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AMACE1: Versatile Aminoacetamide Electrophore Reagent

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Acetamide, 2-amino-*N*-[[3,5-bis(trifluoromethyl)phenyl]-methyl]-*N*-methyl-, monohydrochloride, which we have named AMACE1, was synthesized in three steps starting from *N*-tritylglycine. AMACE1 was coupled via its primary amine group (pK_a 8.2) under aqueous conditions to four model analytes for oxidative sugar damage to DNA: glycolate, 3-hydroxy-2-butanone, 3-phenylbutyraldehyde, and α-hydroxy-γ-butyrolactone, relying on cyanoborohydride for coupling to a keto function and a water-soluble carbodiimide for coupling to a carboxyl function. Further reaction with butyric anhydride led to products that could be detected by gas chromatography/electron capture mass spectrometry when 1 μL of ethyl acetate containing essentially 20 amol of each product was injected, on the basis of selected ion monitoring of the analyte characteristic anion fragment from dissociative loss of the 3,5-bis(trifluoromethyl)phenylmethyl moiety: *m*/*z* 215, 289, 299, and 329, respectively. Since many small, organic analytes contain a keto or carboxylic acid group, AMACE1 should be useful in general in the area of trace organic analysis.

Electrophores are substances that have a high electron affinity in the gas phase. This usually makes them very detectable with an electron capture detector[1,2] or an electron capture mass spectrometer,[2,4] traditionally after gas chromatography (GC-ECD and GC/EC-MS). Commonly an electrophore is a polyhalogenated organic compound, and small analytes that are not electrophores inherently often can become so by derivatization. For example, the detection of DNA adducts by electrophore derivatization-GC/EC-MS is of interest.[5,6]

There is a need to improve the detection of oxidative damage to the sugars of DNA, a class of DNA adducts.[7] In part more sensitive detection is needed so that small biological samples can be analyzed. This could help, for example, to elucidate the metabolic pathways that repair these lesions in humans. The large number of diverse, water-soluble products also makes their analysis challenging.[8] We decided to tackle this detection problem by using electrophore derivatization-GC/EC-MS.

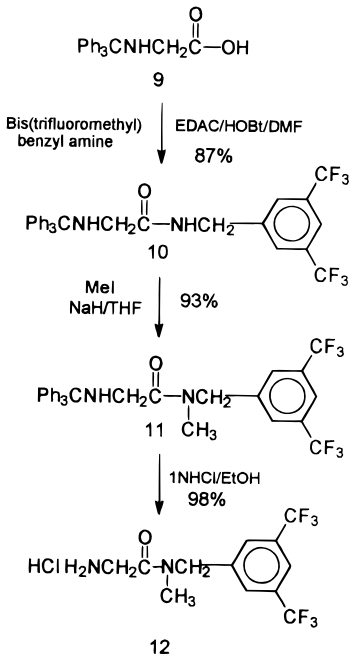

Figure 1. Synthesis of AMACE1 (**12**).

The presence of a keto, carboxylic acid, lactone, or aldehyde moiety on many of the DNA sugar oxidation products motivated us to develop an electrophore reagent containing an amino group with a relatively low pK_a so that all of these functional groups could be derivatized conveniently and efficiently under aqueous conditions. Further, we wanted to generate a reagent possessing an appropriately oriented, internal dissociative electron capture site in order to provide a sensitive, universal, analyte-characteristic response. This latter concept previously was introduced by others.[9] Here we report success in this overall endeavor by the preparation and testing of an aminoacetamide electrophore reagent that we have named "AMACE1" (a-mā′-sē-1).

EXPERIMENTAL SECTION

Reagents. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC) and 2-(*N*-morpholino)ethanesulfonic acid

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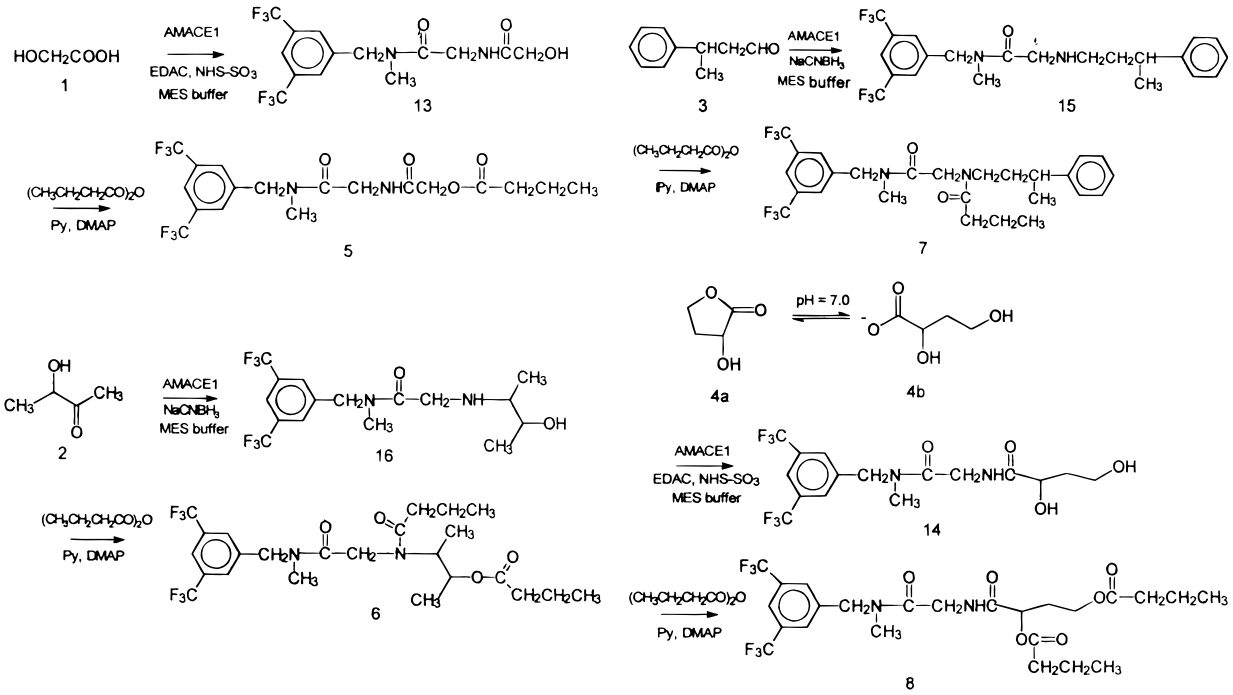

Figure 2. Synthesis of electrophoric derivatives of compounds **1**−**4**.

(MES) were from Sigma (St. Louis, MO). *N*-Hydroxysuccinimide-3-sulfonate (NHS-SO_3) was from Pierce Chemical Co. (Rockford, IL). All solvents were from Fisher Scientific (Pittsburgh, PA), and all reagents were from Aldrich (Milwaukee, WI) unless indicated otherwise. Anhydrous ethanol was prepared by distillation of anhydrous ethanol (Aldrich) from Mg/catalytic I_2, and converted to 1 N HCl/ethanol by HCl bubbling with weight monitoring. The pyridine (Py) was distilled and then stored over KOH. Immunopure epoxy-activated Agarose was from Pierce Chemical Co. (Rockford, IL). AMACE1 will soon be available from Fluka (Buchs, Switzerland) as product number 29245.

Procedures. Flash column chromatography was performed with silica gel 60 (170−400 mesh) from Fisher, and the bed was 19 mm × 45.7 cm ($d \times h$) unless indicated otherwise. ^{1}H and ^{13}C NMR were obtained on a Varian ZL300 NMR. GC/EC-MS spectra were recorded on a Hewlett-Packard 5973 by injecting 1μL of sample in ethyl acetate (Ultra from Fisher). All evaporations or concentrations were done on a rotary evaporator with water aspirator vacuum, and all extractive-washing steps were 3× with 0.1 volume, unless indicated otherwise.

2-Tritylamino-*N*-[(3,5-bis(trifluoromethyl)benzyl]acetamide (10). To a solution of *N*-tritylglycine (0.635 g, 2 mmol), 3,5-bis[(trifluoromethyl)benzyl]amine (0.535 g, 2.2 mmol), and 1-hydroxybenzotriazole hydrate (HOBt, 0.554 g, 4.1 mmol) in 4 mL of dimethylformamide (DMF) was added triethylamine (710 μL, 5.1 mmol) followed by EDAC (0.595 g, 3.1 mmol). The mixture was stirred overnight at room temperature, then quenched with 10 mL of water, extracted with ethyl acetate, and washed with ice-cold 0.5 N HCl, water, ice-cold 0.5 N NaOH, and brine. After neutral pH was confirmed with pH paper, the solution was dried over $MgSO_4$ and the solvent removed by evaporation. The resulting crude product was dissolved in 1 mL of ethyl acetate and purified by flash chromatography using 1:4 acetone/hexanes, monitoring 2-mL fractions by TLC-spotting UV quench, to give an oil (0.943 g, 77%): ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.80−7.60 (d, 3H), 7.55−7.20 (m, 15H), 5.12 (s, 2H), 3.24 (s, 2H), 1.5 (b, 1H).

2-Tritylamino-*N*-[(3,5-bis(trifluoromethyl)benzyl]-*N*-methylacetamide (11). Under N_2, to 0.814 g (1.5 mmol) of **10** in 15 mL of anhydrous (distilled from Na) tetrahydrofuran (THF) was added 0.060 g (1.5 mmol) of NaH (as a 60% dispersion in mineral oil; used as received) at −5 °C. The resulting mixture was stirred for 30 min and then 0.277 g (1.95 mmol) of MeI in 5 mL of THF was added dropwise over 30 min. The mixture was stirred for 4 h and then quenched by pouring into 30 mL of cold water saturated with NH_4Cl. The solution was extracted with ethyl acetate, and the combined organic layers were washed with brine, dried, and concentrated to give an oil that was purified by flash chromatography on silica gel with 1:4 acetone/hexanes to give an oil (0.78 g, 93%): ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.82−7.60 (d, 3H), 7.52−7.16 (m, 15H), 4.09 (s, 2H), 3.25 (s, 2H), 2.65 (s, 3H).

Acetamide, 2-Amino-*N*-[[3,5-bis(trifluoromethyl)phenyl]-methyl]-*N*-methyl-, Monohydrochloride (12) (AMACE1: Aminoacetamide Electrophore 1). To 0.556 g (1 mmol) of **11** was added 2 mL of 1 N HCl/EtOH (see Reagents), and the solution was hand-shaken in a 60 °C bath for 5 min. Cooling to room temperature gave an off-white flocculent precipitate which was scintered-glass filtered and washed with 5 mL of cold diethyl ether to give white cubes (0.344 g, 98%), mp 227−228 °C: ^{1}H NMR (DMSO, 300 MHz) δ 8.15 (b, 2H), 8.05−7.95 (d, 3H), 4.75 (s, 2H), 4.00 (s, 2H), 3.00 (s, 3H); ^{13}C NMR (DMSO, 300 MHz) 168.31, 142.10, 132.15, 131.80, 131.36, 129.66, 126.42, 122.73, 122.55, 51.09, 35.54. Calcd mass for M, 315.24; obsd, 314.9. pK_a = 8.2 by pH titration in 0 or 8% methanol.

Coupling of AMACE1 to Glycolic Acid, Yielding 13. A solution of 0.040 g (0.3 mmol) of glycolic acid, 0.148 g of EDAC (0.775 mmol), and 0.217 g of NHS-SO_3 (1 mmol) in 4 mL of 0.1 M MES buffer (pH 6.0, adjusted with NaOH) was stirred at room temperature for 15 min under N_2, followed by the addition of 0.175

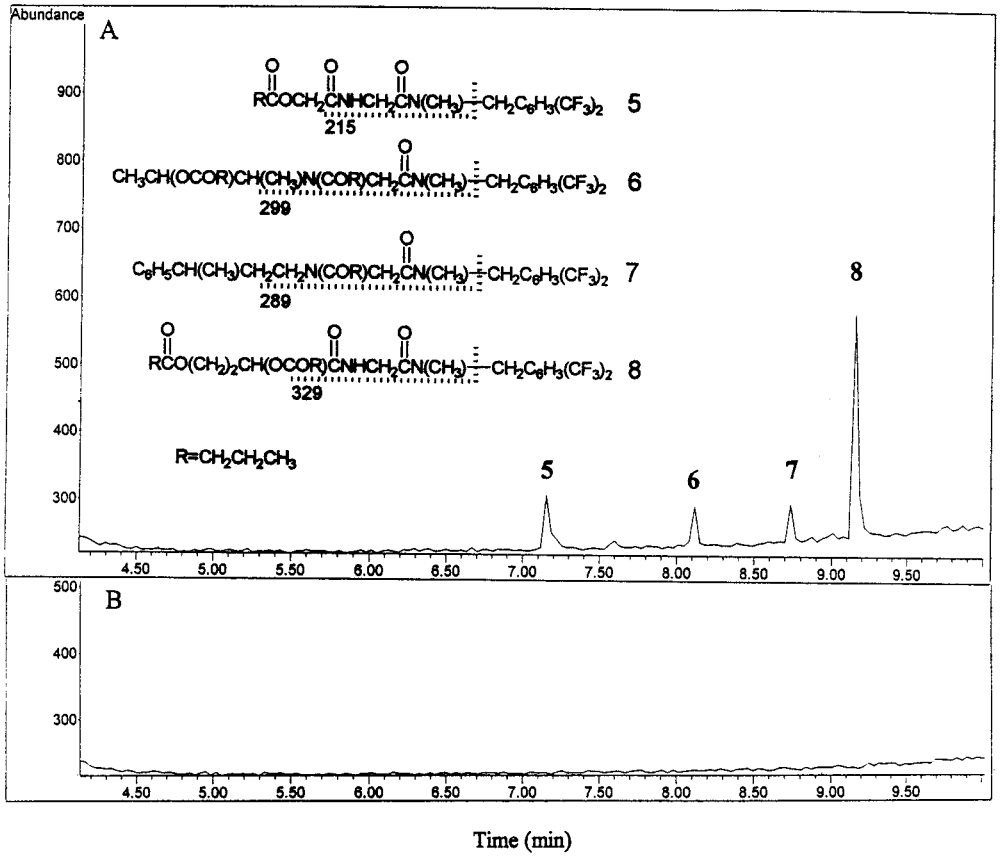

Figure 3. (A) Structures and fragmentation by electron capture of electrophoric derivatives **5**−**8** prepared by separately reacting compounds **1**−**4**, respectively, with AMACE1 followed by butyric anhydride, and mass chromatogram obtained by injecting 1 μL of ethyl acetate containing 10 fg each of these derivatives into a GC/EC-MS. (B) Blank chromatogram obtained by injecting ethyl acetate. GC/EC-MS conditions: model 6890 GC interfaced to a 5973 MS (Hewlett-Packard), Ultra 1 (cross-linked methyl siloxane) column, L = 50 m, i.d. = 0.2 mm, 0.11-μm film thickness; inject with column at 100 °C, then immediately ramp at 20 °C/min up to 300 °C, and hold for 10 min; multiple selected ion monitor *m*/*z* 215, 289, 299, and 329.

g (0.5 mmol) of **12**. After 6 h of further stirring, the reaction mixture was quenched with 15 mL of water and extracted with ethyl acetate. The extract was washed with ice-cold 0.5 N HCl, water, ice-cold 0.5 N NaOH, and brine and dried over $MgSO_4$. Evaporation then flash chromatography with 2:1 acetone/hexanes gave white needles (0.151 g, 81%), mp 138−139.5 °C: ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.91−7.82 (d, 3H), 7.25 (b, 1H), 4.85 (s, 2H), 4.24−4.21 (d, 2H), 4.06−4.02 (d, 2H), 3.15 (s, 3H).

Butyrylation of 13, Yielding 5. To a mixture of 0.149 g of **13** (0.4 mmol), 0.146 g of 4-(dimethylamino)pyridine (DMAP) (1.2 mmol), and 1 mL of pyridine in 5 mL of anhydrous acetonitrile (distilled from P_2O_5) was added 196 μL (1.2 mmol) of butyric anhydride and the mixture was stirred at 45−50 °C for 12 h, when TLC with 1:1 ethyl acetate/hexanes showed reaction completion. The reaction was quenched by pouring into 10 mL of ice water and extracted with ethyl acetate. The combined organic layers were washed with ice-cold 0.5 N NaOH, water, and ice-cold saturated aqueous NH_4Cl and dried over Na_2SO_4. Concentration gave a crude yellow oil which was purified by flash chromatography with 1:1 ethyl acetate/hexanes to give a colorless oil (0.127 g, 72%): ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.87−7.71 (d, 3H), 4.74 (s, 2H), 4.66 (s, 2H), 4.21−4.20 (d, 2H), 3.01 (s, 3H), 2.47−2.44 (t, 2H), 1.78−1.76 (m, 2H), 1.04−0.99 (t, 3H).

Coupling of AMACE1 to α-Hydroxy-γ-Butyrolactone (4a, b), Yielding 14. Compound **14** was prepared in the same way as **13** except pH 7.0 MES buffer was utilized in place of pH 6.0 MES buffer, giving a colorless oil or white plates (0.166 g, 80%), mp 121−123 °C: ^{1}H NMR($CDCl_3$, 300 MHz) δ 8.21−7.96 (d, 3H), 7.78−7.74 (d, 1H), 7.59−7.54 (m, 1H), 7.49−7.25 (m, 1H), 4.83 (s, 2H), 4.09−4.07 (d, 2H), 3.79−3.68 (m, 1H), 3.20 (s, 2H), 2.09 (s, 3H), 2.08−2.03 (m, 2H); ^{13}C NMR ($CDCl_3$, 300 MHz) δ 174.94, 169.18, 139.26, 132.38, 131.93, 128.06, 124.94, 121.81, 121.33, 71.40, 60.21, 50.93, 40.92, 36.07, 34.28.

Butyrylation of 14, Yielding 8. Compound **8** was prepared in the same way as **5** except that the molar amounts of pyridine, butyric anhydride, and DMAP relative to starting material were doubled, yielding a colorless light oil (0.162 g, 73%): ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.84−7.71 (d, 3H), 7.22 (b, 1H), 5.39−5.37 (t, 1H), 4.73 (s, 2H), 4.73−4.12 (m, 6H), 3.00 (s, 3H), 2.48−2.43 (m, 2H), 2.66−2.32 (m, 2H), 2.65−2.18 (m, 2H), 1.79−1.64 (m, 4H), 1.02−0.95 (m, 6H).

Attachment of AMACE1 to 3-Phenylbutyraldehyde (3) by Reductive Amination, Yielding 15. To a solution of 0.525 g (1.5 mmol) of AMACE1 in 4 mL of 0.1 M MES buffer (pH 6.0) was added 74 mg (0.5 mmol) of 3-phenylbutyraldehyde, followed by 33 mg (0.5 mmol) of $NaCNBH_3$. The mixture was stirred at room temperature under N_2 overnight, quenched into 15 mL of ice-cold, saturated, aqueous sodium bicarbonate, and extracted with ethyl acetate. The combined organic layers were washed with brine, dried over Na_2SO_4, and concentrated to give a yellow oil, which

was purified by flash chromatography with 3:5 ethyl acetate/hexanes to give a colorless light oil (0.156 g, 70%): ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.82, 7.70 (d, 3H), 7.34−7.16 (m, 5H), 4.70 (s, 2H), 3.48 (s, 2H), 2.94 (s, 3H), 2.89−2.79 (m, 1H), 2.62−2.48 (m, 2H), 1.9−1.81 (m, 2H), 1.29−1.25 (d, 3H).

Butyrylation of 15, Yielding 7. Compound **7** was prepared from **15** in the same way that **8** was prepared from **14**, yielding product as a colorless light oil (0.136 g, 66%): ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.83−7.70 (d, 3H), 7.32−7.19 (m, 5H), 4.67 (s, 2H), 4.14−3.98 (m, 2H), 3.36−3.25 (m, 2H), 3.62−3.60 (d, 3H), 2.72−2.66 (m, 1H), 2.36−2.31 (t, 2H), 2.10−2.05 (m, 2H), 1.89−1.81 (m, 2H), 1.72−1.57 (m, 2H), 1.32−1.30 (d, 3H), 1.01−0.98 (t, 3H).

Attachment of AMACE1 to 3-Hydroxy-2-butanone (2) by Reductive Amination, Yielding 16. Compound **16** was prepared from **2** in the same way that **15** was prepared from **3**, yielding a colorless light oil (0.115 g, 62%): ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.85−7.72 (d, 3H), 4.74 (s, 2H), 4.15−4.10 (m, 1H), 3.66 (s, 2H), 3.00 (s, 3H), 3.09−2.99 (m, 1H), 1.58−1.55 (d, 3H), 1.46−1.40 (d, 3H).

Butyrylation of 16, Yielding 6. Compound **6** was prepared from **16** in the same way that **8** was prepared from **14** except the reaction temperature was raised to 60−65 °C, yielding a colorless light oil (0.183 g, 61%): ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.85−7.70 (d, 3H), 4.96−4.91 (m, 1H), 4.82−4.59 (q, 2H), 4.32−3.70 (q, 2H), 4.11−4.03 (m, 1H), 3.08−3.05 (d, 3H), 2.99 (s, 3H), 2.46−2.11 (m, 3H), 1.78−1.63 (m, 4H), 1.31−1.28 (d, 4H), 1.03−0.90 (m, 6H).

RESULTS AND DISCUSSION

AMACE1 (**12**) was prepared by the scheme shown in Figure 1. As seen, *N*-tritylglycine was coupled to 3′,5′-bis(trifluoromethyl)-benzylamine using 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride, triethylamine, and 1-hydroxybenzotriazole in dimethylformamide (87% yield), followed by methylation with methyl iodide in NaH/tetrahydrofuran (93%) and deprotection using 1 N HCl anhydrous ethanol (98%).

AMACE1 was designed to have some favorable properties for our purposes, all of which have been realized. First, it possesses a primary amine as a functional group with a low pK_a of 8.2. This enhances the ease of amino coupling AMACE1 onto electrophilic sites (e.g., aldehydes, ketones, activated carboxyls) under aqueous conditions, as about to be demonstrated. Second, aldehyde-substituted scavenger supports are available to remove residual, primary amine reagents.[10,11] Third, this functional amino group exists as an α-aminoketone, which sets up another way to remove residual reagent: immobilized metal ion affinity chromatography.[12,13] Fourth, AMACE1 incorporates a novel electrophoric structure which efficiently yields an *N*-methylamide anion under electron capture conditions (see below). Fifth, the electrophoric 3,5-bis(trifluoromethyl)benzyl moiety makes our reagent easy to handle (UV-active and nonvolatile at room temperature) and is itself chemically inert.

As shown in Figure 2, true or model analytes for oxidative sugar damage, glycolate (**1**), 3-hydroxy-2-butanone (**2**), 3-phenylbutyraldehyde (**3**), and α-hydroxy-γ-butyrolactone (**4a**) were each tagged with AMACE1 by a direct nucleophilic (**4**), Schiff base/$NaCNBH_3$ (**2**, **3**), or carbodiimide-induced (**1**, **4b**) reaction, followed by reaction with butyric anhydride at residual active hydrogen sites, to form electrophores **5**−**8**. The reactions were conducted at the milligram level, and TLC showed essentially complete conversion of starting material to product in each case; isolated yields for the products were ≥60%.

Shown in Figure 3 is a GC/EC-MS chromatogram obtained by injecting a mixture containing 10 fg each of the products (ranging from 18 to 23 amol) as diluted standards. To obtain this multiple selected ion chromatogram, four ions were monitored, one for each of the analytes, on the basis of the intended, dissociative electron capture. In part, high sensitivity is achieved since the electron capture behavior of each derivative (full-scan data not shown) is dominated by the characteristic ion that is monitored in Figure 3.

Since the AMACE1 labeling reactions are all conducted in water under mild, similar conditions, potentially they can be conducted sequentially in the same vial. Work on this is in progress, along with effort to perform the overall analysis on oxidized sugar analytes at the trace level starting with a biological sample.

Prior to selecting butyrylation as a way to remove residual active hydrogens (NH and OH) in the intermediate products derived from the labeling reaction with AMACE1, we explored pivaloylation and acetylation. The former reaction gave high yields of product from compounds **13**−**15**, but the yield from **16** was very low, apparently for steric reasons. Acetylation gave good yields in all cases, but the acetyl products tailed more than the pivaloyl products by GC/MS. Butyrylation provided high yields throughout, and the peak shapes of the products were similar to those of the pivaloyl derivatives.

Other sugar and sugar-related analytes contain one or more of the functional groups that can be labeled by AMACE1, and so do other kinds of small analytes. AMACE1 should be useful in general for trace organic analysis, given the above-cited advantages including the aqueous coupling conditions.

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