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Note

Molar-response factors for the quantitative analysis of fully methylated methyl 2-acetamido-2-deoxyhexopyranosides by gas–liquid chromatography with flame-ionization detection

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Throughout the development in this laboratory of the reductive-cleavage method for the analysis of linkage positions and ring forms of the monosaccharide residues of complex carbohydrates, the effective carbon response (ECR) theory has been used for molar correction of GLC data for the anhydroalditol derivatives so obtained. This correction method, initially advanced by Ackman[1], has been shown to be valid for partially methylated alditol acetates[2] as well as partially methylated anhydroalditol acetates[3]. The ECR method corrects for a compound's response by flame-ionization detection (FID) by additive factors for the contributions of individual or groups of carbon atoms based on their functionality (hydrocarbon, alcohol, aldehyde, ether, etc.). The method has worked well for the derivatives obtained so far by the reductive-cleavage method because they are largely comprised of ether, ester, and aliphatic groups.

During the development of methods for the analysis of 2-acetamido-2-deoxyhexopyranosyl residues by the reductive-cleavage method, derivatives such as compound **1** were obtained[4]. The ECR method cannot be applied to **1** because existing rules for ECR calculations do not include factors for methyl glycosides and *N*-methylacetamido groups. Therefore, GLC-based techniques such as the reductive-cleavage method and standard methylation analysis are reliable only for identification, not quantification.

In order to establish the response factors for methyl glycosides and *N*-methylacetamido groups, mixtures containing known amounts of compounds **1**, **2**, and **3** were separated by GLC, and the relative integrals (peak area counts) of compounds **1** and **2** were compared to those of **3**. A triplicate set of standard solutions containing the three compounds was prepared from stock solutions of the individual compounds. Each of the standard solutions was analyzed in triplicate by GLC using cold on-column injection[5]. Studies by Conchie, et al.[6,7], and Neeser,

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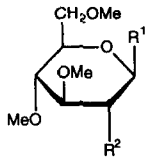
TABLE I

Summary of the GLC-FID and ^{1}H NMR data used to derive effective carbon response (ECR) values for compounds 1 and 2

Compound (x)	1			2		
Standard solution	A	B	C	A	B	C
Relative peak area (x:3) [a]	0.744	0.762	0.733	0.591	0.609	0.589
Molar ratio (x:3) [b]	0.546	0.551	0.538	0.508	0.519	0.487
$mRRF_x$ [c]	1.36	1.38	1.36	1.16	1.17	1.18
ECR_x [d]	0.682	0.691	0.681	0.582	0.587	0.591
Mean (SD)	0.684 (0.005)			0.586 (0.005)		

[a] Ratio of mean areas from three injections per solution. [b] From integrations of ^{1}H NMR spectra as described in the text. [c] $mRRF_x$ (molar relative response factor) = (relative peak area)/(molar ratio). [d] ECR_x (effective carbon response of x) = $ECR_3 \cdot mRRF_x$.

Schweizer, and co-workers[8–10] have demonstrated the superiority of this sampling technique for GLC of carbohydrates, including aminosugar derivatives. The relative peak areas of **1** and **2** were normalized to **3** and are given in Table I.



1 R^1 = OMe R^2 = N(Me)Ac
2 R^1 = OMe R^2 = OMe
3 R^1 = H R^2 = OMe

Because of the difficulty of obtaining accurate weights of **1** and **3**, which were prepared as oils, the relative molar amounts of the compounds in each mixture were determined by ^{1}H NMR spectroscopy by integration of the H-1 resonance of **2**, the H-1e resonance of **3**, and the sum of the integrals of the *N*-acetyl resonances of **1**. Three spectra were recorded for each standard solution for a total of nine spectra. Identical parameters were set for all spectra, including integral ranges. The relative molar ratios derived from these integrations were also normalized to the anhydroglucitol standard (**3**) and are given in Table I.

Empirical molar response factors for compounds **1** and **2** were derived as shown in Table I. The GLC integrals were adjusted for the relative molar amounts of each compound in the standard mixture as determined by NMR spectroscopy to give the molar relative response factor (mRRF). This value was then multiplied by the ECR value (0.500) of the internal standard (**3**), which was calculated by Ackman's method[1], to give the empirical ECR value. The ECR values were calculated from the data obtained for each of the standard solutions, and the means and standard deviations were then computed. The methyl glucoside (**2**) gave an ECR value of 0.586 ± 0.005, whereas the ECR value for the aminosugar (**1**) was

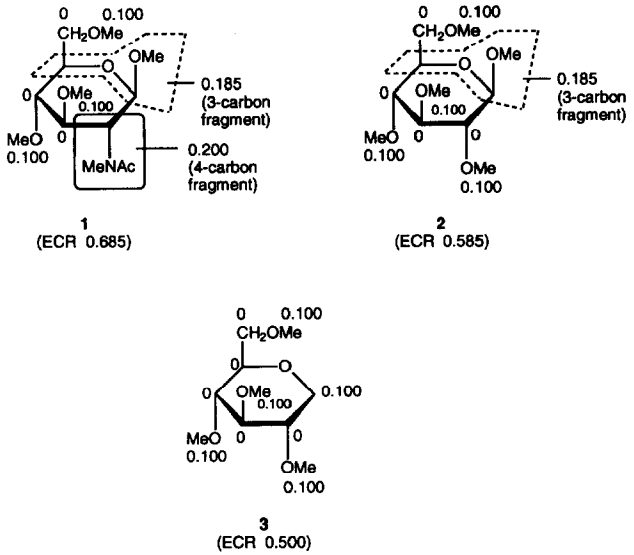


Fig. 1. Functional groups and ECR values for compounds **1–3**.

found to be 0.684 ± 0.005. For purposes of assigning ECR values to functional groups, these values were rounded to 0.585 and 0.685.

Error in the raw data (not shown) was compared as per cent relative deviation (standard deviation/mean). For the GLC data, the injection-to-injection relative deviations were 0.25–1.7%, and among the standard solutions, the deviations were 0.65% for **1**, 1.8% for **2**, and 1.3% for **3**. The NMR integral values gave relative deviations of 0.32–0.67% among spectra for each compound of each standard solution. The standard-to-standard relative deviations were 0.073% for **1**, 1.1% for **2**, and 0.21% for **3**. All sets of three data points for which means were calculated fell within the 90% confidence limit of the Q-test, which defines outlying data points[11]; therefore, no data set required a fourth point.

Functional groups were assigned ECR values as shown in Fig. 1. The difference in the ECR values between compounds **2** and **3** is 0.085. Thus, the ECR value assigned to the 3-carbon acetal group CH—O—CH—OMe is 0.185. The entire acetal group was considered for assignment of a response factor because it was not possible to account for the contribution of each carbon to the response of this group. Similarly, the entire four-carbon *N*-methylacetamidomethine group [CH–N(Me)Ac] of **1** was assigned the value 0.200.

EXPERIMENTAL

General.—^{1}H NMR spectra were recorded at a frequency of 500 MHz on a Varian VXR-500 spectrometer equipped with a VNMR data system. Samples were dissolved in CD_2Cl_2 (Cambridge Isotope Laboratories; dried over anhyd K_2CO_3)

and referenced to the instrument's internally set frequency for residual CH_2Cl_2 (δ 5.32).

Analytical GLC was performed on a Hewlett–Packard model 5890A gas–liquid chromatograph equipped with a Hewlett–Packard model 3392A integrator, dual flame-ionization detectors, a cool on-column inlet, and a split-splitless inlet operated in the split mode. The detector temperature was set at 275°C. The following conditions were used: *Method 1.* On-column injection into a fused-silica capillary column (0.25 mm × 30 m), wall-coated with DB-5 (0.25-μm film thickness; J&W Scientific), programmed from 40 to 250°C at 6°/min. *Method 2.* Split injection (injector temperature, 250°C; split ratio, 40:1) into the DB-5 column, programmed from 80 to 250°C at 6°/min. *Method 3.* Split injection (injector temperature, 250°C; split ratio, 10:1) into the DB-5 column or a fused-silica capillary column (0.25 mm × 30 m) wall-coated with Rt_x-200 (0.25-μm film thickness; Restek), programmed from 80 to 250°C at 2°/min. Each column was fitted with a J&W deactivated fused-silica guard column (0.32 mm × 1 m, *Method 1*; 0.25 mm × 1 m, *Methods 2* and *3*) via a press-tight connector (J&W or Supelco). Chromatography by GLC *Method 3* was performed on the two columns simultaneously by inserting a two-way (Y) press-tight capillary-column splitter (Restek) between the guard column and the analytical columns. Helium was used as the carrier gas at measured linear velocities (methane injection, oven temperature 80°C) of 26.1 cm/s (*Methods 2* and *3*) and 28.3 cm/s (*Method 1*) for the DB-5 column and 27.8 cm/s for the Rt_x-200 column. Retention indices were derived from data acquired by GLC *Method 3* by the linear-temperature-programmmed gas-chromatographic retention index (LTPGCRI) method as described by Elvebak, et al.[12].

Medium-pressure liquid chromatography (MPLC) was performed on 230–400 mesh Silica Gel-60 (E. Merck) on an instrument consisting of a 0.8 × 25-cm glass column, an Eldex model B-100-S-4 pump, a Scientific Systems model LP-21 pulse dampener and a Rheodyne 7125 injector. Thin-layer chromatography was performed on glass plates precoated with Silica Gel GF (Analtech); spots were visualized by charring with 5% H_2SO_4 in EtOH.

Methyl β-D-glucopyranoside was obtained from Eastman Chemical Co. 1,5-Anhydro-D-glucitol[13] and compound **1** (ref 4) were prepared as previously described. Compounds **3** and **2** were prepared by methylation of 1,5-anhydro-D-glucitol and methyl β-D-glucopyranoside, respectively, by the method of Ciucanu and Kerek[14]. The products were purified by MPLC: **2**, 1:4 EtOAc–hexane, R_f 0.32; **3**, 3:7 EtOAc–hexane, R_f 0.23. The purity of compounds **1**, **2**, and **3** was at least 99% by GLC *Method 2*. GLC retention indices (LTPGCRI method): **1** — DB-5, 1824.6; Rt_x-200, 2226.9; **2** — DB-5, 1436.7; Rt_x-200, 1572.8; **3** — DB-5, 1362.1; Rt_x-200, 1572.8.

Standards.—A stock solution of each standard (**1**, **2**, and **3**) was prepared by weighing samples into 5.00-mL screw-cap volumetric flasks. The flasks were filled to their marks with CD_2Cl_2 and then sealed with Teflon-lined caps. The nominal concentrations of these stock solutions, that is, their concentrations based on mass,

ranged from 55 to 115 mM. Standard solutions containing **1**, **2**, and **3** were prepared in triplicate by transferring aliquots (50 μL) of each stock solution via Kirk volumetric micropipettes to 5.00-mL screw-cap volumetric flasks and then diluting to their marks with CD_2Cl_2.

Analysis.—In lieu of accurate mass measurements in the preparation of the stock solutions, the relative molar ratios of **1**, **2**, and **3** in the standard solutions were determined by integration of selected resonances in the ^1H NMR spectra of the mixtures. Three spectra were recorded for each of the three standard solutions under identical conditions, including the following parameters: sweep width, 3500 Hz; sampling rate, 0.25 Hz/point; pulse width, 10 ms; acquisition time, 2.0 s; and recycle delay, 4.0 s. Resonances used for integration were H-1 of **2** (δ 4.11, d, 1 H), H-1e of **3** (δ 4.00, dd, 1 H), and *N*-Ac of **1** (δ 2.08, 2.07, 2 s, rotamers, 3 H total), and their area ratios were normalized to the H-1e resonance of **1**. Each of the standard solutions was analyzed in triplicate by GLC (*Method 1*) using on-column injection. Thus, each compound was analyzed nine times. The integrated areas for the peaks were converted to ECR values as described in Table I.

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