DMD Fast Forward. Published on August 30, 2011 as doi:10.1124/dmd.111.041798

Title page

Complex *N*-acetylation of Triethylenetetramine

Marc Cerrada-Gimenez, Janne Weisell, Mervi T. Hyvönen, Myung Hee Park, Leena Alhonen, Jouko Vepsäläinen and Tuomo A. Keinänen

Department of Medicine (M.C-G.), Laboratory of Chemistry, Department of Biosciences (J.W., J.V., T.A.K.), Biotechnology and Molecular Medicine, A.I. Virtanen Institute (M.T.H., L.A.), Biocenter Kuopio, University of Eastern Finland, Kuopio Campus PO Box 1627, FI-70211 Kuopio, Finland. Oral and Pharyngeal Cancer Branch, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD 20892, USA (M.H.P.).

DMD Fast Forward. Published on August 30, 2011 as DOI: 10.1124/dmd.111.041798
This article has not been copyedited and formatted. The final version may differ from this version.

Running title page

A running title: *N*-acetylation of triethylenetetramine

Address correspondence to: Tuomo A. Keinänen, Laboratory of Chemistry, Department of Biosciences, Biocenter Kuopio, University of Eastern Finland, Kuopio Campus, Yliopistonranta 1 (for courier), PO Box 1627, FI-70211 Kuopio, Finland. Telephone +358-40-3552387; Fax +358-17-163025. E-mail: Tuomo.Keinanen@uef.fi.

Number of text pages 26

Number of tables 4, Supplementary Tables 2

Number of figures 4, Supplementary Figures 6

References 37

Number of words in the Abstract 201

Number of words in the Introduction 500

Number of words in the Discussion 976

Abbreviations

DAH, 1,7-Diaminoheptane; HFBA, heptafluorobutyric acid; HIF-1α, hypoxia-inducible factor 1α; N^1AcTETA, N^1-monoacetyltriethylenetetramine; N^3AcTETA, N^3-monoacetyltriethylenetetramine; N^1N^8DiAcTETA, N^1,N^8-diacetyltriethylenetetramine; NAT2, *N*-acetyltransferase 2; PCA, perchloric acid; Put, putrescine; Spd, spermidine; Spm, spermine; SSA, sulphosalisylic acid; SSAT1, spermidine/spermine N^1-acetyltransferase; SSAT2, thialysine acetyltransferase; TCA, trichloroacetic acid; TETA, triethylenetetramine (1,8-diamino-3,6-diazaoctane)

DMD Fast Forward. Published on August 30, 2011 as DOI: 10.1124/dmd.111.041798
This article has not been copyedited and formatted. The final version may differ from this version.

ABSTRACT:

Triethylenetetramine (TETA) is an efficient copper chelator having versatile clinical potential. We have recently shown that spermidine/spermine-N^1-acetyltransferase (SSAT1), the key polyamine catabolic enzyme, acetylates TETA *in vitro*. Here, we studied the metabolism of TETA in three different mouse lines: syngenic, SSAT1 overexpressing and SSAT1-deficient (SSAT1-KO) mice. The mice were sacrificed at 1, 2 or 4 hours after TETA injection (300 mg/kg i.p.). We found only N^1-acetyltriethylentetramine (N^1AcTETA) and/or TETA in the liver, kidney and plasma samples. As expected, SSAT1 overexpressing mice acetylated TETA at an accelerated rate as compared to syngenic and SSAT1-KO mice. Interestingly, SSAT1-KO mice metabolized TETA as syngenic mice did, probably by thialysine acetyltransferase, which had K_m of 2.5 ± 0.3 mM and k_{cat} of 1.3 s^{-1} for TETA when tested *in vitro* with the human recombinant enzyme. Thus, the present results suggest that there are at least two *N*-acetylases potentially metabolizing TETA. However, their physiological significance for TETA acetylation requires further studies. Furthermore, we detected chemical intramolecular *N*-acetyl migration from N^1- to N^3-position of N^1AcTETA and N^1,N^8-diacetyltriethylenetetramine in acidified HPLC sample matrix. The complex metabolism of TETA together with the intramolecular *N*-acetyl migration may explain the huge individual variations in the acetylation rate of TETA reported earlier.

DMD Fast Forward. Published on August 30, 2011 as DOI: 10.1124/dmd.111.041798
This article has not been copyedited and formatted. The final version may differ from this version.

INTRODUCTION

Triethylenetetraamine (TETA) is a charge-isosteric analogue of spermidine with efficient copper chelating properties (Figure 1). Before 1969, the only practical treatment through copper chelation for Wilson's disease was D-penicillamine, which produces a wide variety of secondary effects, such as hepatotoxicity, fever, aplastic anaemia, leading to intolerance among a number of patients. Currently, TETA is used as a substitute for D-penicillamine (Roberts and Schilsky, 2008), and although it presents some adverse effects as well, they are less severe and less common than those caused by D-penicillamine (Gouider-Khouja, 2009). TETA reacts in a stoichiometric ratio 1:1 with copper and the complex is then excreted in the urine. TETA is also able to chelate and mediate excretion of iron and zinc *in vivo* (Kodama et al., 1997).

More recently, TETA has been found to alleviate secondary complications associated with diabetes. In a streptozotocin-model of type 1 diabetes, a daily TETA treatment for 8 weeks after the induction of diabetes suppressed kidney and glomerular hypertrophy (Gong et al., 2008) or cardiac damage without decreasing the circulating glucose levels (Lu et al., 2010a). In the case of type 2 diabetes, Zucker diabetic fatty rats treated with TETA demonstrated significantly reduced development of diabetic cardiomyopathy (Baynes and Murray, 2009). Although the exact mechanism of these beneficial effects is not known, Lu et al. suggested that copper chelation by TETA induces antioxidant defense mechanims, thus alleviating diabetes-associated complications (Lu et al., 2010a).

Kodama et al. were the first to report that TETA is readily acetylated in humans into N^1-monoacetyltriethylenetetramine (N^1AcTETA, Figure 1) (Kodama et al., 1997). Ten years later, by using LC-MS based methodology, Lu et al. identified that TETA can also be diacetylated in the form of N^1N^8-diacetyltriethylenetetramine (N^1N^8DiAcTETA, Figure 1) (Lu et al., 2007b). Actually, most of the urine-excreted TETA appears in the form of N^1AcTETA and N^1N^8DiAcTETA in humans, with huge individual variations (Lu et al., 2007a). Although TETA is acetylated to form N^1AcTETA and N^1N^8DiAcTETA *in vivo*, to date no enzyme has been definitely identified as responsible for TETA acetylation. Lu et. al. tested

DMD Fast Forward. Published on August 30, 2011 as DOI: 10.1124/dmd.111.041798
This article has not been copyedited and formatted. The final version may differ from this version.

N-acetyltransferase 2 (NAT2), which is known to acetylate aromatic amines, as a possible candidate for TETA acetylation. With no conclusive results with NAT2, they suggested that the rate-limiting enzyme in the polyamine catabolism, spermidine/spermine N^1-acetyltransferase (SSAT1), could be responsible for TETA acetylation (Lu et al., 2010b). SSAT1 is indeed a potential candidate due to the close chemical resemblance between its natural substrate spermidine and TETA (Figure 1). We recently have shown that SSAT1 *N*-acetylates TETA in cell cultures and also *in vitro* by using purified mouse recombinant protein (mSSAT1) (Weisell et al., 2010). In order to test this hypothesis *in vivo*, we have now taken the advantage of two genetically modified mouse lines, a SSAT1 overexpressing line (Pietilä et al., 1997) and another with disrupted SSAT1 expression (SSAT1-KO) (Niiranen et al., 2006).We used these mouse lines to test whether SSAT1 has a function in the TETA acetylation and whether it is the sole acetylating enzyme of TETA *in vivo*.

DMD Fast Forward. Published on August 30, 2011 as DOI: 10.1124/dmd.111.041798
This article has not been copyedited and formatted. The final version may differ from this version.

MATERIALS AND METHODS

Animal experiments

A transgenic mouse line overexpressing the SSAT1 gene under the control of its own promoter (Pietilä et al., 1997), a mouse line with disrupted SSAT1 gene (Niiranen et al., 2006), and their syngenic littermates were all on C57BL/6J background. A pilot study using doses of 100, 200 or 300 mg/kg i.p. of TETA was performed to study the drug tolerance and tissue accumulation and metabolism of TETA at 2 hours. The highest dose used was below the published LD_{50} of 468 mg/kg i.p. of TETA for mouse. In further experiments, the mice were injected with 300 mg/kg i.p. of TETA in PBS (Sigma-Aldrich Finland oy, Helsinki) or PBS alone for the control groups. The mice were sacrificed by CO_2 asphyxiation at one, two and four hours after administration of TETA. Each treatment group contained five age-matched male mice. Blood samples were taken by cardiac puncture and placed on heparinized tubes. Liver and kidney samples were removed and frozen immediately in liquid nitrogen and stored at -70 ºC until processing for analyses. The animal experiments were approved by the Animal Care and Use Committee at the Provincial Government of Southern Finland and carried out in accordance with the Declaration of Helsinki.

Analytical methods

Whole blood samples were processed to obtain plasma. The plasma samples were diluted 9:1 in 50% sulphosalisylic acid (SSA) solution containing 100 µM DAH (1,7-diaminoheptane) as an internal standard. Pieces of liver and kidney samples were homogenized in a buffer containing 25 mM Tris pH 7.4, 0.1 mM EDTA and 1 mM DTT using the TissueLyzer II (Qiagen, Hilden, Germany). The homogenates were diluted 1:9 in 5% SSA solution containing 10 µM DAH, kept on ice for 20 min and centrifuged at 14000 g for 30 min. Polyamines, TETA and its acetylated derivates were analyzed by HPLC following the previously published method by Hyvönen et al (Hyvönen et al., 1992). In the biological sample matrix stability test, aliquots of 200 µl of supernatant fractions were neutralized using 14 µl 2 M Na_2CO_3 in order

DMD Fast Forward. Published on August 30, 2011 as DOI: 10.1124/dmd.111.041798
This article has not been copyedited and formatted. The final version may differ from this version.

to prevent intramolecular *N*-acetyl migration of N^1AcTETA. Since ortho-phtaldialdehyde (OPA) can be used for primary amines only, the dansyl-Cl method was used to detect N^1N^8DiAcTETA in the 4 hour samples (Kabra and Lee, 1986). SSAT1, SSAT2 and the overall *N*-acetyltransferase activities were assayed as described by Coleman et al.(Coleman et al., 2004). Kinetic values of TETA acetylation for hSSAT1 were determined as described in (Weisell et al., 2010). Recombinant human SSAT1 and SSAT2 were kind gift from Professor A.E. Pegg (Pennsylvania State University, U.S.A).

NMR measurements

NMR samples were prepared in 5% SSA in H_2O (100 mM of N^1N^8DiAcTETA or N^1AcTETA) to activate the acid catalyzed N-acetyl rearrangement reaction and spectra were recorded on a Bruker AVANCE DRX spectrometer operating at 500.13 MHz using a double-tube system facilitating locking and chemical shift referencing. The external reference tube (o.d. 2 mm, supported by a Teflon adapter) containing the reference substance (sodium 3-trimethylsilyl[2,2,3,3-d_4]propionate (TSP) 40 mmol/l, $MnSO_4$ 0.6 mmol/l in 99.8% D_2O) was placed coaxially into the NMR sample tube (o.d. 5 mm) containing 400 µl of each sample. ^{1}H and ^{13}C NMR spectra were measured using standard protocols to follow up the reaction. After the reaction was "completed", the NMR samples were dried *in vacuo* and dissolved into DMSO-d_6 to detect the NH protons and long range NH-C couplings in 1D and 2D NMR spectra. ^{1}H-^{1}H homonuclear correlation (gradient-enhanced COSY) experiments were carried out in the magnitude mode. For each FID, four transients were accumulated. The ^{1}H–^{13}C gradient-enhanced heteronuclear single quantum correlation (HSQC) experiments were carried out in the phase-sensitive mode using the Echo/Antiecho-TPPI gradient selection. For each FID, 16 transients were accumulated. ^{1}H–^{13}C heteronuclear multiple bond correlation (HMBC) experiments were carried out with low-pass J-filter to suppress one-bond correlations. For each FID, 16 transients were accumulated. Window functions and j values were according to common practice in each of the experiments.

LC-MS measurements

DMD Fast Forward. Published on August 30, 2011 as DOI: 10.1124/dmd.111.041798
This article has not been copyedited and formatted. The final version may differ from this version.

Fresh standard samples of N^1AcTETA and N^1N^8DiAcTETA (1 mM) in 0.5% heptafluorobutyric acid (HFBA) were prepared prior to LC-MS measurements. The acetyl migration samples from the NMR measurements were diluted to 1 mM solution in 0.5% HFBA. Chromatographic separations were performed using Ultimate/Famos LC system (LC Packings, Amsterdam, The Netherlands) on a Phenomenex Gemini reversed phase C18 column (3 µm 50 mm x 2 mm, 110Å) protected with a Phenomenex C18 guard column (4 mm x 2 mm). A linear gradient was used starting from 98% of 0.1% HFBA in H_2O, 2% 0.1% HFBA in ACN to 50 % of 0.1% HFBA in H_2O, 50 % 0.1% HFBA in ACN in 16 min, at 200 µL/min. The data were recorded on a QSTAR XL hybrid quadrupole TOF instrument (Applied Biosystems, Foster City, CA, USA) in positive ionization mode using information-dependent acquisition (IDA) experiments for obtaining MS/MS data. During each run, TOF MS survey scans 1/s were recorded for mass range m/z 120-600 followed by 4 s MS/MS scans of the two most intense singly and doubly charged ions (mass range m/z 30-600). Ion-spray voltage was 5.5 kV. Nitrogen was used as a curtain- and nebulizer gas, with flow rates of 16 l/min and 14 l/min, respectively. Declustering and focusing potentials were set at 65 and 200 V, respectively. For the TOF scans, ion release time and width of six and five l s were used. For MS/MS IDA experiments, nitrogen was used as a collision gas and the energy was 25 eV. The operation and the spectral processing were performed on Analyst QS v1.1 software (Applied Biosystems).

Statistical analysis

Values are expressed as mean ± S.D. (n = 5 animals or 3 samples /group). The data was analyzed using the non-parametric Kruskal-Wallis test, when the test was significant pairwise comparison was performed by the Mann-Whitney test with the aid of the software package SPSS v 14.0 (SPSS, Chicago, IL, USA)

DMD Fast Forward. Published on August 30, 2011 as DOI: 10.1124/dmd.111.041798
This article has not been copyedited and formatted. The final version may differ from this version.

RESULTS

Metabolism of TETA *in vivo*

We first studied the tissue accumulation of TETA by injecting SSAT1-KO mice with 100, 200 or 300 mg/kg i.p. of the drug and determining the tissue and plasma levels of TETA and N^1AcTETA after two hours. The SSAT1-KO mice were selected for the pilot trials in order to see whether there were any signs of unexpected toxicity and whether TETA remained unacetylated when SSAT1 activity is not present. There was a significant increase in tissue levels of TETA and N^1AcTETA between 100 and 200 mg/kg doses but 300 mg/kg just moderately elevated TETA and N^1AcTETA levels in comparison to 200 mg/kg dose (data not shown). The highest dose of TETA (300 mg/kg) was, however, used in the subsequent metabolic studies because of the short half life of TETA in rodents (about 2-4 hours) (Lu et al., 2010b). Hepatic TETA and N^1AcTETA levels reached the maximum at 1 hour after injection, and hepatic N^1AcTETA levels were the highest in SSAT1 overexpressing mice (Table 1), suggesting that SSAT1 acted as *in vivo* acetylating enzyme of TETA. After one hour, no N^1AcTETA was detected in kidneys suggesting that the metabolism of TETA is active in liver (Table 2). Interestingly, SSAT1-KO mice metabolized TETA similarly to syngenic mice, suggesting that SSAT1 contributes only little to TETA acetylation in mice not overexpressing the enzyme (Table 1 and 2). In all genotypes, TETA treatment reduced both Spd and Spm levels in liver and kidney (Table 1 and 2).

Of the genotypes, SSAT1 mice had the lowest plasma levels of TETA, indicating the fastest clearance of the drug (Table 3). When the 4-hour time point samples from liver, kidney and plasma were subjected to precolumn dansyl-Cl derivatization and further analysis (Kabra and Lee, 1986), we were not able to detect N^1N^8DiAcTETA in any sample, although N^1N^8DiAcTETA-dansyl derivative was detectable in 20 pmol/50 µl level in standard assay mixtures. We also verified the applicability of the used method by mixing control mouse plasma sample with TETA, N^1AcTETA, and N^1N^8DiAcTETA and derivatized with dansyl-

DMD Fast Forward. Published on August 30, 2011 as DOI: 10.1124/dmd.111.041798
This article has not been copyedited and formatted. The final version may differ from this version.

Cl in order to detect the added drugs in a biological sample matrix. All the added drugs were detectable in the biological sample matrix at similar levels as in the standards (data not shown).

TETA as a substrate for thialysine acetyltransferase *in vitro*

Once we found that SSAT1-KO mice were able to efficiently *N*-acetylate TETA, we performed literature (PUBMED) and enzyme database (BRENDA; http://www.brenda-enzymes.info/) search in order to find potential *N*-acetylases capable of metabolizing TETA. Thialysine acetyltransferase (SSAT2) was selected as one potential candidate because of its ability to metabolize ethylenediamine and its structural similarity to SSAT1 (Abo-Dalo et al., 2004). SSAT2 has been cloned and is available as a recombinant human protein (Coleman et al., 2004). We performed enzyme kinetic studies with TETA and N^1AcTETA as substrates for SSAT2. SSAT2 had lower affinity for TETA (K_m 2.5 ± 0.3 mM) than for thialysine (K_m 0.29 mM) and the V_{max} was 3.96 ± 0.15 µmol/min/mg SSAT2 as determined by using the P81 disc method (Della Ragione and Pegg, 1982). This method could not be used for thialysine but by using spectrophotometric method the acetylation rate of TETA by SSAT2 was the same as determined for thialysine at 10 mM substrate concentration under the same conditions (Coleman et al., 2004). The acetylation rate of N^1AcTETA was about 10% at 10 mM as compared with TETA at 10 mM using spectrophotometric method (Coleman et al., 2004) (data not shown). Furthermore, we determined the kinetic values for acetylation of TETA using hSSAT1 in order to compare the properties of the human and mouse recombinant enzymes that show high structural homology (Hegde et al., 2007; Montemayor and Hoffman, 2008). hSSAT1 had K_m of 83 ± 7 µM and V_{max} of 0.90 ± 0.02 µmol/min/mg (Spd reference V_{max} of 9.09 ± 0.30 µmol/min/mg) that were similar to those determined earlier for mSSAT1 K_m of 169 ± 9 µM and V_{max} 1.37 ± 0.02 µmol/min/mg (Spd reference at 2500 µm V_{max} of 8.85 ± 0.40 µmol/min/mg) under the same conditions (Weisell et al., 2010).

Effect of TETA treatment on liver *N*-acetyltransferase activity

DMD Fast Forward. Published on August 30, 2011 as DOI: 10.1124/dmd.111.041798
This article has not been copyedited and formatted. The final version may differ from this version.

In order to dissect the *N*-acetylating activity in response to TETA treatment, *N*-acetyltransferase activities were determined from liver homogenates by using the P81 disc method (Della Ragione and Pegg, 1982) with 1 and 10 mM spermidine or TETA as a substrate. As shown in Table 4, TETA treatment induced *N*-acetylating activity similarly in syngenic and SSAT1-KO mice as analyzed with both 1 and 10 mM Spd or TETA. SSAT1 overexpressing mice exhibited the highest induction in *N*-acetylation activity but the relative induction was similar in all genotypes.

Acid-catalyzed chemical rearrangement of N^1AcTETA

Charge-deficient polyamine analogues, like 1,12-diamino-3,6,9-triazadodecane (SpmTrien) and TETA, are interesting because they resemble the natural polyamines spermine and spermidine (Figure 1), respectively, and are metabolized by SSAT1 *in vitro* to the corresponding acetylated derivatives (Weisell et al., 2010). When testing the substrate properties of N^1N^8DiAcTETA for acetyl polyamine oxidase [EC 1.5.3.13; APAO], we observed that the treatment of N^1N^8DiAcTETA reaction mixture with 5% (w/v) SSA (used to terminate enzymatic reactions) in a non-enzymatic control sample resulted in the formation of a novel compound (retention time of 18.1 min) containing a primary amine group (data not shown). Furthermore, the treatment of N^1AcTETA HPLC standard for 20 h with 5% SSA gave rise to two additional products, i.e. TETA and N^3-monoactetyltriethylenetetramine (N^3AcTETA), which had different retention times than N^1AcTETA when analyzed with HPLC using polyamine protocol (Figure 2). Testing the effect of trichloroacetic acid, perchloric acid and heptafluorobutyric acid, the commonly used acids for HPLC sample preparation or as ion pairing reagents, revealed that they all induced the chemical rearrangement of N^1AcTETA (Supplementary Table S1). This led us to hypothesize a possible mechanism of an *N*-acetyl group migration from the terminal position to the neighboring secondary amine group (Figure 1).

Stability of N^1AcTETA in biological sample matrix

DMD Fast Forward. Published on August 30, 2011 as DOI: 10.1124/dmd.111.041798
This article has not been copyedited and formatted. The final version may differ from this version.

We checked the stability of N^1AcTETA and TETA in a biological sample matrix by supplementing liver homogenate with the drugs (results shown in Supplementary Table S2). TETA was relatively stable for 24 hours and N^1AcTETA was slowly converted into N^3AcTETA as detected by an increase in the peak at the elution position of Spd since N^3AcTETA co-elutes with Spd that is present in liver homogenates. N^3AcTETA was quantitated using a standard curve for Spd that clearly seemed to overestimate the formation of N^3AcTETA. Neutralization of the sample matrix strongly retarded the rearrangement but hampered the quantitation of TETA and N^1AcTETA and could not be used for stabilizing HPLC samples containing TETA or its acetylated metabolites. Furthermore, 100 µM N^1N^8DiAcTETA in 5% SSA was estimated to be fully converted into N^1N^6DiAcTETA in 40 h at ambient temperature (data not shown). We quantitated N^1N^6DiAcTETA formation by using N^1AcTETA standard curve, because we did not have N^1N^6DiAcTETA as a synthesized drug. Rearrangement and relatively low fluorescent yield in dansyl-Cl derivatization may have limited the detection of N^1N^8DiAcTETA in biological samples.

NMR and LC-MS/MS analysis of intramolecular *N-N'* rearrangement

After the initial HPLC studies, a series of NMR and mass spectrometry measurements under acidic conditions were carried out to monitor the acetyl migration for both N^1AcTETA and N^1N^8DiAcTETA. The easiest method was ^{1}H NMR spectroscopy, since a new methyl signal appears at ca. 0.2 ppm downfield from the original acetyl methyl signal (Figure 3, Supplementary Figure S1). The novel methyl signal was split into two peaks, which is typical of tertiary amides due to *cis* and *trans* conformers in the structure arising from the partial double bond character of the C-N bond (Supplementary Figure S1). Similar doublets have been observed also for N^4AcSpd (Lurdes et al., 1989). The rest of the ^{1}H NMR spectra for the rearranged compounds N^3AcTETA and N^1N^6DiAcTETA were complicated due to same reason, but all of the novel signals which appeared during the migration process at NCH_2 region (3.75-3.15 ppm) were confirmed to belong to the rearranged products based on 2D NMR techniques (Supplementary Figure S3-

DMD Fast Forward. Published on August 30, 2011 as DOI: 10.1124/dmd.111.041798
This article has not been copyedited and formatted. The final version may differ from this version.

S5). In addition, the ^{13}C NMR spectra gave the same *cis* and *trans* conformers for the compounds N^3AcTETA and N^1N^6DiAcTETA (Supplementary Figure S2, data not shown).

After monitoring the acetyl group migration of N^1AcTETA by NMR for 12 days, a LC-MS sample was prepared and immediately analyzed. In this sample, two peaks were separated with the same molecular weight, supporting the hypothesis that the acetyl group has intramolecularly migrated from the terminal position to the nearby secondary amine (Figure 4, Supplementary Figure S6). Distinct daughter ions were also observed in the MS/MS spectra, and their expected structures based on the semi exact masses are shown in Figure 4. Furthermore, only a single peak at m/z 189 was observed in the freshly prepared sample of N^1AcTETA. To rule out any possible bias created by LC-MS instrumentation, the first sample was also spiked with N^1AcTETA and measured, as Lu et al. reported unresolved shoulder peaks in their LC-MS measurements from human urine sample after the per os administration of TETA (Lu et al., 2007b).

DMD Fast Forward. Published on August 30, 2011 as DOI: 10.1124/dmd.111.041798
This article has not been copyedited and formatted. The final version may differ from this version.

DISCUSSION

TETA has proven to be a very interesting drug molecule having potential in the treatment of several diseases (Lu, 2010). N^1AcTETA and N^1N^8DiAcTETA are characterized as the major metabolites of TETA in humans (Lu et al., 2010b), but the *N*-acetylase(s) responsible for TETA metabolism have remained uncharacterized (Lu, 2010). Here we demonstrated that SSAT1 acetylated TETA *in vivo*, as SSAT1 overexpressing mice metabolized TETA at an accelerated rate as compared to syngenic mice. Furthermore, we showed that in addition to SSAT1, TETA was monoacetylated *in vivo* also by other acetylases, as SSAT1-KO mice metabolized TETA in a similar way than the syngenic mice.

Although N^1AcTETA and N^1N^8DiAcTETA are the major metabolites of TETA in humans (Lu et al., 2010b), we detected only N^1AcTETA from mouse tissue samples. Thus, the metabolism of TETA may be different in rodents as compared to humans. Therefore, we will expand our future studies to rats and analyze serum/urine for any N^1N^8DiAcTETA in order validate the animal models in relation to metabolism of TETA in humans. Furthermore, our HPLC method is able to detect primary amines and our efforts to detect secondary amine containing compounds using the dansyl-Cl method may have failed because of low level of N^1N^8DiAcTETA in biological samples treated with 5% SSA. In order to reliably measure TETA, N^1AcTETA, N^1N^8DiAcTETA and their acetylated rearrangement products we are developing a feasible LC-MS/MS methodology using deuterium-labeled internal standards (Häkkinen et al., 2008). However, the current data clearly supports the view that SSAT1 is not the only enzyme potentially acetylating TETA. Although, SSAT1 posses similar substrate properties for TETA as for natural substrate spermidine the physiological relevance of SSAT1-mediated acetylation of TETA requires some further studies.

We found that human recombinant SSAT2 efficiently acetylates of TETA *in vitro*. SSAT2 enzyme has just recently been characterized, and its exact physiological role is still unknown (Cavallini et al., 1991; Coleman et al., 2004; Cooper, 2004; Han et al., 2006). Based on our present data, TETA is clearly a

DMD Fast Forward. Published on August 30, 2011 as DOI: 10.1124/dmd.111.041798
This article has not been copyedited and formatted. The final version may differ from this version.

substrate for human SSAT2 and may thus interfere with the enzyme protein and with the connected metabolic pathway *in vivo* by competing with the natural substrate thialysine. Interestingly, both SSAT2 and SSAT1 have been associated with the regulation of hypoxia-inducible factor 1α, which functions as a master regulator of oxygen homeostasis (Vogel et al., 2006; Baek et al., 2007a; Baek et al., 2007b). In a few comparative trials, TETA has exhibited enhanced activity as compared to other common metal chelators (Yoshii et al., 2001; Yu et al., 2006; Lu et al., 2010a), which may be attributed to its higher copper chelation selectivity and potency as compared to other tested chelators. However, the novel cellular targets of TETA are expected to explain the physiological background of its therapeutic potential in many diseases. Thus, detailed structure-activity studies using structural analogs closely resembling TETA are definitely warranted in distinct disease models in order to understand its drug action (Feng et al., 2009).

We have recently shown that accelerated polyamine flux by the induction of SSAT1 and simultaneous activation of ODC increases energy consumption and insulin sensitivity (Pirinen et al., 2007), while SSAT1-KO mice develop insulin resistance upon ageing (Niiranen et al., 2006). Furthermore, recent studies on TETA metabolism show that its acetylation is accelerated in type 2 diabetic patients (Lu et al., 2007a). Thus, it is tempting to speculate that type 2 diabetic patients display increased SSAT1 activity and consequently activated ODC, i.e. accelerated polyamine flux, as a physiological compensatory mechanism in order to enhance insulin sensitivity (Kramer et al., 2008). Furthermore, many stressful conditions have been shown to activate SSAT1/polyamine flux (Pegg, 2008), which leads to depletion of cellular ATP and generation of reactive oxygen species (ROS). Acetylation of TETA by SSAT1, may reduce ROS generation by polyamine-metabolizing enzymes (Babbar et al., 2007; Weisell et al., 2010), thus alleviating peripheral tissue damage among type 2 diabetic patients (Cerrada-Gimenez et al., 2011). In addition, copper dependent amino oxidases are inhibited by TETA. Copper and/or iron chelation affects to the regulation and the functions of HIF-1α and its potential interaction with SSAT1/2 renders TETA action very complicated (Baek et al., 2007a; Baek et al., 2007b; Feng et al., 2009). In the light of the previous

DMD Fast Forward. Published on August 30, 2011 as DOI: 10.1124/dmd.111.041798
This article has not been copyedited and formatted. The final version may differ from this version.

and present findings, it is clear that further studies with TETA and SpmTrien in animal models of type 2 diabetes and cancer models are warranted (Yu et al., 2006; Gupte and Mumper, 2009; Lu, 2010; Lu et al., 2010a).

In addition to the biochemical findings, we report here for the first time a intramolecular acetyl group migration from the less hindered nitrogen to the more hindered one in the terminally *N*-monoacetylated N^1AcTETA and *N*,*N*'-diacetylated N^1N^8DiAcTETA under acidic conditions. The observed intramolecular acetyl group migration was confirmed to proceed faster in 0.6 M HCl than in 5% SSA supporting the theory that the rearrangement is acid-catalysed. Furthermore, the rearrangement reaction was extremely slow when tri- or dihydrochlorides of N^1AcTETA or N^1N^8DiAcTETA, respectively, were incubated in water as compared to the samples incubated in 5% SSA. Moreover, the prolonged incubation of N^1N^8DiAcTETA in 5% SSA resulted in the formation of N^1AcTETA, N^3AcTETA, and finally TETA after deacetylations due to the lower stability of the tertiary amide in acidic water solution. Similarly, N^1AcTETA was decomposed to TETA via the same reaction pathway as N^1N^8DiAcTETA. The studies clearly implied that neutralization after acid treatment is required to retard or prevent N^1AcTETA and N^1N^8DiAcTETA rearrangement in HPLC samples. Thus, the observed rearrangement interferes with the quantitative measurements of TETA and its metabolites N^1AcTETA and N^1N^8DiAcTETA in biological samples. The detected chemical rearrangement during HPLC sample analysis may partly explain the huge individual differences reported earlier in TETA metabolism. Our data suggests that the acetyl migration can be retarded or prevented in samples when they are neutralized immediately after protein precipitation, but neutralization with Na_2CO_3 hampers TETA quantification by using HPLC.

Acknowledgments

We thank Professor A.E. Pegg (Pennsylvania State University, U.S.A) for providing human recombinant SSAT1 and SSAT2 enzymes. The authors thank Ms. Sisko Juutinen, Ms. Anne Karppinen, Ms. Arja Korhonen, Ms. Tuula Reponen and Ms. Maritta Salminkoski for skillful technical assistance.

Author contribution

Participated in research design: Cerrada-Gimenez, Weisell, Vepsäläinen and Keinänen.

Conducted experiments: Cerrada-Gimenez, Weisell, Hyvönen, Park and Keinänen.

Contributed to new reagents or analytic tool: Weisell, Alhonen and Vepsäläinen.

Performed data analysis: Cerrada-Gimenez, Weisell, Hyvönen, Vepsäläinen and Keinänen.

Wrote or contributed to the writing of manuscript: Cerrada-Gimenez, Weisell, Hyvönen, Park, Alhonen, Vepsäläinen and Keinänen.

DMD Fast Forward. Published on August 30, 2011 as DOI: 10.1124/dmd.111.041798
This article has not been copyedited and formatted. The final version may differ from this version.

References

Abo-Dalo B, Ndjonka D, Pinnen F, Liebau E and Luersen K (2004) A novel member of the GCN5-related N-acetyltransferase superfamily from Caenorhabditis elegans preferentially catalyses the N-acetylation of thialysine [S-(2-aminoethyl)-L-cysteine]. *Biochem J* **384:**129-137.

Babbar N, Murray-Stewart T and Casero RA, Jr. (2007) Inflammation and polyamine catabolism: the good, the bad and the ugly. *Biochem Soc Trans* **35:**300-304.

Baek JH, Liu YV, McDonald KR, Wesley JB, Hubbi ME, Byun H and Semenza GL (2007a) Spermidine/spermine-N1-acetyltransferase 2 is an essential component of the ubiquitin ligase complex that regulates hypoxia-inducible factor 1alpha. *J Biol Chem* **282:**23572-23580.

Baek JH, Liu YV, McDonald KR, Wesley JB, Zhang H and Semenza GL (2007b) Spermidine/spermine N(1)-acetyltransferase-1 binds to hypoxia-inducible factor-1alpha (HIF-1alpha) and RACK1 and promotes ubiquitination and degradation of HIF-1alpha. *J Biol Chem* **282:**33358-33366.

Baynes JW and Murray DB (2009) The metal chelators, trientine and citrate, inhibit the development of cardiac pathology in the Zucker diabetic rat. *Exp Diabetes Res* **2009:**696378.

Cavallini D, Ricci G, Dupre S, Pecci L, Costa M, Matarese RM, Pensa B, Antonucci A, Solinas SP and Fontana M (1991) Sulfur-containing cyclic ketimines and imino acids. A novel family of endogenous products in the search for a role. *Eur J Biochem* **202:**217-223.

Cerrada-Gimenez M, Pietilä M, Loimas S, Pirinen E, Hyvönen MT, Keinänen TA, Jänne J and Alhonen L (2011) Continuous oxidative stress due to activation of polyamine catabolism accelerates aging and protects against hepatotoxic insults. *Transgenic Res* **20:**387-396.

Coleman CS, Stanley BA, Jones AD and Pegg AE (2004) Spermidine/spermine-N^1-acetyltransferase-2 (SSAT2) acetylates thialysine and is not involved in polyamine metabolism. *Biochem. J.* **384:**139-148.

Cooper AJ (2004) The role of glutamine transaminase K (GTK) in sulfur and alpha-keto acid metabolism in the brain, and in the possible bioactivation of neurotoxicants. *Neurochem Int* **44:**557-577.

Della Ragione F and Pegg AE (1982) Purification and characterization of spermidine/spermine N1-acetyltransferase from rat liver. *Biochemistry* **21:**6152-6158.

Feng W, Ye F, Xue W, Zhou Z and Kang YJ (2009) Copper regulation of hypoxia-inducible factor-1 activity. *Mol Pharmacol* **75:**174-182.

Gong D, Lu J, Chen X, Reddy S, Crossman DJ, Glyn-Jones S, Choong YS, Kennedy J, Barry B, Zhang S, Chan YK, Ruggiero K, Phillips AR and Cooper GJ (2008) A copper(II)-selective chelator ameliorates diabetes-evoked renal fibrosis and albuminuria, and suppresses pathogenic TGF-beta activation in the kidneys of rats used as a model of diabetes. *Diabetologia* **51:**1741-1751.

Gouider-Khouja N (2009) Wilson's disease. *Parkinsonism Relat Disord* **15 Suppl 3:**S126-129.

Gupte A and Mumper RJ (2009) Elevated copper and oxidative stress in cancer cells as a target for cancer treatment. *Cancer Treat Rev* **35:**32-46.

Han BW, Bingman CA, Wesenberg GE and Phillips GN, Jr. (2006) Crystal structure of Homo sapiens thialysine Nepsilon-acetyltransferase (HsSSAT2) in complex with acetyl coenzyme A. *Proteins* **64:**288-293.

Hegde SS, Chandler J, Vetting MW, Yu M and Blanchard JS (2007) Mechanistic and structural analysis of human spermidine/spermine N1-acetyltransferase. *Biochemistry* **46:**7187-7195.

Hyvönen T, Keinänen TA, Khomutov AR, Khomutov RM and Eloranta TO (1992) Monitoring of the uptake and metabolism of aminooxy analogues of polyamines in cultured cells by high-performance liquid chromatography. *J. Chromatogr.* **574:**17-21.

Häkkinen MR, Keinänen TA, Vepsäläinen J, Khomutov AR, Alhonen L, Jänne J and Auriola S (2008) Quantitative determination of underivatized polyamines by using isotope dilution RP-LC-ESI-MS/MS. *J Pharm Biomed Anal* **48:**414-421.

Kabra PM and Lee HK (1986) Solid-phase extraction and determination of dansyl derivatives of unconjugated and acetylated polyamines by reversed-phase liquid chromatography: improved separation systems for polyamines in cerebrospinal fluid, urine and tissue. *J. Chromatogr.* **380:**19-32.

Kodama H, Murata Y, Iitsuka T and Abe T (1997) Metabolism of administered triethylene tetramine dihydrochloride in humans. *Life Sci* **61:**899-907.

Kramer DL, Diegelman P, Jell J, Vujcic S, Merali S and Porter CW (2008) Polyamine acetylation modulates polyamine metabolic flux, a prelude to broader metabolic consequences. *J Biol Chem* **283:**4241-4251.

Lu J (2010) Triethylenetetramine pharmacology and its clinical applications. *Mol Cancer Ther* **9:**2458-2467.

Lu J, Chan YK, Gamble GD, Poppitt SD, Othman AA and Cooper GJ (2007a) Triethylenetetramine and metabolites: levels in relation to copper and zinc excretion in urine of healthy volunteers and type 2 diabetic patients. *Drug Metab Dispos* **35:**221-227.

Lu J, Chan YK, Poppitt SD and Cooper GJ (2007b) Determination of triethylenetetramine (TETA) and its metabolites in human plasma and urine by liquid chromatography-mass spectrometry (LC-MS). *J Chromatogr B Analyt Technol Biomed Life Sci* **859:**62-68.

Lu J, Gong D, Choong SY, Xu H, Chan YK, Chen X, Fitzpatrick S, Glyn-Jones S, Zhang S, Nakamura T, Ruggiero K, Obolonkin V, Poppitt SD, Phillips AR and Cooper GJ (2010a) Copper(II)-selective chelation improves function and antioxidant defences in cardiovascular tissues of rats as a model of diabetes: comparisons between triethylenetetramine and three less copper-selective transition-metal-targeted treatments. *Diabetologia* **53:**1217-1226.

Lu J, Poppitt SD, Othman AA, Sunderland T, Ruggiero K, Willett MS, Diamond LE, Garcia WD, Roesch BG and Cooper GJ (2010b) Pharmacokinetics, pharmacodynamics, and metabolism of triethylenetetramine in healthy human participants: an open-label trial. *J Clin Pharmacol* **50:**647-658.

Lurdes M, Almeida S, Grehn L and Ragnarsson U (1989) Facile synthesis of monoacetylated spermidines, illustrating selective deacetylation and application of a common precursor. *Acta Chem. Scand.* **43:**990-994.

Montemayor EJ and Hoffman DW (2008) The crystal structure of spermidine/spermine N1-acetyltransferase in complex with spermine provides insights into substrate binding and catalysis. *Biochemistry* **47:**9145-9153.

Niiranen K, Keinänen TA, Pirinen E, Heikkinen S, Tusa M, Fatrai S, Suppola S, Pietilä M, Uimari A, Laakso M, Alhonen L and Jänne J (2006) Mice with targeted disruption of spermidine/spermine N1-acetyltransferase gene maintain nearly normal tissue polyamine homeostasis but show signs of insulin resistance upon aging. *J. Cell. Mol. Med.* **10:**933-945.

Pegg AE (2008) Spermidine/spermine-N(1)-acetyltransferase: a key metabolic regulator. *Am J Physiol Endocrinol Metab* **294:**E995-1010.

Pietilä M, Alhonen L, Halmekytö M, Kanter P, Jänne J and Porter CW (1997) Activation of polyamine catabolism profoundly alters tissue polyamine pools and affects hair growth and female fertility in transgenic mice overexpressing spermidine/spermine N1- acetyltransferase. *J. Biol. Chem.* **272:**18746-18751.

Pirinen E, Kuulasmaa T, Pietilä M, Heikkinen S, Tusa M, Itkonen P, Boman S, Skommer J, Virkamäki A, Hohtola E, Kettunen M, Fatrai S, Kansanen E, Koota S, Niiranen K, Parkkinen J, Levonen AL, Ylä-Herttuala S, Hiltunen JK, Alhonen L, Smith U, Jänne J and Laakso M (2007) Enhanced polyamine catabolism alters homeostatic control of white adipose tissue, and energy, and glucose metabolism. *Mol. Cell. Biol.*

Roberts EA and Schilsky ML (2008) Diagnosis and treatment of Wilson disease: an update. *Hepatology* **47:**2089-2111.

Weisell J, Hyvönen MT, Häkkinen MR, Grigorenko NA, Pietilä M, Lampinen A, Kochetkov SN, Alhonen L, Vepsäläinen J, Keinänen TA and Khomutov AR (2010) Synthesis and biological characterization of novel charge-deficient spermine analogues. *J Med Chem* **53:**5738-5748.

Vogel NL, Boeke M and Ashburner BP (2006) Spermidine/Spermine N1-Acetyltransferase 2 (SSAT2) functions as a coactivator for NF-kappaB and cooperates with CBP and P/CAF to enhance NF-kappaB-dependent transcription. *Biochim Biophys Acta* **1759:**470-477.

Yoshii J, Yoshiji H, Kuriyama S, Ikenaka Y, Noguchi R, Okuda H, Tsujinoue H, Nakatani T, Kishida H, Nakae D, Gomez DE, De Lorenzo MS, Tejera AM and Fukui H (2001) The copper-chelating agent, trientine, suppresses tumor development and angiogenesis in the murine hepatocellular carcinoma cells. *Int J Cancer* **94:**768-773.

Yu Y, Wong J, Lovejoy DB, Kalinowski DS and Richardson DR (2006) Chelators at the cancer coalface: desferrioxamine to Triapine and beyond. *Clin Cancer Res* **12:**6876-6883.

Footnotes

[†]Equal contribution of M. C-G. and J.W.

[‡]The research was supported in part by grants from the Intramural Research Program of National Institute of Dental and Craniofacial Research (NIDCR), NIH; the Academy of Finland; the North Savo Regional Fund of the Finnish Cultural Foundation, and by the University of Eastern Finland strategic funding.

[§]The authors declare no competing financial interest

DMD Fast Forward. Published on August 30, 2011 as DOI: 10.1124/dmd.111.041798
This article has not been copyedited and formatted. The final version may differ from this version.

FIGURE LEGENDS

Figure 1. Chemical structures of polyamines, SpmTrien, TETA and its metabolites, and postulated pathway for chemical intramolecular *N*-acetyl rearrangement of N^1AcTETA and N^1N^8DiAcTETA.

Figure 2. HPLC graphs of A) 100 µM N^1AcTETA in 5% SSA 0 h, B) 100 µM N^1AcTETA in 5% SSA after 20 h at room temperature. Table containing respective area analysis (µV · s) for individual analyte are shown below B. ND, not detectable.

Figure 3. Accumulation of the rearrangement products during the incubation of the compounds N^1AcTETA (left) and N^1N^8DiAcTETA (right) in aqueous 5% SSA.

Figure 4. LC-MS/MS analysis of N^1AcTETA. A) The extracted ion chromatogram (m/z 189) of N^1AcTETA after 12 days of incubation in aqueous 5% SSA showing separation and detection of N^1AcTETA and N^3AcTETA; B) The MS/MS spectrum of N^3AcTETA; C) The MS/MS spectrum of N^1AcTETA.

DMD Fast Forward. Published on August 30, 2011 as DOI: 10.1124/dmd.111.041798
This article has not been copyedited and formatted. The final version may differ from this version.

Table 1 Hepatic polyamine levels after administration of TETA

Mice were injected with TETA (300 mg/kg i.p. in PBS) or PBS only, and sacrificed at indicated time points. ND = not detectable (< 10 pmol/μg protein). Statistical analysis comparing genotypes (vs syngenic) with the same treatment group, *, $p < 0.05$; and **, $p < 0.01$.

Genotype	Time and treatment	Put	N^1AcSpd	Spd	Spm	N^1AcTETA	TETA
		(pmol/μg protein)					
Syngenic	Control	26 ± 0	ND	230 ± 36	322 ± 26	ND	ND
	TETA 1h	34 ± 3	ND	185 ± 4	247 ± 19	352 ± 85	796 ± 63
	TETA 2h	ND	ND	145 ± 42	287 ± 38	328 ± 37	244 ± 150
	TETA 4h	ND	ND	145 ± 52	306 ± 19	339 ± 81	135 ± 122
SSAT1	Control	156 ± 50	82 ± 26	345 ± 66*	167 ± 19**	ND	ND
	TETA 1h	126 ± 19	71 ± 6	311 ± 71**	142 ± 14**	625 ± 77*	934 ± 74*
	TETA 2h	247 ± 154	485 ± 363	364 ± 172*	136 ± 15**	945 ± 552	423 ± 170
	TETA 4h	253 ± 335	217 ± 93	233 ± 248	162 ± 29**	244 ± 56	137 ± 133
SSAT-KO	Control	10 ± 0	14 ± 4	284 ± 50	269 ± 31*	ND	ND
	TETA 1h	ND	ND	176 ± 9	238 ± 19	400 ± 60	796 ± 29
	TETA 2h	ND	ND	145 ± 28	302 ± 55	347 ± 44	194 ± 135
	TETA 4h	ND	ND	154 ± 53	302 ± 29	364 ± 90	146 ± 158

DMD Fast Forward. Published on August 30, 2011 as DOI: 10.1124/dmd.111.041798
This article has not been copyedited and formatted. The final version may differ from this version.

Table 2 Kidney polyamine levels after administration of TETA

Mice were injected with TETA (300 mg/kg i.p. in PBS) or PBS only, and sacrificed at indicated time points. ND = not detectable (< 10 pmol/μg protein). Statistical analysis comparing genotypes (vs syngenic) with the same treatment group, *, $p < 0.05$; and **, $p < 0.01$.

Genotype	Time and treatment	Put	N^1AcSpd	Spd	Spm	N^1AcTETA	TETA
		(pmol/μg protein)					
Syngenic	Control	59 ± 20	ND	118 ± 24	314 ± 47	ND	ND
	TETA 1h	49 ± 4	ND	124 ± 12	285 ± 24	ND	1348 ± 147
	TETA 2h	11 ± 1	ND	90 ± 18	255 ± 36	129 ± 38	422 ± 530
	TETA 4h	34± 29	ND	83 ± 26	263 ± 18	169 ± 26	265 ± 135
SSAT1	Control	138 ± 20	36 ± 11	103 ± 11	260 ± 19*	ND	ND
	TETA 1h	115 ± 12	47 ± 2	57 ± 18*	267 ± 18	ND	1015 ± 223
	TETA 2h	88 ± 17	44 ± 8	57 ± 23*	248 ± 12	144 ± 20	240 ± 244
	TETA 4h	115 ± 39	36 ± 12	57 ± 23	200 ± 24**	124 ± 21	265 ± 98
SSAT-KO	Control	39 ± 23	ND	154 ± 10*	290 ± 25	ND	ND
	TETA 1h	ND	ND	128 ± 14	263 ± 25	ND	1137 ± 262
	TETA 2h	30 ± 23	ND	95 ± 25	264 ± 21	152 ± 56	236 ± 283
	TETA 4h	37 ± 24	ND	98 ± 30	248 ± 27	173 ± 50	277 ± 141

DMD Fast Forward. Published on August 30, 2011 as DOI: 10.1124/dmd.111.041798
This article has not been copyedited and formatted. The final version may differ from this version.

Table 3 Plasma levels of TETA and N^1AcTETA after administration of TETA

Mice were injected with TETA (300 mg/kg i.p. in PBS) or PBS only, and sacrificed at indicated time points. Plasma samples were processed as described in analytical methods and TETA and N^1AcTETA concentrations, average μM ± SD (n= 5 animals) were determined with HPLC. Statistical analysis comparing genotypes (vs syngenic) with the same treatment group, **, $p < 0.01$.

Genotype	Time and treatment	N^1AcTETA *(μM)*	TETA
Syngenic	TETA 1h	171 ± 42	1255 ± 272
	TETA 2h	356 ± 78	1261 ± 730
	TETA 4h	174 ± 147	205 ± 97
SSAT1	TETA 1h	224 ± 173	625 ± 211**
	TETA 2h	279 ± 49	650 ± 159
	TETA 4h	133 ± 59	103 ± 72
SSAT-KO	TETA 1h	195 ± 108	800 ± 393
	TETA 2h	401 ± 36	1519 ± 523
	TETA 4h	162 ± 149	228 ± 111

DMD Fast Forward. Published on August 30, 2011 as DOI: 10.1124/dmd.111.041798
This article has not been copyedited and formatted. The final version may differ from this version.

Table 4 Effect of TETA on the activity of hepatic *N*-acetylases

Mice were injected with TETA (300 mg/kg i.p. in PBS) or PBS only, and sacrificed after 1h, 2h or 4h. *N*-acetylase activities were determined by using the P81 disc method using 1 and 10 mM Spd or TETA as a substrate. Statistical analysis comparing the different treatments within each genotype (0h control versus 1h, 2h or 4h TETA), *, $p < 0.05$, and **, $p < 0.01$.

Genotype	Time and treatment	Substrate			
		Spd, 1mM	Spd, 10mM	TETA, 1mM	TETA, 10mM
		pmol/10 min/mg liver wet weight			
Syngenic	Control	0.4 ± 0.2	1.3 ± 0.9	0.1 ± 0.1	2.6 ± 2.2
	TETA 1h	2.6 ± 0.8**	5.6 ± 1.8**	1.3 ± 0.4*	3.9 ± 1.6
	TETA 2h	2.7 ± 1.3**	5.5 ± 2.4**	1.0 ± 1.0	5.9 ± 2.8
	TETA 4h	1.8 ± 0.6**	4.2 ± 0.9**	1.6 ± 0.5*	4.4 ± 1.2
SSAT1	Control	4.8 ± 1.0	9.0 ± 1.6	1.5 ± 0.5	5.2 ± 1.2
	TETA 1h	16.6 ± 4.7**	25.7 ± 5.6**	4.0 ± 1.2**	8.2 ± 2.4
	TETA 2h	28.2 ± 9.0**	45.1 ± 10.1**	4.9 ± 1.6**	11.4 ± 2.3**
	TETA 4h	15.4 ± 12.5*	23.8 ± 17.3*	3.0 ± 1.7	6.9 ± 3.4
SSAT1-KO	Control	0.3 ± 0.1	1.5 ± 0.3	0.4 ± 0.3	2.9 ± 0.5
	TETA 1h	1.8 ± 1.2*	4.6 ± 2.5*	1.4 ± 0.9*	3.9 ± 2.2
	TETA 2h	2.5 ± 1.0**	2.3 ± 1.6	2.5 ± 2.1*	5.1 ± 2.2
	TETA 4h	1.7 ± 0.4**	4.5 ± 1.3**	1.6 ± 0.2**	4.3 ± 1.2

Figure 1

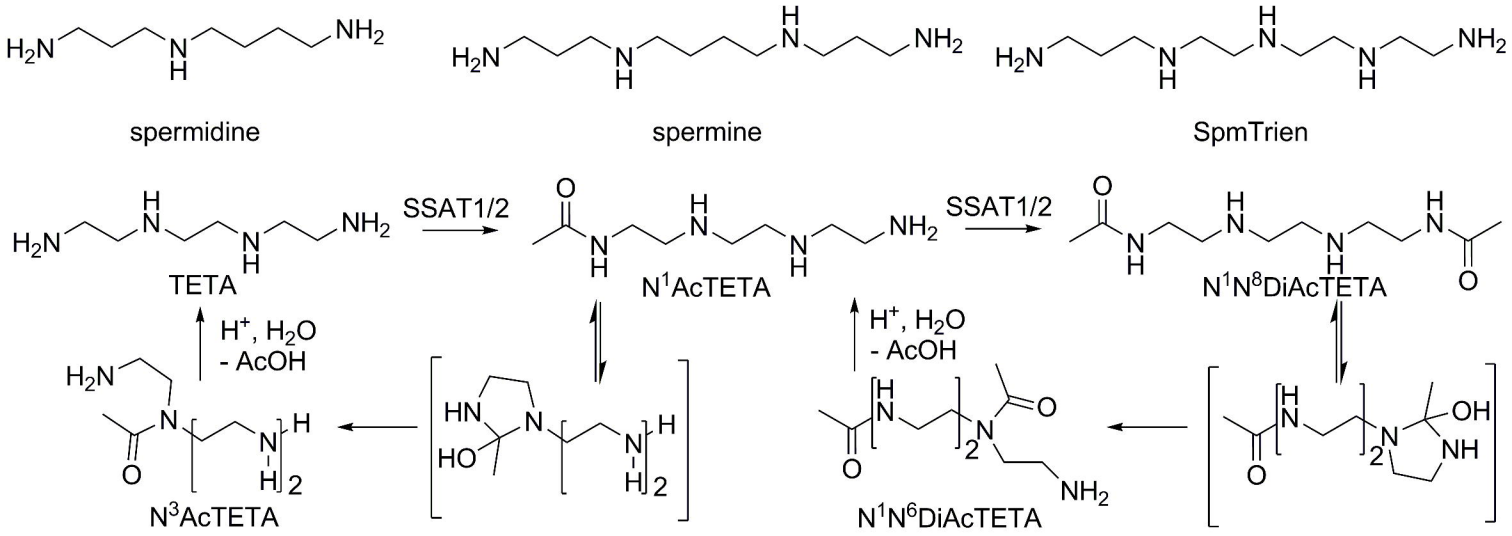
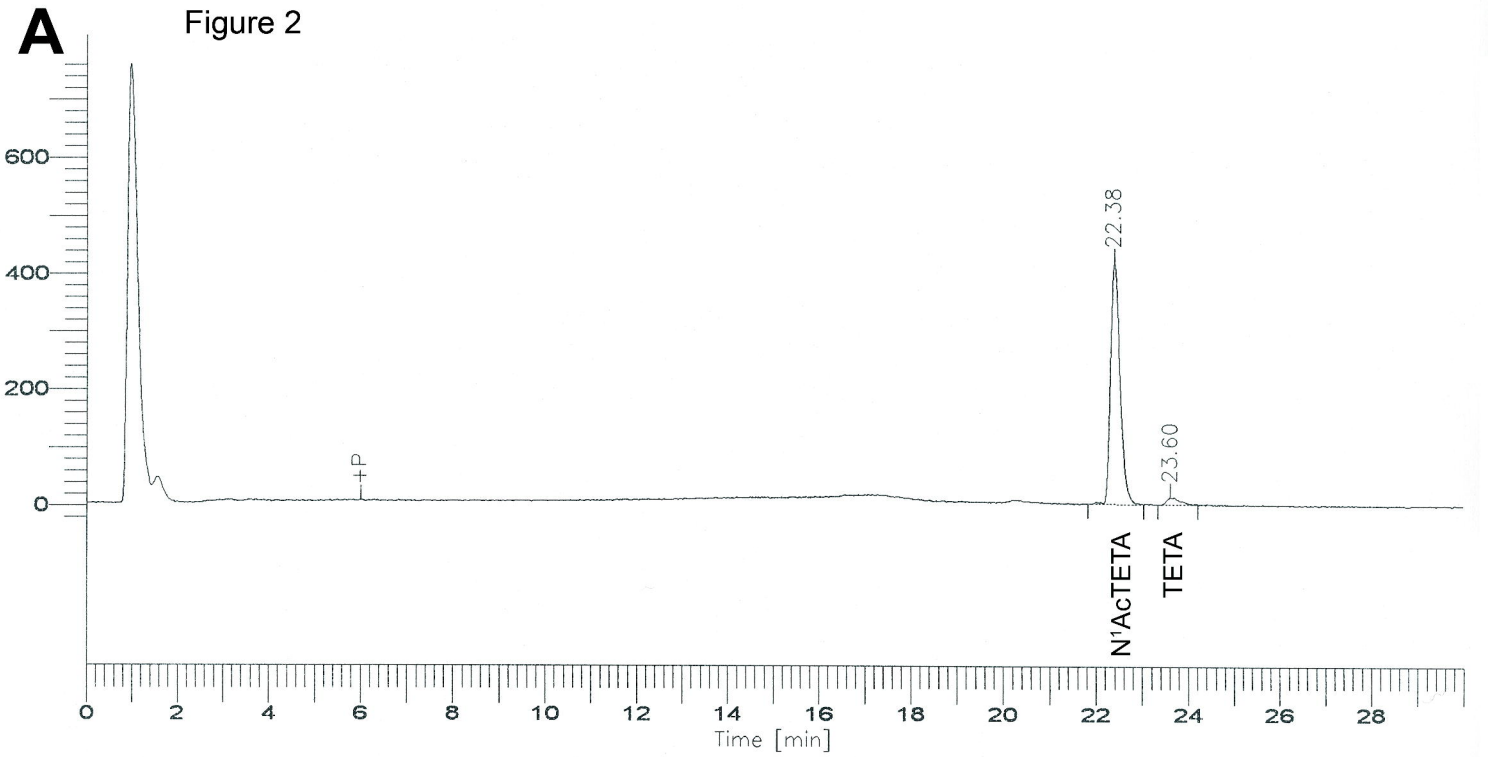
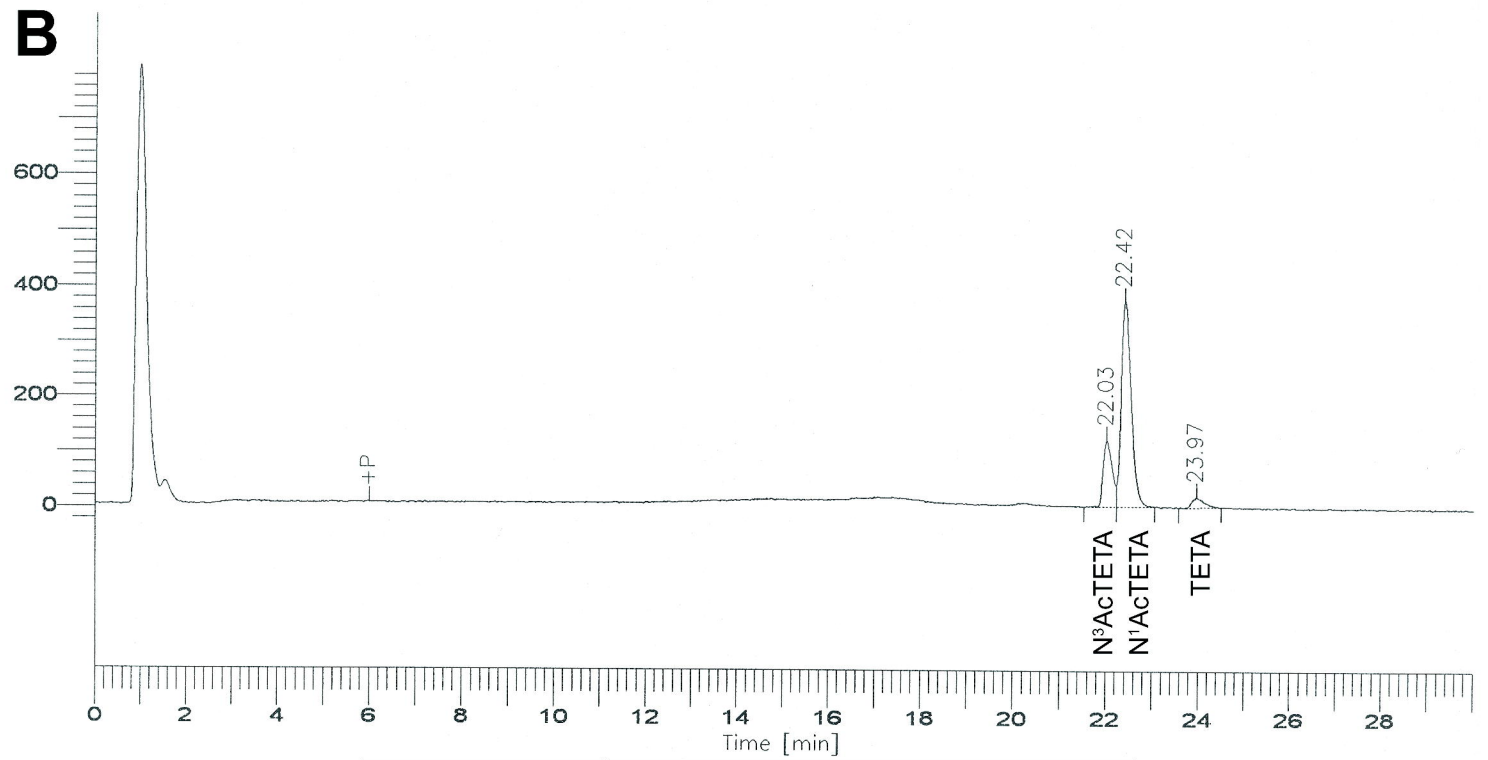

spermidine
spermine
SpmTrien
TETA
SSAT1/2
N1AcTETA
SSAT1/2
N1N8DiAcTETA
H+, H2O
- AcOH
H+, H2O
- AcOH
N3AcTETA
N1N6DiAcTETA

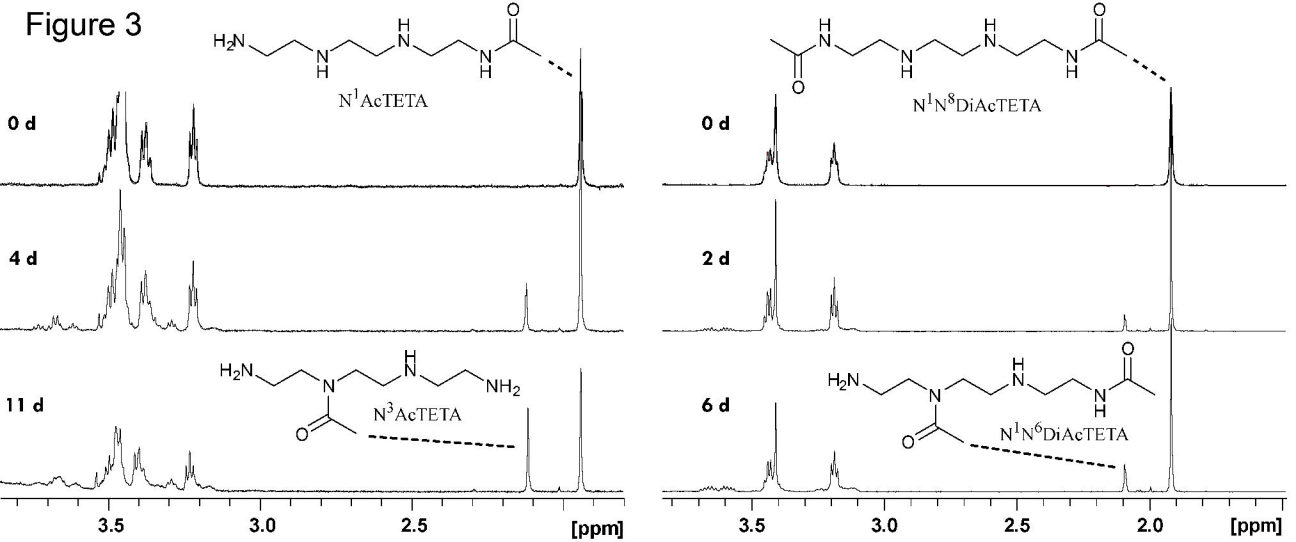
Figure 2

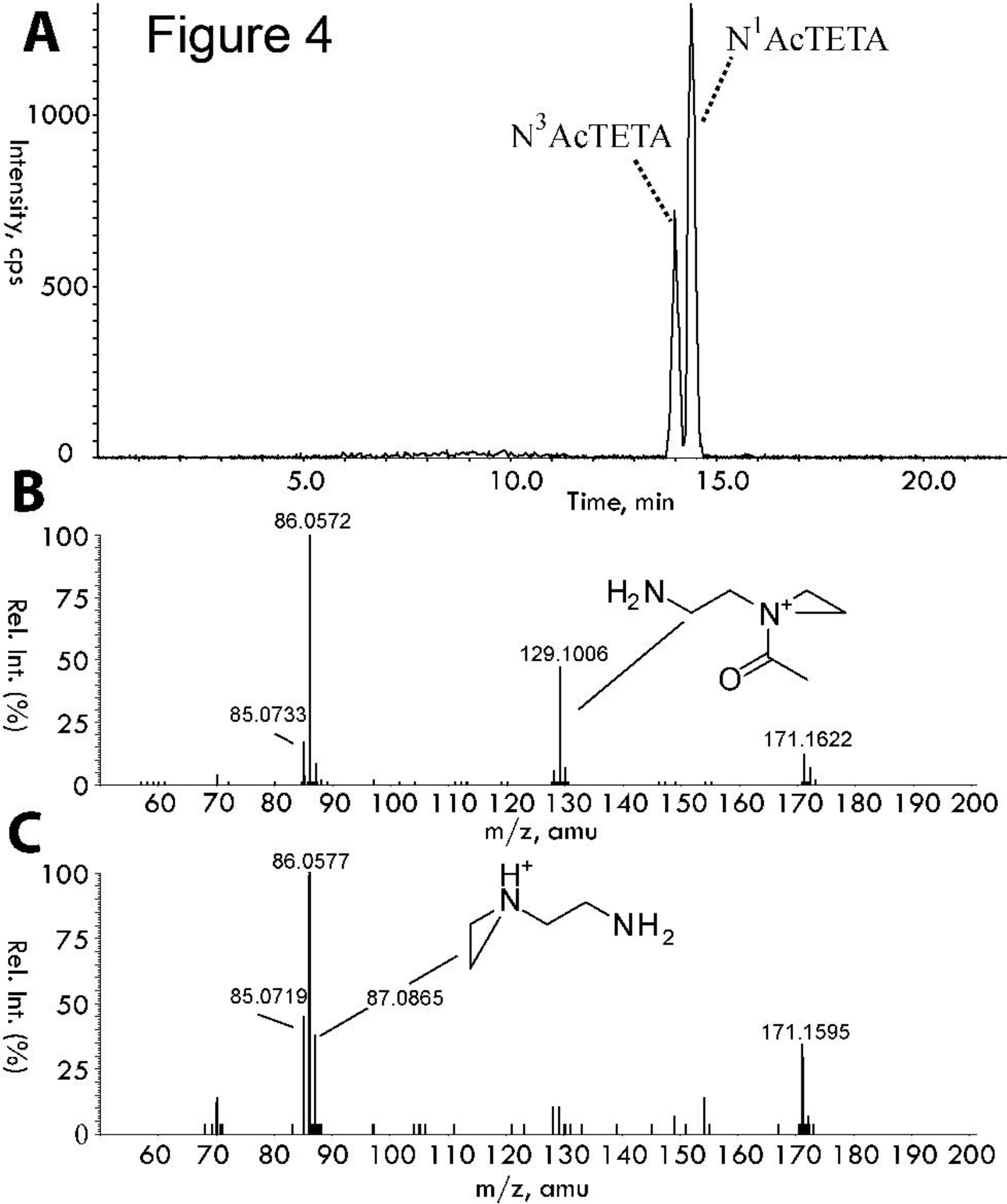




Analyte	A 0 h	B 20 h
	Area [µV·s]	
N^3AcTETA	ND	1609897
N^1AcTETA	6001708	5530137
TETA	259904	350970

Figure 3


N1AcTETA
0 d
4 d
11 d
N3AcTETA
3.5
3.0
2.5
[ppm]
N1N8DiAcTETA
0 d
2 d
6 d
N1N6DiAcTETA
3.5
3.0
2.5
2.0
[ppm]


A
Figure 4
N1AcTETA
N3AcTETA
1000
500
0
Intensity, cps
5.0
10.0
15.0
20.0
Time, min
B
86.0572
100
75
50
25
0
Rel. Int. (%)
H2N
N+
O
129.1006
85.0733
171.1622
60 70 80 90 100 110 120 130 140 150 160 170 180 190 200
m/z, amu
C
86.0577
100
75
50
25
0
Rel. Int. (%)
H+
N
NH2
85.0719
87.0865
171.1595
60 70 80 90 100 110 120 130 140 150 160 170 180 190 200
m/z, amu