A gas chromatographic/mass spectrometric method for tracing the microbial conversion of glucose into amino sugars in soil

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Amino sugars in soils are heterogeneous and have been used as microbial residue biomarkers to investigate the microbial contribution to soil organic matter. However, it is not clear what the available carbon source is and how glucose is utilized for the synthesis of soil amino sugars. This paper presents a new gas chromatography/mass spectrometry (GC/MS) approach for the identification of $^{13}$C incorporation into three amino sugars, D-glucosamine, D-galactosamine, and muramic acid, in soil incubated with U-$^{13}$C-glucose. Method evaluation showed that the chemical ionization (CI) mode was suitable for all these amino sugars, but that electron impact (EI) mode was applicable only to glucosamine and galactosamine. The $^{13}$C conversion rate was estimated based on the abundance ratio of the ions corresponding to the masses of the ions $F + n$ and $F$ (where $n$ is the skeleton carbon number in the fragment ions $F$ of the amino sugars) and calculated as atom percentage excess. The reproducibility of the method was excellent and clearly adequate for the present purpose. In addition, the new approach is highly accurate as tested with mixtures of U-$^{13}$C-glucose and natural glucose. Copyright © 2005 John Wiley & Sons, Ltd.

The stable isotope technique has been applied to measure soil organic matter (SOM) turnover, especially for carbon (C) and nitrogen (N) transformation. Doping with $^{15}$N-labeled NH₄⁺ has been a popular approach for studying the internal N-cycle in soil and characterizing the N-transformation dynamics of amino compounds.¹ Up to now, the rapid translocation from $^{15}$NH₄⁺ to plant amino acids has been investigated by a gas chromatography/mass spectrometry (GC/MS) technique.²–⁵ Following the same principles, we have traced the microbial transformation from $^{15}$NH₄⁺ into soil amino sugars in soil (unpublished data).⁶ The dynamics of the amino compounds provided significant information on the transformation of soil N, because amino sugars in soils are microbial residues that can be used to investigate the microbial contribution to SOM turnover.⁷–¹¹ The soil amino sugars detected quantitatively by the GC/MS method are D-glucosamine (GluN), D-galactosamine (GalN), mannosamine (ManN) and muramic acid (MurN).⁶,¹² Chitin, a major component of fungal cell walls, is a polymer of N-acetylglucosamine. The peptidoglycan of bacterial cell walls contains N-acetylglucosamine and N-acetylmuramic acid, and most of the GalN in soils is also derived from bacteria. Although amino sugars in soils are thus strongly related to microbial processes, there is no suitable technique to characterize the transformation dynamics, especially for C translocation into amino sugars.

The N assimilation was always associated with the utilization of soil organic C. Glucose serves as both the energy and C sources most available for soil microorganisms, and therefore some studies on the transformation and utilization of glucose have been conducted by monitoring the $^{13}$C content in microbial biomass after treatment with $^{13}$C-labeled glucose.¹³,¹⁴ However, the physiological pathways of glucose utilization for microbial metabolism were too complicated to be represented by microbial biomass C, and none of the research to date has addressed the dynamics of the $^{13}$C transformation into specific compounds with biochemical function; the latter aspect is more significant for tracing the fate of C than simply biomass. It appeared to us that the stable isotope technique should be effective for this purpose, and therefore our objective was to develop a new method combining stable isotope incubation using glucose as a substrate with GC/MS detection to trace the translocation from glucose-C into amino sugar C. This new technique can be used further to search for insights into the microbial process of amino sugar transformation.

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EXPERIMENTAL

Soil samples
Surface Udoll samples (0–20 cm) were collected from Gongzhuling, Jilin, China. After collection the soil samples were air-dried and sieved (<1 mm) for simulated incubation in the laboratory.

Reagents
U-13C-glucose (13C atom 99%) was purchased from Cambridge Isotope Laboratories Inc., USA. (NH4)2SO4, d-glucose and all amino sugars were obtained from Sigma-Aldrich Chemical Co., USA. The derivatization reagents, i.e. hydroxylamine hydrochloride, 4-(dimethylamino)pyridine and acetic anhydride, were also from Sigma. All the chemicals were of either analytical or GC grade.

Laboratory incubation
The soil samples were weighed (ca. 8 g) into glass bottles in triplicate and incubated at 25°C. During incubation the water content was held at 20% on the basis of oven-dried soil. Distilled water was added to adjust moisture content by weighing each container. (NH4)2SO4 plus U-13C-glucose solution as substrate was added once per week. The added amounts were 0.1 mg for N and 1.0 mg for C, per gram of soil. The incubated soils were sampled at the 2nd and 4th weeks for analysis and the original soil was used as a control.

Analysis of soil amino sugars by GC/MS
The incubated samples were air-dried and ground (<0.25 mm) after sampling, and a 0.5 g portion out of the 8 g total was weighed for analysis. The determination of amino sugars, from hydrolyses and purification to derivatization, was performed according to the method of Zhang and Amelung.12 Briefly, soil samples were heated with 6 mol/L HCl for 8 h at 105°C. After cooling, the hydrolysate was filtered and dried by rotary evaporator in vacuum. Then, the residue was dissolved with methanol and centrifuged, and then the supernatant was freeze-dried. The residue was dissolved with water, and the pH was adjusted to 6.6–6.8. The precipitate was removed by centrifugation and the supernatant was freeze-dried by a stream of N2 gas. Finally, after dissolution in 1 mL water and freezing in an ethanol bath, the residue was dried completely in vacuum in preparation for the derivatization prior to GC analysis. The derivatization procedure included two steps: first a mixture of hydroxylamine hydrochloride and 4-(dimethylamino)pyridine was added to reduce the aldehyde group to a nitrile group, and then acetic anhydride was added to form the acetate, so, finally, the amino sugars were transformed into volatile aldononitrile acetates (Fig. 1).

According to extraction, the derivatives were separated on a DB-5MS column (30 m × 0.25 mm × 0.25 μm; Agilent Technologies Inc., USA) and the incorporation of labeled C into amino sugars was identified by GC/MS (Finnigan Trace, Thermo Electron-Finnigan Co. Ltd., USA) with both electron impact (EI) and chemical ionization (CI) sources. The injector temperature was 250°C and helium was used as carrier gas at a flow rate of 0.8 mL/min. The EI source temperature was 200°C and the GC temperature program was set as described by Zhang and Amelung12 the initial column temperature of 120°C was held for 1 min and then temperature was increased at 10°C/min to 250°C for 2.5 min. Thereafter, the temperature was increased again at 20°C/min to 270°C, and held there for 2 min. All three amino sugars were separated perfectly in EI mode. When using the negative chemical ionization (CI⁻) mode, the temperature program was changed slightly, i.e. the temperature between 230–250°C was increased at 5°C/min and the split ratio was raised to 40:1 from the previous value of 30:1 to ensure the baseline separation between GalN and MurN. The CI source temperature was set to 180°C and CH4 flow was 1.5 mL/min. The electron energy in both EI and CI sources was set to 70 eV. The mass spectrometer was programmed in full scan mode (m/z 40–500), and the dominant fragment ions as well as their corresponding isotopic variants were monitored.

Analysis of glucose derivatives by GC/MS
Mixtures with different ratios of U-13C-glucose to d-glucose were prepared and the derivatization of the mixture was performed using the same procedure as described above for the amino sugars, so that glucose was transformed into glucononitrile acetate and then determined by the same GC/MS method in both EI and CI modes.

RESULTS AND DISCUSSION
Fragmentation pattern of amino sugar derivatives
The fragmentation patterns were similar for the two epimeric hexosamines, GluN and GalN. In EI mode both GluN and GalN derivatives dissociated into three major fragments at m/z 98, 127, and 187. The rupture of the C–C bond between the 2nd and 3rd C atoms in the amino sugar skeleton contrib-
uted to the fragmentation pattern. The ion at m/z 98 contained two skeleton C atoms and one N atom (unpublished data), while the other two ions at m/z 127 and 187 each contained four C atoms (most likely from the 3rd to 6th in the skeleton, inclusive).

$^{13}$C-Labeled amino sugars should be used to confirm the dissociation characteristics and the isotope distribution in the derivative molecules. Unfortunately, there are no $^{13}$C-labeled amino sugars available commercially, and we were forced to select U-$^{13}$C-glucose as an alternative to interpret the fragmentation pattern of the amino sugars. This is acceptable because there is a strong similarity in structure between hexosamine and glucose, i.e. the difference, for instance, between glucose and GluN molecules is the group at the 2nd C atom, i.e. -OH for the former and -NH$_2$ for the latter; the fragmentation pattern from the 3rd to 6th skeleton C atoms was the same. Comparison of the mass spectra of glucose with that of the U-$^{13}$C-glucose indicated both ions at m/z 127 and 187 contained four skeleton C atoms (see Fig. 2).

Compared with the EI spectra, the number of fragment ions as well as of interfering ions in the CI$^{-}$ mode was decreased significantly. Ions at m/z 148 and 206 were dominant fragments for GluN and GalN. By applying the same principle a comparison with the glucose derivatives was conducted to clarify the number of the skeleton C atoms. The abundance of m/z 165 was predominant in glucose derivatives, and this ion contains six skeleton C atoms (it shifts to m/z 171 for U-$^{13}$C-glucose). For the two hexamine derivatives, m/z 164 was the corresponding ion, which should have been derived from the ion m/z 206 after the loss of RCO$^+$ (significant abundance of the complementary ion at m/z 43 in the mass spectra). The ion at m/z 148 was most probably due to loss of an acetyl group as C$_2$H$_2$O$_2$ (58 Da) from the ion at m/z 206. This implies that the skeleton of six C atoms in amino sugar molecules was preserved during CI (Fig. 3).

Satisfactory fragmentation information could be obtained for GluN and GalN in both CI and EI modes, but it was difficult for MurN in EI mode because there were no significant fragment ions recorded due to the low sensitivity in EI mode, as well as to the low content of MurN in soils. However, in CI$^{-}$ mode the spectral quality for the MurN derivative was improved significantly, and m/z 264 was the characteristic base peak that should contain all skeleton C atoms according to the background knowledge summarized above (Fig. 3(b)).

Reproducibility of fragmentation

Because of the presence of natural abundances of heavy isotopes of C, N, H, and O, each of the main fragment ions was accompanied by some minor peaks corresponding to isotopic variants, as in the earlier mass spectra of amino acids in plant samples determined by GC/MS. In the present work the natural abundance ratios of the fragment ions F$^+$ and F + 2 relative to F were highly reproducible (relative uncertainties were lower than 1%), while the higher isotopic variants were not detectable due to their low natural abundance. As reported by Hama et al., the excellent reproducibility of these spectral characteristics of the non-labeled samples provides a essential basis for practical use of the new method. Thus, if there is an observed increase in intensity of peak F$^+$ relative to that of F, it represents a contribution from the transformation of the U-$^{13}$C-glucose into amino sugars.

Accuracy of the method evaluated by using U-$^{13}$C-glucose

For the reason mentioned above, we were forced to use mixtures with different ratios of U-$^{13}$C-glucose to d-glucose to estimate the accuracy of the present technique, rather than the amino sugars themselves. Five test solutions were made by adding 0, 10, 25, 50, and 100 µL U-$^{13}$C-glucose solution (1 mg/mL) to 100 µL of d-glucose solution at the same concentration. The $^{13}$C-glucose content was calculated using the isotope abundance of the U-$^{13}$C-glucose (99% atom) and the natural $^{13}$C abundance (1.11% atom) of the d-glucose.

When analyzed by EI-MS, fragment ions at m/z 127 and 187 were observed for the natural glucose derivative, and these correspond to the same ions obtained for the hexosamine derivatives; thus the F + 4 peaks can be considered to have

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**Figure 2.** Mass spectra of the derivatives of two hexosamines and glucose in EI mode: (a) glucosamine, (b) glucose, and (c) U-$^{13}$C-glucose.

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arisen from the $^{13}$C-labeled derivatized glucose via rupture of the C–C bond between the 2nd and 3rd C atoms (see above). When detected in CI$^{-}$/C$^0$ mode, m/z 165 was the characteristic peak, corresponding to a fragment ion that contains the six skeleton C atoms, and therefore m/z 171 was used to calculate the abundance of $^{13}$C. On comparing $^{13}$C concentrations in the prepared solutions with the values measured using the new GC/MS method, the results from both ionization sources showed good agreement (Table 1) with rather low relative errors (<3%).

**Conversion of $^{13}$C-labeled glucose into soil amino sugars**

After U-$^{13}$C-glucose was added to soils, the relative abundances of all target F$^+$ ions of the amino sugars became larger as detected by both modes, i.e. for the two hexosamines, the F$^+2$ for m/z 98 and F$^+4$ for m/z 127 and 187 in EI mode, and F$^+6$ for the dominant ions in CI$^{-}$ mode. The corresponding ion abundance ratio of F$^+n$ to F was applied to calculate the $^{13}$C enrichment. In the same manner, the abundance ratio of m/z 270 to 264 was used to compute the $^{13}$C incorporation into MurN for incubated soil samples (Fig. 4). Additionally, for amino sugars the molecular ions of [M$^+$1]$^+$ in EI and [M–1]$^-$ in CI behaved in the same way, and thus all the corresponding isotope ions arising from $^{13}$C incorporation were shifted by 6 Th and their abundances increased during incubation. Our results all indicated that the increase in the abundance of the corresponding isotope ions arose from U-$^{13}$C-glucose incorporation into the skeleton of soil amino sugars.

In previous research into the transformation of NH$_4^+$ into amino acids in plants, the relevant fragment ion (F) as well as the isotopic variants (F$^+$1) or (F$^+$2) were monitored for each amino compound according to the number of amino groups, Table 1. Comparison of $^{13}$C content in the prepared solutions of labeled and unlabeled glucose with those measured by the new method (%)

<table>
<thead>
<tr>
<th>Calculated $^{13}$C</th>
<th>Measured $^{13}$C by EI</th>
<th>Measured $^{13}$C by CI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>127$^+$ (4C)</td>
<td>187$^+$ (4C)</td>
</tr>
<tr>
<td>10.01</td>
<td>9.83 (0.17)$^{*}$</td>
<td>9.64 (0.18)</td>
</tr>
<tr>
<td>20.67</td>
<td>21.47 (0.48)</td>
<td>21.02 (0.35)</td>
</tr>
<tr>
<td>33.74</td>
<td>34.58 (0.67)</td>
<td>34.82 (0.23)</td>
</tr>
<tr>
<td>50.06</td>
<td>50.91 (1.14)</td>
<td>49.56 (1.01)</td>
</tr>
</tbody>
</table>

$^{*}$ m/z values of ions monitored.

$^{*}$ The values in parentheses are standard deviations.

and the abundance ratio \((F+1)/F\) or \((F+2)/F\) was used to estimate the rapid translocation.\(^3\),\(^4\) The present work shows that, during the microbial synthesis process of GluN and MurN from glucose, the glucose skeleton was not broken. Moreover, all the acetyl groups in the derivatized amino sugar were introduced without contributing to the isotope enrichment at \(F+4\) and \(F+6\). Therefore, the abundances for fragment ion \(F\) and also the corresponding isotope variant \((F+n)\) (where \(n\) is the number of skeleton C atoms in the fragment) were measured to investigate the microbial conversion of glucose into amino sugars. The \(^{13}\)C incorporation was evaluated from the abundance ratio of \(F+n\) to \(F\), and was expressed by the atom percentage excess (APE) calculated as follows:

\[
APE = \frac{(R_e - R_c)}{[1 + (R_e - R_c)]} \times 100
\]

where \(R_e\) is the enriched ratio \([F+n]/F\) for \(^{13}\)C incorporation and \(R_c\) is the corresponding ratio obtained for samples obtained using unlabeled glucose and analyzed in the same GC/MS session.

The APE values and standard deviations (SD) of the whole determination procedure for the incubated samples were measured. For the two hexosamines the average value of APE was preferred as several fragment ions were used independently, while, for muramic acid, the abundance ratio of \(m/z\) 270 to 264 was used to calculate \(^{13}\)C conversion. The APE (\(^{13}\)C) values determined for the incubated samples are listed in Table 2. For each individual sample, the APE values obtained for each of GluN and GalN using the two ionization modes agreed well, indicating that the present method is reliable. Also, the reproducibility was excellent despite the different APE values among amino sugars (presumably reflecting microbial heterogeneity, more research on this is needed in the future) and the relative error was lower than 5% for all sugars determined in both modes. Although the sensitivity for the two hexosamines detected in CI mode was lower than that in EI mode, the results were acceptable for soil samples.

**CONCLUSIONS**

A new GC/MS approach was developed for tracing the conversion of glucose into three amino sugars in soil. The conversion rate of \(^{13}\)C in glucose into the amino sugars can be estimated precisely by measuring the abundance ratio of the heavy isotope ion from the labeled glucose substrate \((F+n)\) to the corresponding light isotope ion \((F)\), where \(n\) is the skeleton carbon number in the fragments. Both electron impact (EI) and negative chemical ionization (CI) modes were tested. The findings indicate that the CI mode was suitable for all three amino sugars, while EI-MS was not applicable to muramic acid due to low sensitivity in EI mode as well as low concentration in soil. The determination was confirmed to be reproducible and reliable, and the accuracy of the method was excellent as tested by analyzing different

<table>
<thead>
<tr>
<th>Ionization mode</th>
<th>Incubation weeks</th>
<th>Glucosamine</th>
<th>Galactosamine</th>
<th>Muramic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>EI</td>
<td>6.49 (0.15)*</td>
<td>11.53 (0.24)</td>
<td>3.30 (0.11)</td>
<td>4.65 (0.15)</td>
</tr>
<tr>
<td>CI</td>
<td>6.37 (0.28)</td>
<td>12.04 (0.33)</td>
<td>3.51 (0.14)</td>
<td>4.83 (0.22)</td>
</tr>
</tbody>
</table>

* The values in parentheses are standard deviations.

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abundances of U-$^{13}$C-glucose to natural glucose (unfortu-
nately $^{13}$C-labeled amino sugars are not available). The pre-
sent method can be used to investigate biotransformation or
renewal of amino sugars in soil.

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