally, both HMTD and TMDDD form products or adducts with amines. At this point, it was not certain whether the amine adduct could be used to improve the APCI signal for TMDDD.

Attempts to trap and dissociate TMDDD-amine adduct peaks in ESI provided no fragmentation at all, just depletion of the parent ion. Krawczyk also reported that attempting to fragment metal adducts of TMDDD produced no observable fragments [18]. When larger amines (see list in Table 1) were studied, where the protonated amine had a *m/z* greater than the 50 Da cut-off of the Orbitrap, we observed only the

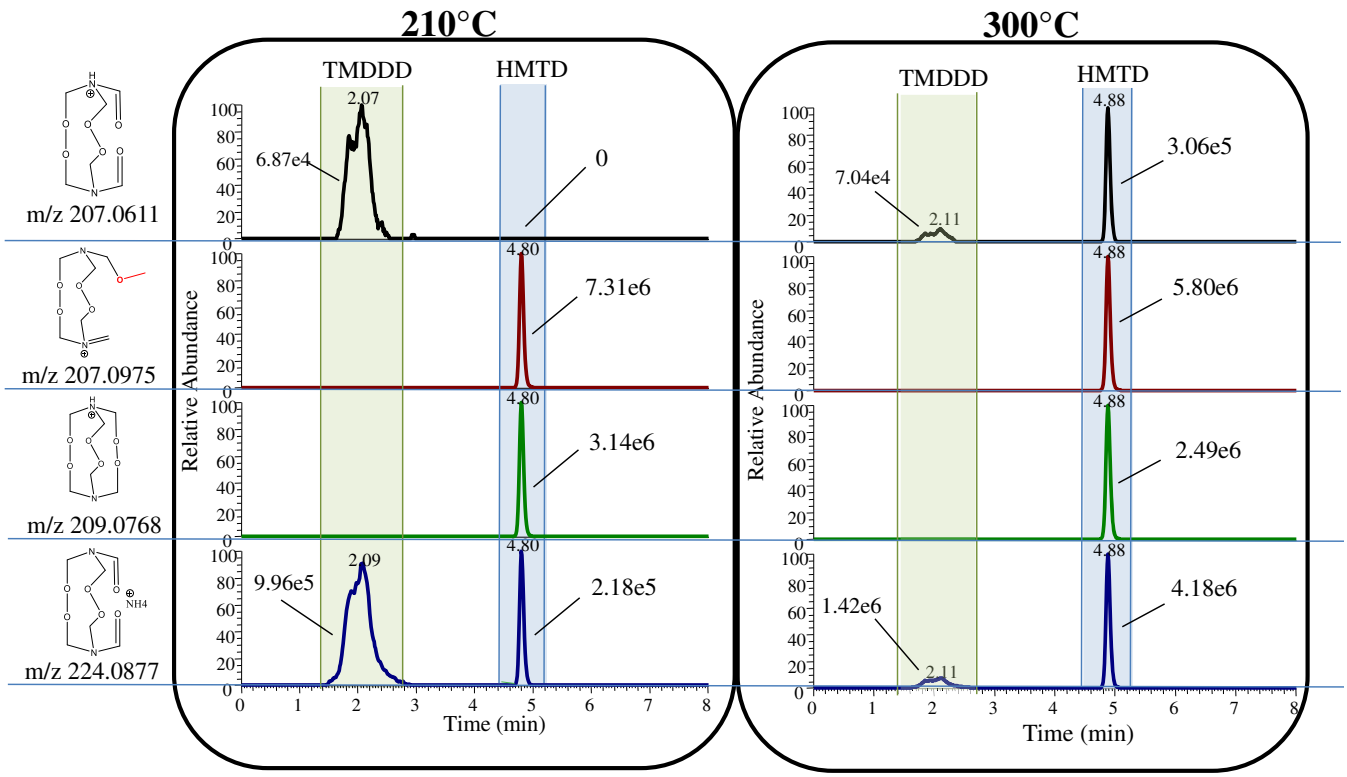


Figure 2. TMDDD and HMTD with 100 ng of HMTD injected onto a 5 cm PFP column with APCI source at 210 °C and 300 °C. (Numbers displayed by each peak is integrated area counts)

fragment corresponding to the protonated amine. This suggests that the amine sequestered all charge and explains the reason no fragments were observed during CID of the TMDDD adduct. It appears that the affinity of TMDDD for amines is related to the basicity of the amine, with the more basic amine producing a larger adduct signal. Post-column addition of organic amines indicated that the formation of organic amine adducts with TMDDD are favored over ammonium. Unfortunately, the signal observed for the amine was equivalent in intensity to the ammonium adduct, but not significantly better.

Unlike TMDDD, trapping and dissociation of the HMTD amine adducts in APCI produced multiple stable fragments (see Figure 5, Table 2). CID spectra and proposed fragments for many of the amines tested can be viewed in the Online Resource Figures S1–6. Interpretation of the spectra for each of the amines showed that although each exhibited a fragment of 209.0768 (product **4**, Table 2) suggesting adduct formation, they all lost water (product **2**, Table 2) and H_2O_2 (product **3**, Table 2). Notably, in all spectra was the formation of the fragment *m/z* 197.0768 (product **5**, loss of exactly 12.000 Da from

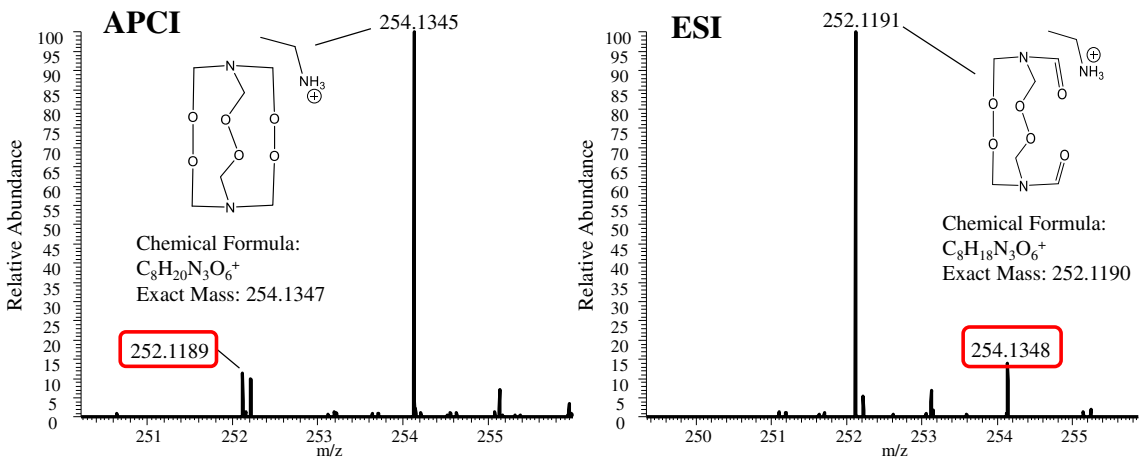


Figure 3. APCI and ESI spectra for the infusion of HMTD with 1 mM ethylamine

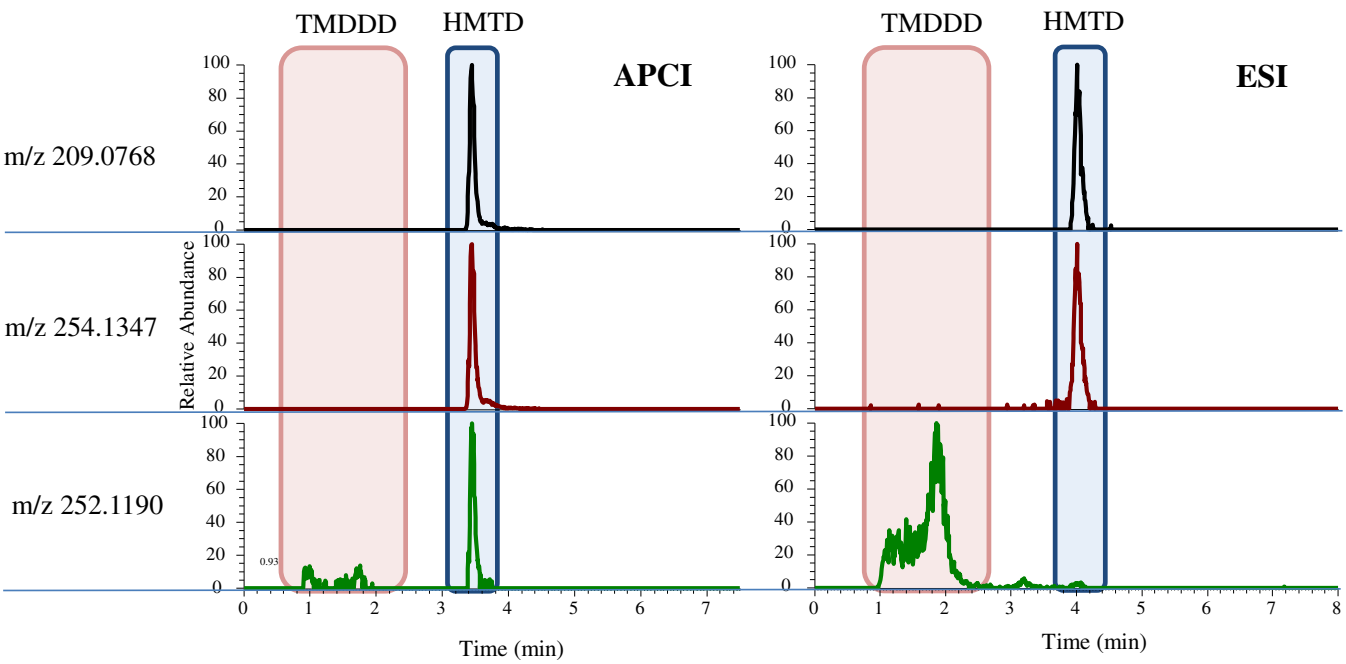


Figure 4. Chromatogram of HMTD in APCI and ESI with post-column addition of ethylamine

HMTD) and a fragment corresponding to the parent amine increasing by exactly 1 carbon (product **10**, Table 2). This suggested that the amine performed a nucleophilic attack on one of the methylene groups of the HMTD molecule, similar to the reaction with alcohols [1]. To confirm this result, d_{12}-HMTD was synthesized and full hydrogen/deuterium exchange (HDX) studies were performed using isopropylamine. Results are shown in Table 2 and Online Resource Figures S-7 and S-8. All fragments produced were in agreement with the proposed structures (within ±5.6 ppm for fragments > 100 *m/z* and ± 10 ppm for fragments <100 *m/z*). This nucleophilic attack was observed for both primary and secondary amines. Only triethylamine formed an HMTD adduct, and the dissociation of this product generated only a small protonated HMTD fragment and a large fragment at *m/z* 102.1277

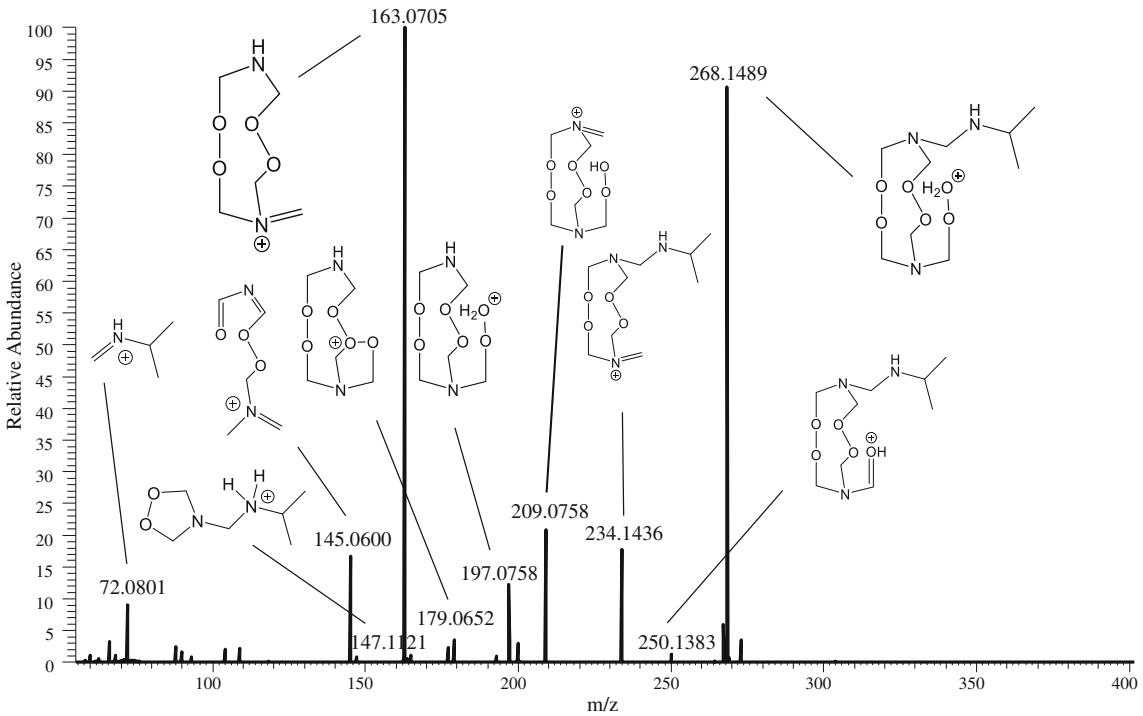
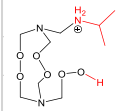
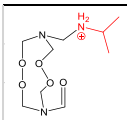
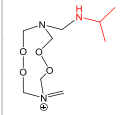
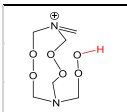
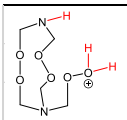
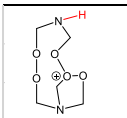
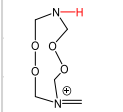
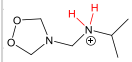
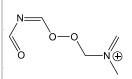
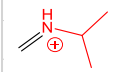


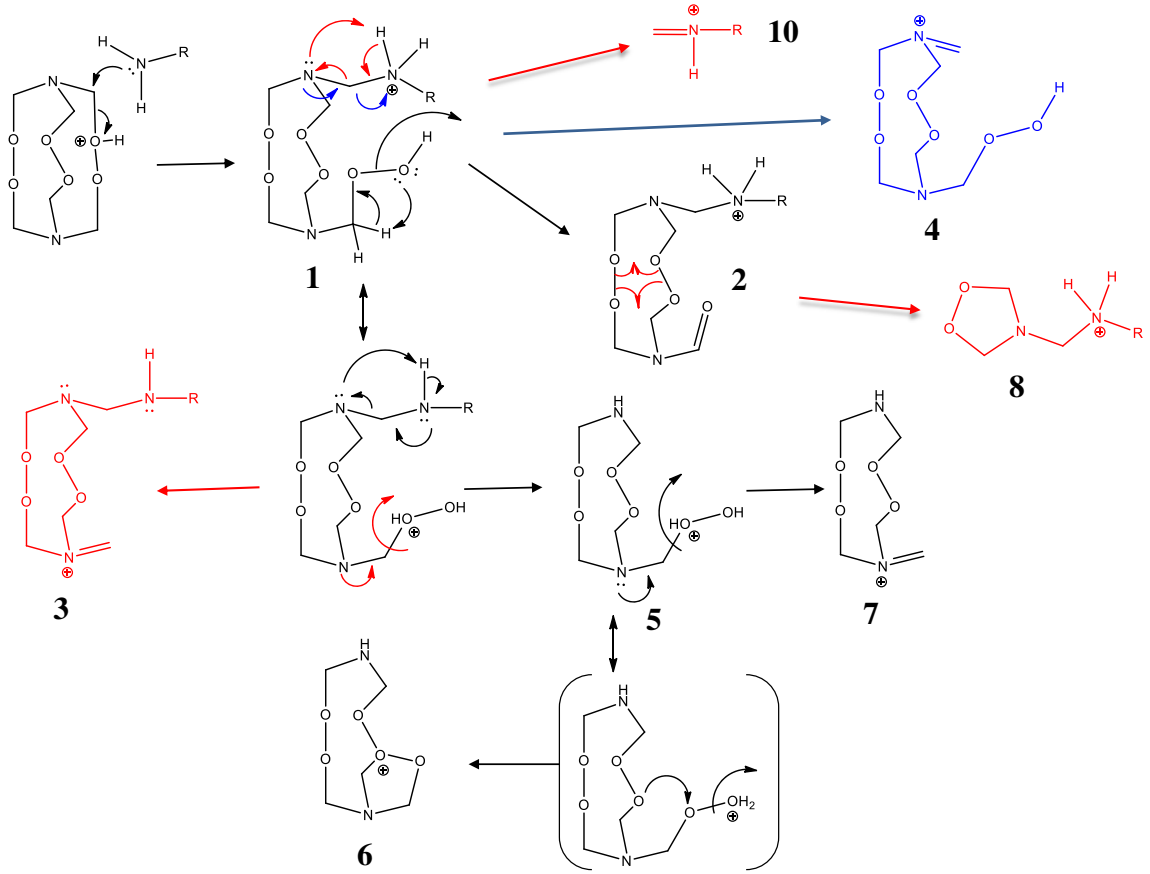
Figure 5. Fragmentation of t HMTD/isopropylamine product formed in APCI at *m/z* 268.1489

Table 2. Fragments Associated with HMTD Gas-Phase Reaction (Including HDX) with Isopropylamine

Proposed fragment	Product	Material/solvent	Exact Mass	Molecular formula	Observed Mass	ΔPPM
	**1**	U/US	268.1503	C9H22N3O6+	268.1489	-5.2
		D18/US	280.2256	C9H10D12N3O6+	280.2247	-3.2
		U/DS	271.1691	C9H19D3N3O6+	271.1686	-1.8
	**2**	U/US	250.1397	C9H20N3O5+	250.1383	-5.6
		D18/US	261.2088	C9H9D11N3O5+	261.2091	1.1
		U/DS	252.1523	C9H18D2N3O5+	252.1516	-2.8
	**3**	U/US	234.1448	C9H20N3O4+	234.1436	-5.1
		D18/US	246.2202	C9H8D12N3O4+	246.2194	-3.2
		U/DS	235.1511	C9H19DN3O4+	235.1506	-2.1
	**4**	U/US	209.0768	C6H13N2O6+	209.0758	-4.8
		D18/US	221.1521	C6HD12N2O6+	221.1514	-3.2
		U/DS	210.0831	C6H12DN2O6+	210.0826	-2.4
	**5**	U/US	197.0768	C5H13N2O6+	197.0758	-5.1
		D18/US	207.1396	C5H3D10N2O6+	207.1389	-3.4
		U/DS	200.0956	C5H10D3N2O6+	200.0951	-2.5
	**6**	U/US	179.0662	C5H11N2O5+	179.0652	-5.6
		D18/US	189.1290	C5HD10N2O5+	189.1284	-3.2
		U/DS	180.0725	C5H10DN2O5+	180.0720	-2.8
	**7**	U/US	163.0713	C5H11N2O4+	163.0705	-4.9
		D18/US	173.1341	C5HD10N2O4+	173.1334	-4.0
		U/DS	164.0776	C5H10DN2O4+	164.0772	-2.4
	**8**	U/US	147.1128	C6H15N2O2+	147.1121	-4.8
		D18/US	153.1505	C6H9D6N2O2+	153.1498	-4.6
		U/DS	149.1254	C6H13D2N2O2+	149.1249	-3.4
	**9**	U/US	145.0608	C5H9N2O3+	145.06	-5.5
		D18/US	154.1173	C5D9N2O3+	154.1167	-3.9
		U/DS	145.0608	C5H9N2O3+	145.0604	-2.8
	**10**	U/US	72.0808	C4H10N+	72.0801	-9.7
		D18/US	74.0933	C4H8D2N+	74.0927	-8.1
		U/DS	73.0871	C4H9DN+	73.0865	-8.2

corresponding to protonated triethylamine. The proposed CID fragmentation mechanism for the HMTD-amine products (confirmed by HDX) is shown in Scheme 1.

To improve detection of HMTD, post-column addition of organic amines (with or without neutralization) was examined with increasing temperature in the APCI source. This attempt at in-source conversion of HMTD to TMDDD was successful, but the signal for the TMDDD-amine adduct was not greater than HMTD-methanol product (*m/z* 207.0975). In another attempt to improve the HMTD signal, we tried to attach a charged quaternary amine, producing a permanently charged ion. Two organic quaternary amines, one with a pendent primary amine [(2-aminoethyl) trimethylammonium] and the other with a pendent primary



Scheme 1. (Arrow colors correspond to formed structure color).

alcohol (choline), were infused in a MeOH/H_2O solution of HMTD. No reaction or reaction product (including multiply charged products) was observed in either ESI or APCI. We speculate that the quaternary amine pulled the electron density from the alcohol or amine tail which created a species that was not a strong enough electrophile

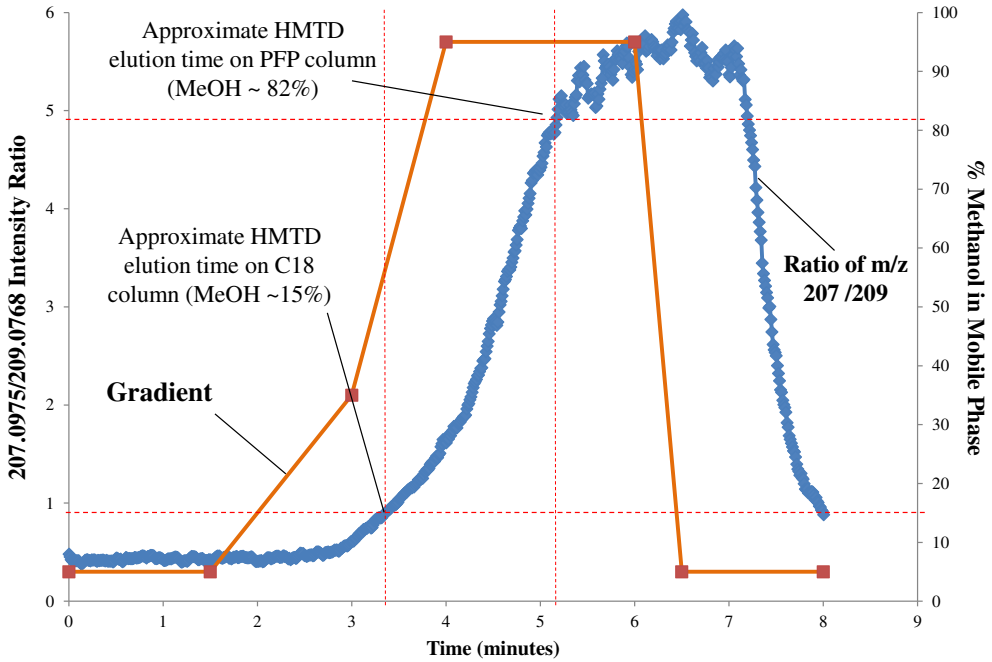


Figure 6. HMTD MP infusion into gradient of methanol (right axis) and observed *m/z* 207/209 ratio (left axis). Red dotted lines identify the approximate in-source MeOH concentration seen by HMTD on the C18 versus the PFP column

to attack the methylene group. Additionally, the electron rich oxygens and nitrogen surrounding the methyl groups can draw the quaternary group toward molecule thus preventing the nucleophilic group from proper approach for reactivity. Similar experiments using amines were attempted with other peroxides including TATP and MEKP. No reaction products were detected in either ESI or APCI for these compounds [7].

Using the ammonium acetate/methanol mobile phase, chromatographic data frequently showed $[HMTD+MeOH_2–H_2O_2]^+$ = *m/z* 207.0975 ion produced the most intense signal under APCI conditions. Inexplicably, the ratio of the $[M+H]^+$ and $[M+MeOH_2–H_2O_2]^+$ varied from analysis to analysis. In order to use the MeOH incorporated product for HMTD quantification, it was necessary to understand the variability of this signal. Direct infusion of HMTD (10 μg/mL at 20 μL/min) while increasing the N_2 gas flow in the APCI source favored the formation of the parent $[M+H]^+$ over the formation of $[M+MeOH_2–H_2O_2]^+$. The effect of increased gas flow rate (also observed for TATP [7]) suggests that higher gas flow does not give the alcohol as much time to react with the HMTD ion/molecule in either the corona discharge or the vaporizer region of the source. Direct infusion experiments (even in very high MeOH concentrations) almost always produced more of the $[M+H]^+$ ion. However, MP infusion studies of HMTD show significantly more of the alcohol incorporated product with increasing MeOH concentration. Figure 6 depicts the ratio of *m/z* 207.0975/209.0768 in the standard LC gradient and demonstrates that this can be leveraged for column optimization. Use of a PFP column (t_R HMTD ~5.1 min) over the C18 column (t_R HMTD ~3.5 min) favors the formation of *m/z* 207.0975. Optimizing gas flow was performed by injecting the same 10 μg/mL sample onto the PFP column at 250 °C vaporizer temperature under various flow conditions (Table S-3, Online Resource). Although a higher sheath than auxiliary gas provided more intense signal, it was also associated with the most variability. The best stability with the most intense signal for the $[M+MeOH_2–H_2O_2]^+$ product was achieved when the sheath and auxiliary gasses were both set to 15 AU. Additionally, HMTD peak shape is strongly affected by the amount of organic in the sample plug. More than 20% ACN in the sample plug can produce sever fronting of the peak, reducing our ability to detect low levels of HMTD (see Figure 7). Since HMTD is not volatile and we are using a deuterated IS, samples can be evaporated to dryness and reconstituted in 90% water/10% ACN. Although concerns about solubility did arise, the assay linear range spanned from 10 ng/mL (48nM) to 20000 ng/mL (96 μM), demonstrating that this was not an issue. This also allows for the concentration of higher volume samples to push detection limits even lower. Using optimized conditions for HMTD, we have detected HMTD as low as 100 pg on column with a robust analysis of 300 pg on column.

Conclusions

Although TATP and HMTD have been observed to undergo a gas-phase reaction with alcohols [1, 7], no corresponding reaction has been observed for TMDDD. While enhancing detection of HMTD we discovered that TMDDD is frequently a contaminant in purified HMTD and can also be formed in the gas phase within the APCI source. TMDDD $[M+H]^+$ (*m/z* 207.0612) produces a much better signal in ESI than APCI, and has tremendous affinity for ammonium or organic amine

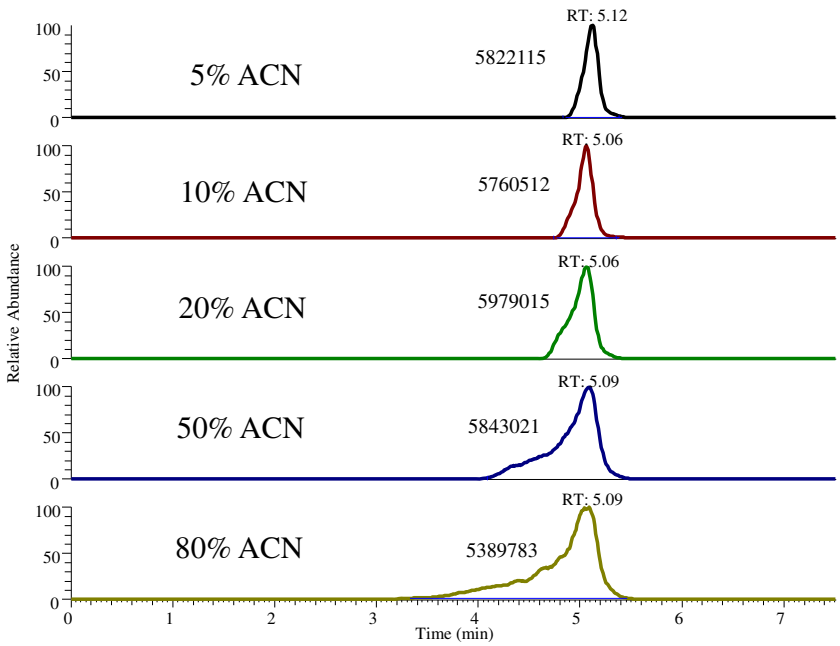


Figure 7. Injection of 20 μL of 10 μg/mL HMTD in various combinations of ACN/water in the sample plug. Peak integration was performed manually, but t_R and areas (number left of peak) are quite similar

ions. HMTD and cyclic peroxides have exhibited significantly better ionization by APCI, while linear peroxides (MEKP) and TMDDD respond best to ESI [7]. We speculate that the open nature of TMDDD (a 10-membered ring) may allow this to behave more like a linear peroxide. Our attempts to create TMDDD in-situ were a success, in that HMTD is oxidized in a temperature-dependent fashion within the APCI source. Unfortunately, we were unable to exploit the high TMDDD affinity for amines to improve HMTD detection limits. Off-line conversion of HMTD to TMDDD as suggested by Krawczyk [18] might improve detection limits using post-column addition of basic volatile organic amines instead of metals. Additionally, we discovered a direct reaction of organic amines with the methylene carbon of HMTD, analogous to the reaction of alcohols. This behavior may be exploited for other areas of research, but did not appear to enhance detection limits as did the MeOH. Current linear dynamic range for HMTD analysis (using the $[M+MeOH_2–H_2O_2]^+$ product) is 10 ng/mL (48 nM) to 20,000 ng/mL (96 μM) using APCI with a vaporizer temperature of 250 °C and a mobile phase of MeOH/200 μM NH_4OAc on a PFP column. If MeOH is used as the mobile phase organic modifier, there are two different compounds with a nominal mass of *m/z* 207 being produced. This information combined with the HMTD conversion to TMDDD in storage solution [7] substantiate the need for good separation and high resolution MS for proper identification and quantification.

Acknowledgments

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