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Determination of N^9/N^7-isomer ratio of alkyl (guaninyl)acetates by electrospray ionization tandem mass spectrometry

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N^9- and N^7-Substituted (guaninyl)acetic esters were studied by electrospray ionization tandem mass spectrometry (ESI-MS/MS) in order to determine their ratio in alkylation reactions. The loss of ammonia is significantly different for the N^9- and N^7-alkylated guanine regioisomer pairs. More importantly, the abundance of the $[MH-17]^+$ ion is in linear correlation with the N^9-isomer content. Therefore, the ratio of regioisomers can be determined in a mixture containing these compounds. Copyright © 2004 John Wiley & Sons, Ltd.

N^9-Substituted analogs of guanine nucleosides have been extensively investigated for their biological activity especially as anticancer and antiviral compounds.[1] They are important as building blocks ((guanin-9-yl)acetic acid) of antisense and antigene oligonucleotide analogs, e.g. peptide nucleic acids (PNAs).[2] Unfortunately, the glycosylation and alkylation of guanine and related compounds, e.g. 2-amino-6-chloropurine, is not regioselective, forming the unwanted N^7-regioisomers as side products.[3] The yield of the product can be improved by optimization of reaction conditions, the effectiveness of which can be improved by a fast analytical method avoiding separation of regioisomers. The sensitivity of the method is also important in order to optimize the alkylation conditions on a few mg scale accompanied by a fast thin layer chromatography (TLC) purification.

The site of the alkylation can be ascertained most conveniently by using two-dimensional nuclear magnetic resonance (2D-NMR) (^{1}H, ^{13}C) techniques, but the chemical shift differences of some diagnostic ^{13}C signals[4] and/or chemical shift of N-3[5] are also very informative. Unfortunately, the latter two methods, unlike ^{1}H NMR, give only qualitative information and at least 5 mg of pure solvent free sample is required, usually preceded by chromatographic purification (e.g. TLC). Both the purification and the NMR analysis take at least 10–20 min. Furthermore, expensive deuterated solvents are required for the latter procedure.

High-performance liquid chromatography (HPLC) analysis would be another possible approach but this should be preceded by NMR measurement to have a standard sample. Furthermore, a gradient system for the separation of regioisomers is required and a HPLC run takes 10–20 min/sample, (thus in the case of 10 parallel reactions 100–200 min) and a large volume of high purity solvent. This is not the case with a tandem mass spectrometry (MS/MS) method, in which a few μL of test reaction mixture is sufficient after dilution. The criteria of the applicability are a significant difference in the fragmentation pathways of the two regioisomers and a product ion whose abundance is proportional to the ratio of the isomers. Details of the derivatives of N^7- and N^9-alkylated guanine derivatives studied by MS/MS are collected in Table 1.

Usually, N^7- and N^9-isomer pairs are identified by their product ion spectra but in some cases there is only a slight difference between the spectra. The most unambiguous differentiation between 9- and 7-methylguanines was achieved by chemical ionization (CI)-MS/MS using NH_3 as the CI gas in which the loss of CH_3NH_2 was characteristic exclusively for the N^7-isomer.[6] In the case of β-*O*-activated alkyl groups, e.g. $CH_2OCHR^1R^2$, the abundance of ions derived from the side chains was much higher for the N^9-isomer. Therefore, the identification was based on this fact.[10] These studies have, however, afforded only qualitative information.

The fragmentation of the protonated guanine backbone was confirmed by ^{15}N and ^{16}O labelling[13] and H/D exchange,[12] proving that losses of ammonia and cyanamide are independent and principal pathways, followed by loss of CO and HCN.

(N^2-Acyl-guanin-9-yl)acetic esters **1** (9IbuMe), **3** (9IbutBu)[4] and **5** (9PnttBu)[14] have been synthesized in our laboratory as PNA monomer building blocks and studied by electrospray ionization tandem mass spectrometry (ESI-MS/MS) along with their N^7-regioisomer pairs **2** (7IbuMe) and **4** (7IbutBu), respectively[4] (Fig. 1). The side chain of these compounds is an acetic ester derivative, which is different from those previously studied. It is therefore interesting to consider how this will influence the fragmentation of the regioisomers. Our aim was to determine the N^9/N^7-isomer ratio by a simple and time- and cost-effective MS/MS method finding optimal collision-induced dissociation (CID) conditions where a significant difference can be observed in the fragmentation of regioisomers.

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Table 1. Structures of alkylated guanine derivatives studied by MS/MS (CI-MS/MS,[6] ESI-MS/MS,[7,12] FAB-MS/MS[8–11]) methods

Alkyl side chain[a]	The site of the alkyl group[a]
Me,[6–8] Et,[8] Pr[9]	7,[6–9] 9[6,8]
CH_2CRHOH; R = H, CH_3, CH_2OH	7[9]
$CH_2OCH_2CH_2OR$; R = H, Ac	7,[10,11] 9,[10,11] 9[12] (R = H)
$CH_2OCH(CH_2OR)_2$; R = H, Bn	7[11], 9[11]

[a] Refs. in superscripts.

EXPERIMENTAL

Mass spectrometric measurements were obtained on a Finnigan TSQ-7000 triple quadrupole mass spectrometer (Finnigan-MAT, San Jose, CA, USA) equipped with a Finnigan ESI source. The instrument was scanned in positive ion mode over the mass range *m/z* 160–230 with a scan time of 0.1 s. Samples of 5 μL were injected into the eluent stream of a pump (Applied Biosystems 140 C (Foster City, CA, USA); flow rate: 150 μL/min, eluent: methanol/water/acetic acid 50:50:1 (v/v/v), sample concentration: 0.1 mg/mL).

The electrospray needle was adjusted to 4.5 kV and N_2 (pressure: 3.45 bar) was used as the nebulizer gas. The collision energy was varied between −10 and −70 eV to find the optimal value (−35 eV for compounds **1** (9IbuMe) and **2** (7IbuMe); −45 eV for compounds **3**–**5** (9IbutBu, 7IbutBu and 9PnttBu)). At this value the abundance of the $[M_f]^+$ ion was 100% and that for the $[M_f-17]^+$ ion was almost the same. The collision gas was argon and the pressure in the collision cell region was set at 2 ± 0.1 mTorr.

RESULTS AND DISCUSSION

Our compounds (see Fig. 1) containing different N^2-acyl protecting groups (isobutyryl in **1** (9IbuMe), **2** (7IbuMe),

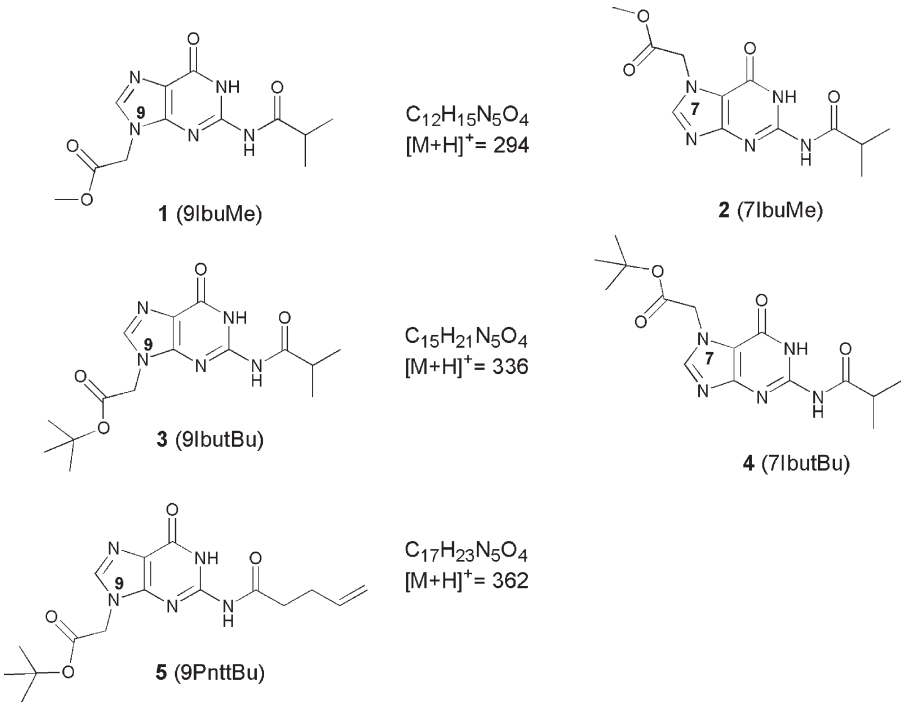

Figure 1. Structures of alkyl (guaninyl)acetates (**1**–**5**, 9IbuMe-9PnttBu) studied by ESI-MS/MS.

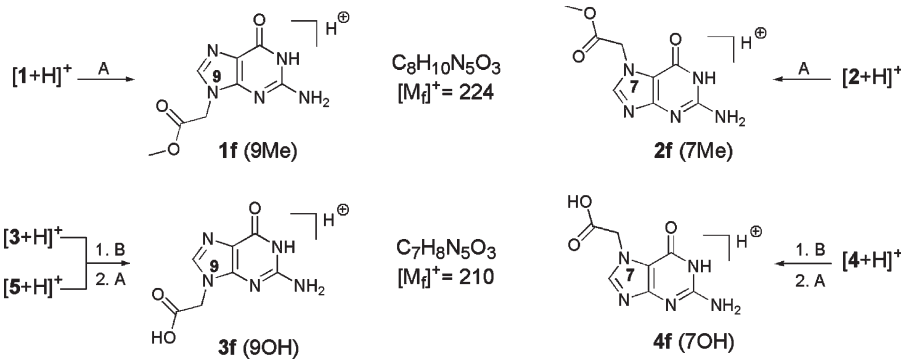

Figure 2. Structures of compounds **1f** (9Me), **2f** (7Me), **3f** (9OH) and **4f** (7OH) formed from alkyl (guaninyl)acetates (**1**–**5**) in MS/MS experiments at −30 eV. Process A: deacylation of the N^2-amino group, process B: hydrolysis of the *tert*-butyl ester. $[M_f]^+$ denotes the *m/z* value of the product ion (**1f**–**4f**).

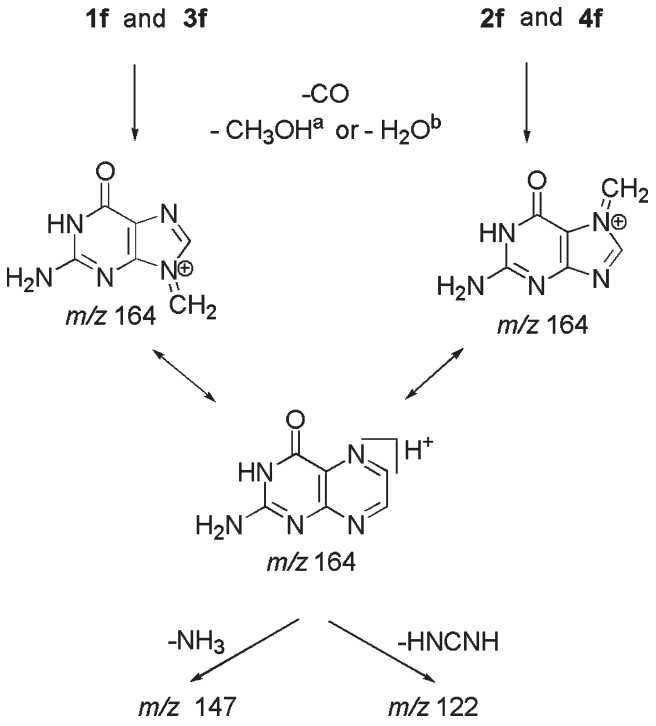

Figure 3. Formation and fragmentation of the ion at *m/z* 164 (a) from compounds **1f** (9Me) and **2f** (7Me); and (b) from compounds **3f** (9OH) and **4f** (7OH).

3 (9IbutBu), and **4** (7IbutBu) and pent-4-enoyl in **5** (9PnttBu)) and alkyl moieties in ester groups (methyl in **1** (9IbuMe) and **2** (7IbuMe) and *tert*-butyl in **3** (9IbutBu) and **4** (7IbutBu)) could give useful information on whether these groups have an effect on fragmentation pathways. The loss of the *tert*-butyl ester (Fig. 2, process B) was followed by dissociation of the N^2-acyl group (Fig. 2, process A). Thus the difference caused by these groups disappeared to such an extent that compounds **3** (9IbutBu) and **5** (9PnttBu) led to the same acid **3f** (9OH). Therefore, the fragmentation of differently protected compounds was similar. The free acid form (**3f** (9OH) and **4f** (7OH)) rendered the side chains unstable at higher energies in an MS/MS study.

The product ions **1f** (9Me), **2f** (7Me), **3f** (9OH) and **4f** (7OH) were the most abundant at −30 eV collision energy (for structures, see Fig. 2). The **1f**–**4f** ions are precursors of lower mass product ions; therefore, the further fragmentation steps can be more easily compared if we derive them from the f series (*m/z* values of the f series are indicated by $[M_f]^+$).

The major fragmentation pathways (losses of ammonia and cyanamide) of ions **1f** (9Me), **2f** (7Me), **3f** (9OH) and **4f** (7OH) are in good agreement with observations by Gregson and McCloskey[13] and Kamel and Munson.[12] Losses of methanol (**1f** (9Me), **2f** (7Me)) or water (**3f** (9OH) and **4f** (7OH)) and carbon monoxide also occurred, depending on the nature of side chain, leading to the ion at *m/z* 164, the rearrangement of which to pterine was suggested by Kralj *et al.*[10] (see Fig. 3). The *m/z* 164 ion loses NH_3 and cyanamide,

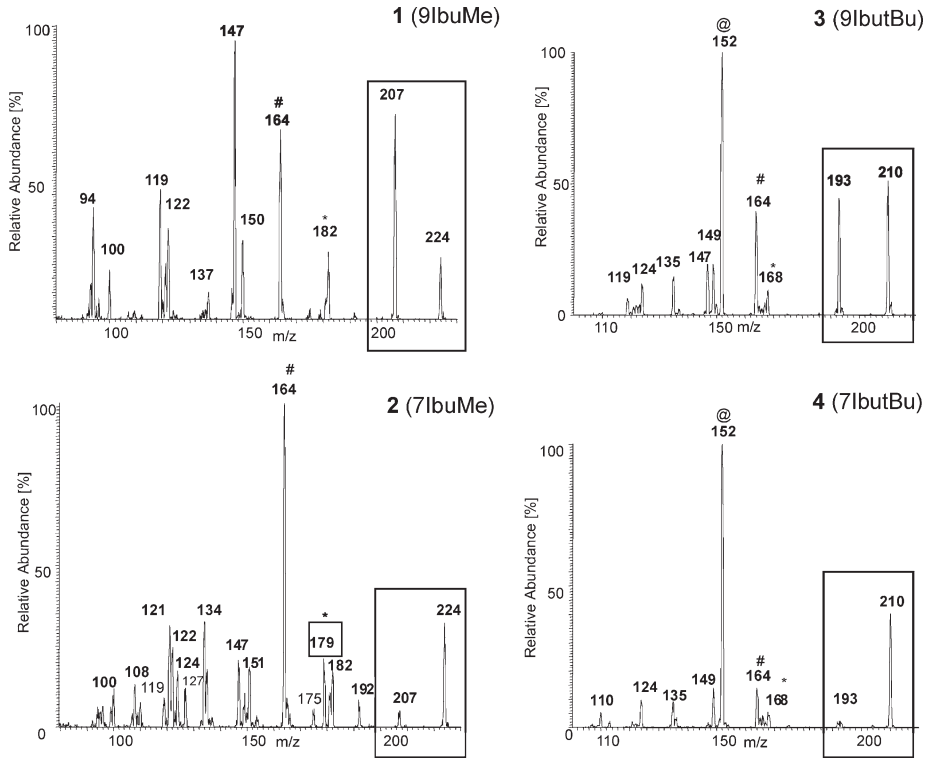

Figure 4. MS/MS spectra of regioisomer pairs **1** (9IbuMe), **2** (7IbuMe) and **3** (9IbutBu), **4** (7IbutBu) studied. The loss of ammonia, the most important fragmentation pathway, is highlighted by boxes. Other important peaks are labelled as follows: *: $[M_f\text{-}42]^+$, #: *m/z* 164 (for structure, see Fig. 3) and @: *m/z* 152. *m/z* 179 is also framed as it could be potentially interesting in future studies of N^7-isomers.

Table 2. Ions of compounds **1** (9IbuMe), **2** (7IbuMe), **3** (9IbutBu) and **4** (7IbutBu) and their masses based on studies by McCloskey *et al.*[13] and Kamel *et al.*[12] Compounds **1f**–**4f** (Fig. 2) originate from initial fragmentation of compounds **1**–**5** [substances **3** and **5** gave an identical acid (**3f**)] The most important, deammoniated fragment (set in bold) was applied for the determination of isomer ratio. Structures of ions corresponding to italicized and underlined peaks have not been confirmed. The underlined one could be important for the characterization of N^7-isomers but further studies are required

1f (9Me), **2f** (7Me) ($[M_f]^+ = 224$)				**3f** (9OH), **4f** (7OH) ($[M_f]^+ = 210$)			
Product ion	Precursor ion	Mass difference	Fragment(s) lost	Product ion	Precursor ion	Mass difference	Fragment(s) lost
207	**224**	**17**	**NH_3**	**193**	**210**	**17**	**NH_3**
192	*224*	*32*	*MeOH*	168	210	42	NH_2CN
182	224	42	NH_2CN	*164*	*210*	*46*	*H_2O+CO*
179	207	28	CO	*152*	*210*	*58*	*$C_2H_2O_2$*
164	*224*	*60*	*MeOH+CO*	*149*	*193*	*44*	*CO_2*
150	*182*	*32*	*MeOH*	147	164	17	NH_3
147	164	17	NH_3	135	152	17	NH_3
137	164	27	HCN	124	152	28	CO
124	151	27	HCN	119	147	28	CO
122	164	42	NH_2CN	110	152	42	NH_2CN
121	164	43	NHCO				
119	147	28	CO				
94	122	28	CO				

as do the precursor ions (**1f** (9Me), **2f** (7Me), **3f** (9OH) and **4f** (7OH)), to yield ions at *m/z* 147 and 122, respectively.

When comparing the MS/MS spectra of compounds **1** (9IbuMe), **2** (7IbuMe), **3** (9IbutBu) and **4** (7IbutBu), it was found that, in both cases, the N^9-isomers (**1** (9IbuMe) and **3** (9IbutBu)) gave abundant $[M_f–NH_3]^+$ ions at *m/z* 207 and 193 that were of negligible abundance in the case of the N^7-isomers (**2** (7IbuMe) and **4** (7IbutBu)) (see boxes in Fig. 4). Therefore, the loss of ammonia from the $[M_f]^+$ ions is a potential candidate for the quantitative determination of the regioisomer ratio. The *m/z* 179 ion in the spectrum of compound **2** (7IbuMe) is interesting as it could be a product of *m/z* 207 formed by loss of CO. This ion could be characteristic for the N^7-isomer but further studies are required involving other alkyl (guaninyl)acetates containing stable ester bonds.

The abundant ion at *m/z* 152, corresponding to protonated guanine, is the result of total side-chain loss from the free acid form of **3f** (9OH) and **4f** (7OH). This ion then loses NH_3, CO and cyanamide to yield ions at *m/z* 135, 124 and 110, respectively.

The N^9-isomer **3f** (9OH) gives a characteristic loss of ammonia as the parallel process of the side-chain loss; therefore, the ratio of regioisomers can also be determined in the case of *tert*-butyl esters.

It appears conclusive that the loss of ammonia is characteristic for N^9-regioisomers (**1** (9IbuMe) and **3** (9IbutBu)). Next, the abundance of the $[M_f\text{-}17]^+$ ion as a function of regioisomer ratio was studied.

The fragmentation pathways confirmed by Gregson McCloskey[13] and Kamel and Munson[12] have been collected in Table 2 for easier interpretation of MS/MS spectra described herein.

A series of solutions containing the isomers in different ratios has been studied by MS/MS in order to obtain a calibration curve. The abundances of the three main product ions, $[M_f\text{-}17]^+$, $[M_f\text{-}42]^+$ and *m/z* 164, were plotted as a function of the isomer ratio (see Fig. 5). The correlation was linear in all three cases but with different slopes. The abundance of the ion $[M_f\text{-}42]^+$ is almost constant; furthermore, its correlation coefficient (R^2) value shows large uncertainty for compounds **3** (9IbutBu) and **4** (7IbutBu) (see Table 3). It is not therefore appropriate for the determination of the isomer ratio.

Based on their high correlation coefficients (see Table 3), the abundances of ions $[M_f\text{-}17]^+$ and *m/z* 164 are both convenient for the determination of isomer ratios; however, as the change in abundance is twice as large for the $[M_f\text{-}17]^+$ ion than for *m/z* 164, its application would lead to more accurate results. The optimal collision energy at which the abundance of the $[M_f\text{-}17]^+$ ion is similar to that of the $[M_f]^+$ ion for the N^9-isomer and negligible for the N^7-isomer (less than 10% relative abundance) is also important for the same reason.

The slope of the calibration curves depends strongly on the collision gas pressure (see Fig. 6). Therefore, the CID pressure should be adjusted accurately to obtain comparable ion abundances. For example, a change of 0.4 mTorr led to a

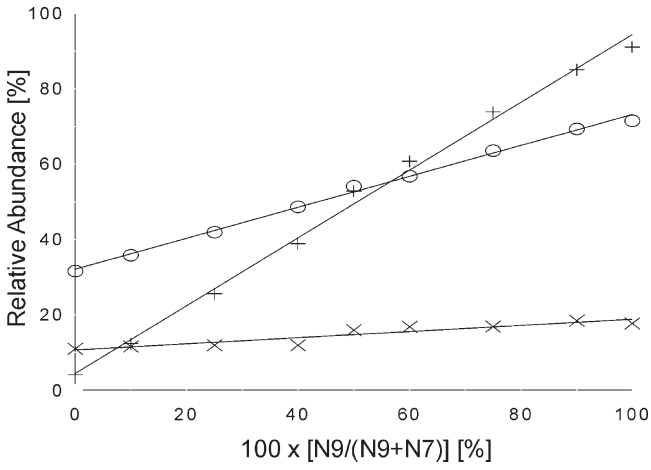

Figure 5. The linear regression study of product ion abundance as a function of isomer ratio in a mixture containing compounds **3** (9IbutBu) and **4** (7IbutBu). + series: *m/z* 193; x series: *m/z* 168 and o series: *m/z* 164.

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Table 3. Correlation coefficient (R^2) values from linear regression analysis of the abundances of three ions ($[M_f\text{-}17]^+$, $[M_f\text{-}42]^+$ and *m/z* 164 (for structure, see Fig. 3)) as a function of the regioisomer ratio for both pairs of regioisomers

Sample constituents	$[M_f\text{-}17]^+$	R^2	$[M_f\text{-}42]^+$	R^2	R^2 for *m/z* 164
1 (9IbuMe), **2** (7IbuMe)	*m/z* 207	0.997	*m/z* 182	0.981	0.994
3 (9IbutBu), **4** (7IbutBu)	*m/z* 193	0.995	*m/z* 168	0.875	0.996

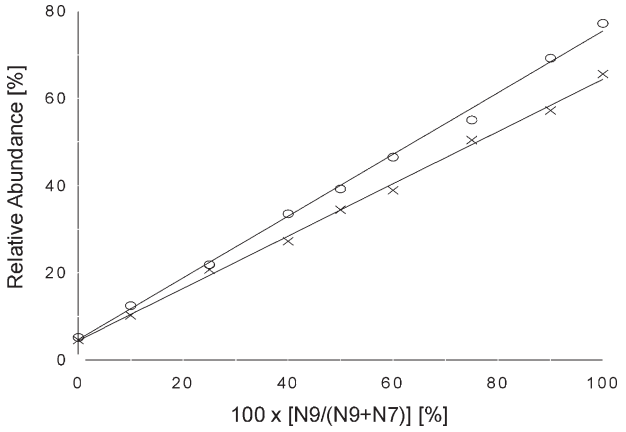

Figure 6. Calibration at two different CID gas pressures of compounds **1** (9IbuMe), **2** (7IbuMe) at 2.1 mTorr (o series, $y = 4.68 + 0.71x$, $R^2 = 0.997$) and 1.7 mTorr (x series, $y = 4.45 + 0.60x$, $R^2 = 0.997$).

0.1 difference in the slope, meaning that the relative abundance of the $[M_f\text{-}17]^+$ ion changed ca. 10% in the case of the pure N^9-isomer. This problem is increased for compounds **3** (9IbutBu) and **4** (7IbutBu), resulting in a 20% change in abundance (data not shown). To obtain accurate results every isomer determination should start with a calibration (maximum 15 min) to guarantee identical conditions in the analysis.

Alternatively, the calibration before each analysis could be avoided by use of a normalization based on an ion whose abundance is independent of the isomer ratio. Unfortunately, there was no such ion in the *m/z* 160–230 range.

The accuracy of the measurement is ca. 2.2–4.4% (**1** (9IbuMe) and **2** (7IbuMe)) and 4.8–5.0% (**3** (9IbutBu) and **4** (7IbutBu)) depending on the isomer ratio (Table 4). These values are acceptable as the aim of the optimization of reaction conditions is to increase the N^9-isomer content from ca. 50% to 90–100%. Almost the same accuracy could be achieved with ^{1}H NMR but this latter method requires cleaner and larger amounts of sample, deuterated solvents and 10–20 min/sample acquisition time. If the isomers were well resolved by HPLC then higher accuracy could be obtained, but even then there is a need for high-purity solvents and again 10-20 min/sample analysis time.

Table 4. Error of the measurement based on two points: 50 and 90% N^9-isomer content. y = the measured relative abundance of the ion $[M_f\text{-}17]^+$; x = the determined value of the isomer ratio; e.g. $x = (y - 3.28)/0.75$ for compounds **1** (9IbuMe) and **2** (7IbuMe). Error: the relative error of the determination; $\delta x = (x\text{-}x_c)/x_c$

	1 (9IbuMe) and **2** (7IbuMe) [%]		**3** (9IbutBu) and **4** (7IbutBu) [%]	
x_c	50	90	50	90
y	39.3	69.3	51.2	81.1
x	47.8	88.0	52.5	85.7
$100\delta x$	−4.4	−2.2	5.0	−4.8
Calibration curves	$y = 3.28 + 0.75x$		$y = 3.99 + 0.9x$	

CONCLUSIONS

In conclusion, we have managed to find a rapid method for the determination of the ratio of regioisomer pairs of alkyl (guaninyl)acetates. Alkylated N^9- and N^7-regioisomer pairs have significantly different fragmentation pathways: deammoniation is characteristic for N^9-isomers, the abundance of which process is in linear correlation with the N^9 content in a mixture of regioisomers.

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