Approach to the Biosynthesis of Atisine-Type Diterpenoid Alkaloids

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Received October 17, 2008

To determine the biosynthesis pathway of the atisine-type diterpenoid alkaloids spiramines A/B and C/D, feeding experiments in *in vitro* cultured plantlets and enzymatic transformations in cell-free extracts were performed in combination with LCMS and tandem MS analyses. L-[2-^{13}C,^{15}N]Serine was used in the feeding experiments and enzymatic transformations, and the diterpene spiraminol was identified as a biosynthetic precursor of spiramine alkaloids. The LCMS and tandem MS spectra of the extracts from these experiments indicated that L-[2-^{13}C,^{15}N]serine was incorporated into spiramines A/B and C/D. The labeled reaction products show that L-serine is the one possible nitrogen source involved in the biosynthesis of atisine-type DAs.

Diterpenoid alkaloids (DAs) are thought to be biosynthesized from the C_{20} diterpene precursor geranylgeranyl diphosphate (GGPP).[1] Generally, diterpenes are considered to originate in the plastidic compartment of plant cells via the recently discovered nonmevalonate pathway.[2,3] Therefore, it appears likely that the isoprenoid moiety of DAs is also synthesized via the nonmevalonate pathway. The hypothetical biosynthesis pathway for DAs described by Ichinohe[1] involves the following conversions: GGPP → diterpene → C_{20}-type DAs containing an oxazolidine ring → other kinds of C_{20}-type DAs → C_{19}-type DAs. However, it is unclear how the C_{20}-type DAs are derived from the corresponding diterpenes. It has been speculated that DAs might be derived from tetracyclic or pentacyclic diterpenes and that their nitrogen might come from methylamine, ethanolamine, or aminoethanol.[4] So far, there has been no evidence to support this hypothesis.

The DAs from *Spiraea japonica* have been described since 1964[5] and are structurally classified into atisine- or hetisine-type DAs (Figure 1A).[6] However, only atisane-type diterpenes corresponding to the basic skeleton of the atisine-type DAs have been isolated from the *S. japonica* complex.[6] The typical atisane-type diterpene spiraminol (Figure 1A) isolated from this complex contains one hemiacetal and one acetal group at C-19 and C-20,[7] respectively. Compared with the structures of spiramine C/D, the main DAs (Figure 1A) distributed in the plant, spiraminol lacks one unit of ethanolamine. This characteristic of spiraminol prompted us to investigate the chemical relationship between the diterpenes and DAs of our previous studies. Interestingly, in our previous experiments we found that spiraminol can be reacted with ethanolamine to transform spiramine C/D almost quantitatively in refluxing THF (Figure 1B).[6,7] The mechanism of this reaction includes two condensation steps of spiraminol with ethanolamine (Figure 1B). From this result, double-condensation reactions can also be inferred to be the key steps in the biosynthesis pathway of atisine-type DAs, although the "driving force" for this reaction remains obscure.

The results of biomimetic reactions allowed us to approach the biosynthesis of DAs. The first key point was to predict the nitrogen source for the alkaloids. According to reports, pyrophosphoric ethanolamine or L-serine could be used as nitrogen source for the biosynthesis studies of alkaloids: pyrophosphoric ethanolamine occurs widely in animals and plants, enabling the organism to use pyrophosphoric ethanolamine (or ethanolamine),[8] and there is evidence that L-serine is used as a nitrogen source for alkaloid biosynthesis in nature.[8,9] Therefore, we provisionally assumed that L-serine was the nitrogen source for the atisine-type DAs, so we selected L-[2-^{13}C,^{15}N]serine as the labeled precursor of the DAs in this study.

Feeding experiments are generally used in biosynthesis studies of the secondary metabolites of plants,[10] and induction with elicitors has been an effective technique for enhancing the secondary metabolite contents of plants.[11] Cell-free systems can also be considered an alternative method in investigations of the biosynthesis of plant secondary metabolites.[12]

In this paper, we report an analysis of the biosynthesis of the atisine-type DAs spiramine C/D from the atisane-type diterpene spiraminol using feeding experiments in *in vitro* cultured plantlets and enzymatic transformation in cell-free extracts. In both experiments, L-[2-^{13}C,^{15}N]serine was used as the biosynthesis precursor nitrogen source. Spiramines A/B and C/D, the main constituent DAs of *Spiraea*,[6] were used to quantitatively determine the formation of particular products, such as labeled *Spiraea* DAs. The labeled DAs were identified by comparing their retention times and tandem mass spectra with those of standard spiramines A/B and C/D.

Results and Discussion

Effect of Elicitors on Spiramine A/B Production in in Vitro Cultured Plantlets. Batches of chopped plantlets from *S. japonica* cultured *in vitro* were aseptically incubated with three elicitors at various concentrations to optimize the concentrations used to enhance the accumulation of spiramine A/B. As shown in Figure 2, the maximum accumulation of spiramine A/B (29.9 mg/L) was detected after the 200 mg/L chitosan treatment, whereas the accumulation of spiramine A/B elicited by methyl jasmonate (Me-JA) or salicylic acid (SA) at the optimal concentrations was only half that elicited with chitosan. The spiramine A/B yield obtained with chitosan was about 9 times greater than the control yield (3.3 mg/L). Therefore, treatment with 200 mg/L chitosan was selected for further study.

LCMS Analysis of the Products in Feeding Experiments. Stable isotopes and LCMS were used to assess how L-[2-^{13}C,^{15}N]serine reacts with a precursor (perhaps spiraminol) to form DAs. Doubly labeled L-serine (100 mg/L) and chitosan (200 mg/L) were added to the precultured plantlets. After incubation for one week, the products were separated and detected (see Experimental Section). The retention times of spiramines A/B and C/D produced with this method were 13.82/12.30 and 11.02/9.80 min, respectively. The selected ion monitoring (SIM) peak of spiramine A/B was at *m/z* 400 $[M + H]^+$ (Figure 3A, SIM400) and the SIM peak of spiramine C/D at *m/z* 358 $[M + H]^+$ (Figure 3A, SIM358). The corresponding LCMS profiles of the control extracts are shown in Figure 3B.

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10.1021/np800657j CCC: $40.75
Published on Web 03/10/2009

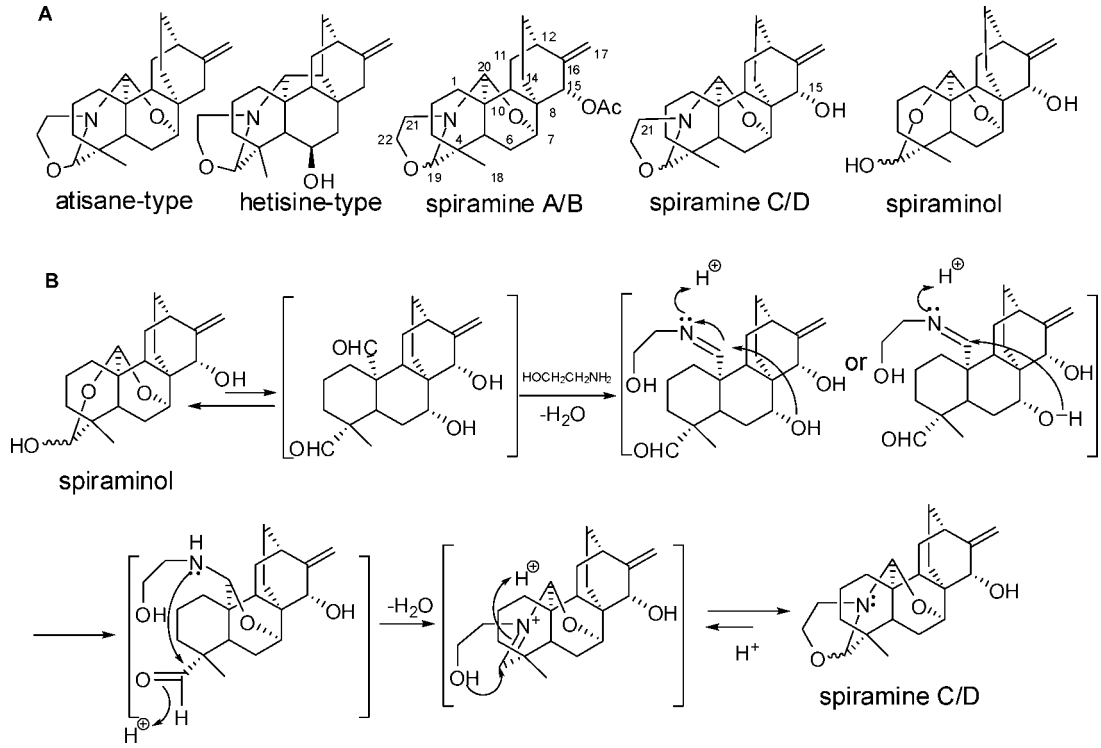

Figure 1. Typical atisane-type diterpene and atisine-type DAs and the hypothetical pathway from atisane-type diterpene to DAs. (A) Typical atisane-type diterpene and atisine-type DAs from *S. japonica*. (B) Hypothetical pathway from atisane-type diterpene to DAs.

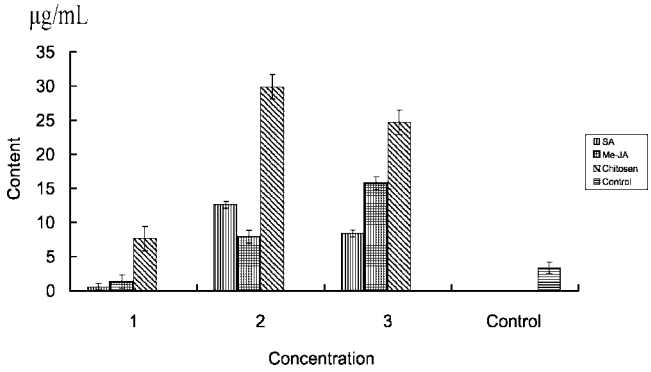

Figure 2. Changes in spiramine A/B contents were measured with LCMS after treatment with three types of elicitors. The data shown are the concentrations of spiramine A/B in the culture medium. Each elicitor was used at three different concentrations: SA was selected for use at 50 μM (1), 200 μM (2), 800 μM (3); Me-JA was used at 8 μM (1), 40 μM (2), 200 μM (3); chitosan was used at 50 mg/L (1), 200 mg/L (2), 800 mg/L (3). Data represent the means (±standard deviations: SA, 0.50; Me-JA, 0.95; chitosan, 1.80; control, 0.82) from three independent experiments.

If the doubly labeled L-serine is incorporated into the corresponding diterpene, the molecular weight of the product should increase by two atomic mass units (amu). The C-21 atom as well as the N atom will be simultaneously replaced with the stable isotopes ^{13}C and ^{15}N, respectively, resulting in the target products detected with SIM peaks at *m/z* 402 $[M + H]^+$ and *m/z* 360 $[M + H]^+$, respectively. In agreement with our original assumption, SIM peaks at *m/z* 402 (Figure 3A, SIM402) and *m/z* 360 (Figure 3A, SIM360) were observed in the LCMS profiles of the doubly labeled products. Moreover, their respective retention times were the same as those for spiramines A/B and C/D, respectively (Figure 3). According to the integral ratios of the spiramine A/B (*m/z* 400 $[M + H]^+$) and the target products (*m/z* 402 $[M + H]^+$) (Figure 3), it suggested about 30% labeled spiramine A/B in the total spiramine A/B. These results confirm that the labeled L-serine was incorporated into the corresponding diterpene to produce spiramines A/B and C/D. Tandem MS was used for further identification.

Incorporation of L-[2-^{13}C,^{15}N]Serine into Spiraea DAs Identified by Tandem MS. From their molecular mass increase of 2 amu (Figure 3), the DAs were considered to be labeled from L-[2-^{13}C,^{15}N]serine. After initial separation by medium-pressure chromatography, the incorporation of L-[2-^{13}C,^{15}N]serine into individual *Spiraea* DAs was further analyzed by tandem MS (Figure 4). The results show that the quasimolecular ion peaks of the standard compounds (*m/z* 400 and 358) had lost a fragment of acetic acid or H_2O to produce the daughter ion peak (*m/z* 340). Furthermore, in the MS/MS/MS experiment, the parent ion peak at *m/z* 340 had lost a neutral ion (C_2H_4) to produce the MS/MS/MS daughter ion peak at *m/z* 312 (Figures 4A and 4C). Likewise, the tandem MS mode of the [2-^{13}C,^{15}N]serine-labeled products revealed that the same fragment was lost in their quasimolecular ion peaks (*m/z* 402 and 360), as in the case of spiramines A/B and C/D (Figures 4B and 4D). The same neutral fragment (C_2H_4) was also lost in the subsequent MS/MS/MS experiment. Taken together, the MS/MS modes of the labeled products were the same in total as those of spiramines A/B and C/D, except for the 2 amu increases in the corresponding ions (Figure 4). These results suggest that the doubly labeled products are structurally identical to the standard DAs (spiramines A/B and C/D), except that the carbon atom at C-21 and the N atom have been replaced by their stable isotopes ^{13}C and ^{15}N, respectively.

Enzymatic Transformation of L-[2-^{13}C,^{15}N]Serine in a Cell-Free System. To further clarify the nitrogen source and to investigate the potential diterpene precursor, an enzymatic transformation of L-[2-^{13}C,^{15}N]serine was performed in cell-free extracts using spiraminol and L-serine or doubly labeled L-serine as the substrates. Quasimolecular peaks at *m/z* 358 $[M + H]^+$ and *m/z* 360 $[M + H]^+$ were observed in the MS spectra. In the spectra of the cell-free extracts fed L-serine and spiraminol (Figure 5B), quasimolecular peaks were observed at *m/z* 358 $[M + H]^+$, and in the spectra of cell-free extracts fed L-[2-^{13}C,^{15}N]serine and spi-

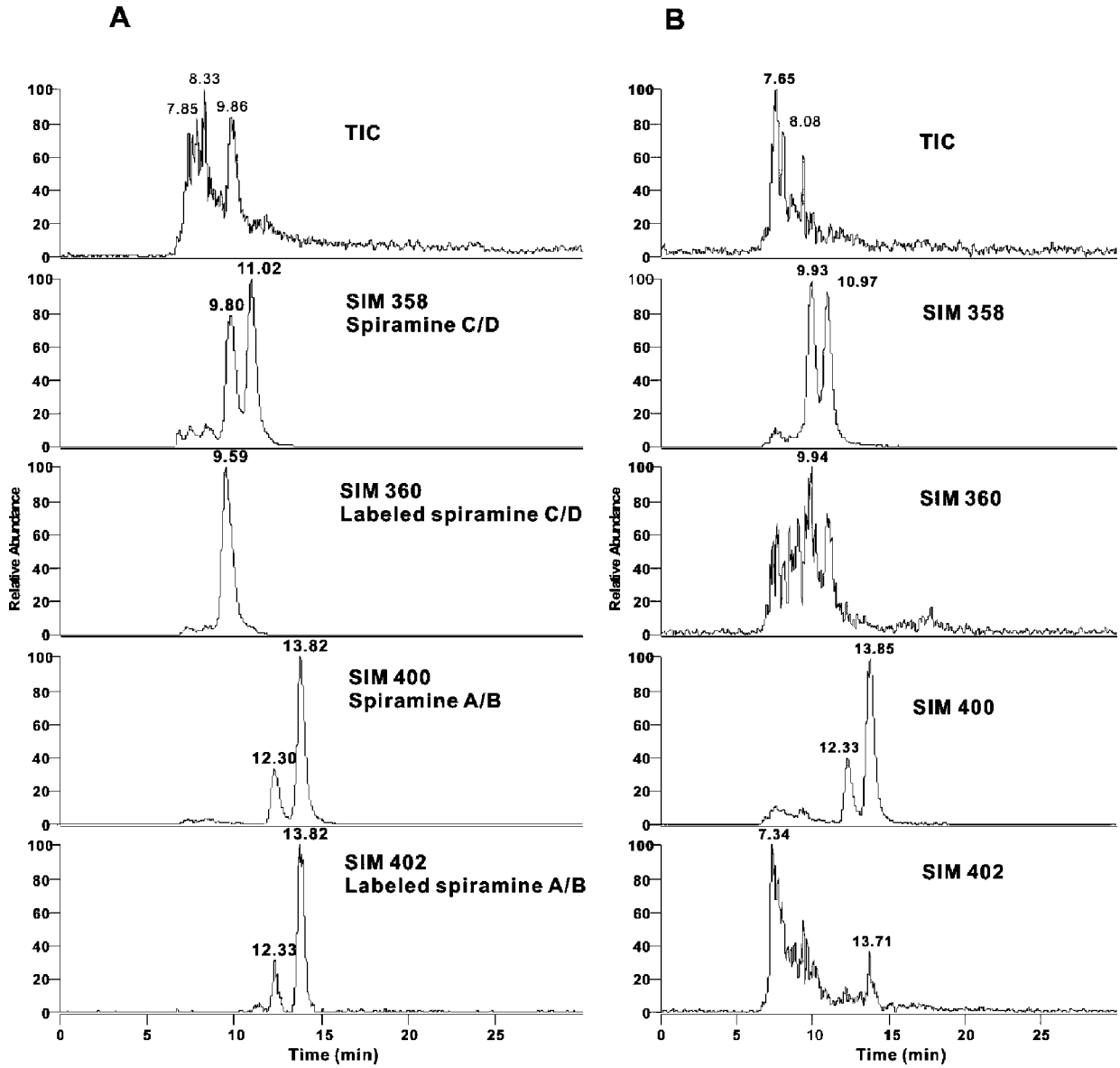

Figure 3. Chromatograms of the products of L-[2-^{13}C,^{15}N]serine. (A) Chromatograms of the products of L-[2-^{13}C,^{15}N]serine. (B) Chromatograms of the products of the control. TIC shows the total ion chromatogram; SIM358 shows the specific ion of spiramine C/D SIM peak at *m/z* 358 $[M + H]^+$; SIM360 shows the specific ion of the target compound SIM peak at *m/z* 360 $[M + H]^+$; SIM400 shows the specific ion of spiramine A/B SIM peak at *m/z* 400 $[M + H]^+$; and SIM402 shows the specific ion of the target compound SIM peak at *m/z* 402 $[M + H]^+$.

raminol (Figure 5C), quasimolecular peaks were observed at *m/z* 360 $[M + H]^+$. In the control cell-free extracts, neither the quasimolecular peak at *m/z* 358 $[M + H]^+$ nor that at *m/z* 360 was detected (Figure 5A). These results show that L-serine may act as the nitrogen source in the biosynthesis pathway of the atisine-type DAs and that spiraminol is possibly a precursor involved in the biosynthesis of DAs such as spiramine C/D.

In conclusion, atisine-type DAs with various biological activities have been studied for a long time as one kind of important natural product,[6] although their biosynthesis pathway is not fully understood. Our results shed light on the biosynthesis pathway of atisine-type DAs by showing that L-[2-^{13}C,^{15}N]serine was incorporated into *Spiraea* DAs as the nitrogen source and that spiraminol can be considered a potential precursor of *Spiraea* DA transformation. From the results of plantlet feeding experiments and cell-free extract reactions combined with LCMS and tandem MS analyses, we infer that *Spiraea* diterpenes are the biosynthesis precursors of *Spiraea* DAs. Because of the close structural relationship of the atisane-type diterpenes in *S. japonica*, it is unclear which diterpenes indeed act as direct precursors.[6]

Experimental Section

General Experimental Procedures. L-Serine, L-[2-^{13}C,^{15}N]serine, methyl jasmonate (Me-JA), and chitosan were purchased from Aldrich Chemical Co. Salicylic acid (SA) was purchased from Tianjin Chemical Reagent Factory in China. Triethylamine was purchased from BDH Laboratory Supplies (Poole, UK). SA and Me-JA were dissolved in EtOH. Chitosan (30%, v/v) was dissolved in HOAc. Standard DAs spiramines A/B and C/D were purified from the roots of *S. japonica*, as described previously,[6] and their structures were confirmed by NMR and MS analyses. Tandem MS and quantitative analyses are presented in the Supporting Information.

Plant Material and Culture Conditions. Plants of *S. japonica* var. o*valifolia* were collected in December 2001 from Kunming Xishan Mountain, Yunnan Province, People's Republic of China. A voucher specimen (no. ZZY030) was identified by Dr. Zhao-yang Zhang and deposited at the Herbarium of the Kunming Institute of Botany, Chinese Academy of Sciences. The *in vitro* cultured plantlets for the feeding

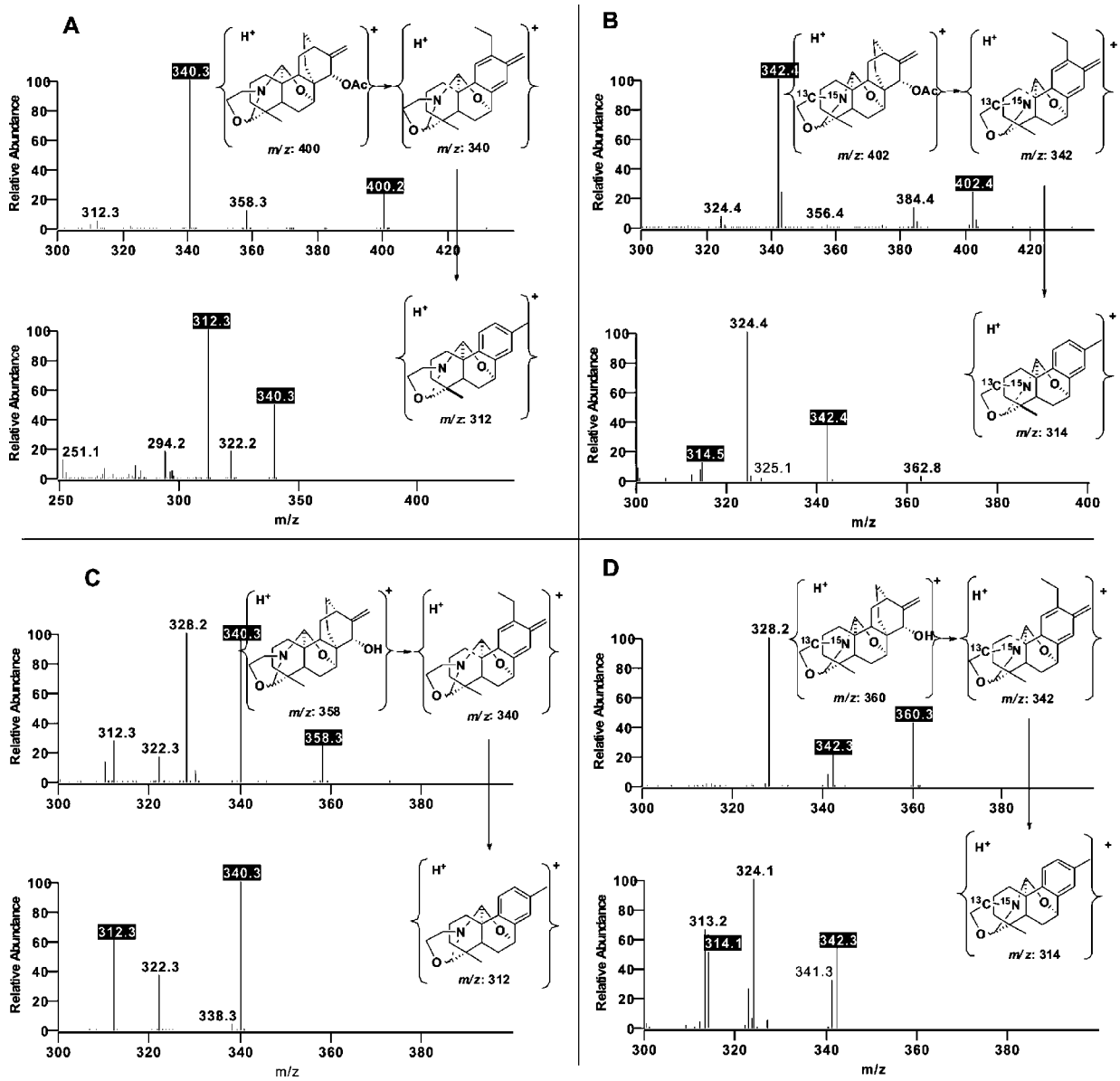

Figure 4. Tandem MS profiles and fragmentation of spiramines A/B and C/D. (A) MS/MS and MS/MS/MS of spiramine A/B; (B) MS/MS and MS/MS/MS of [21-^{13}C,^{15}N]spiramine A/B; (C) MS/MS and MS/MS/MS of spiramine C/D; (D) MS/MS and MS/MS/MS of [21-^{13}C,^{15}N]spiramine C/D.

experiments and cell-free extracts were obtained from tissue culture. Multiple shoots were induced from the explants of shoot tips and stem nodes on Murashige-Skoog solid medium[13] supplemented with 1.0 mg/L 6-benzyladenine (6-BA) and then proliferated on Murashige-Skoog basic medium with 2.0 mg/L 6-BA and 0.1 mg/L 1-naphthalene acetic acid (NAA).[14] The micropropagated shoots were rooted on Murashige-Skoog medium with 0.1 mg/L NAA and formed robust plantlets; the illuminance was 1500−2000 lx.

Preculture and Elicitor Treatment. *In vitro* cultured plantlets (0.5 g, whole plantlet), grown for four weeks, were cut into 2 cm pieces and placed into 300 mL flasks that contained 30 mL of liquid Murashige-Skoog medium plus 0.1 mg/L NAA. The flasks were then kept in the dark at 25 °C with shaking at 120 rpm. After preculture for two weeks (two-week preculture was demonstrated to be more effective in producing DAs than one-week preculture in our previous experiments [data not shown]), each of the three elicitors was added at three different concentrations (50, 200, or 800 μM for SA; 8, 40, or 200 μM for Me-JA; and 50, 200, or 800 mg/L for chitosan). All flasks were then incubated in the dark at 25 °C with shaking at 120 rpm for one week.

The cultured material in each flask was then filtered through qualitative filter paper after elicitor treatment for one week. The filtrates were extracted with an equal volume of EtOAc (×3), and the *in vitro* cultured plantlets were extracted with MeOH (×3). Because most of the alkaloids were concentrated in the liquid medium, the extracts of the filtrates were pooled and dried under vacuum. The remaining residues were dissolved in MeOH and transferred to a 2 mL volumetric flask for the analysis of their alkaloid contents.

After preculture for two weeks at 25 °C with shaking at 120 rpm, the chopped plants had proliferated, with obvious shoot elongation. Their leaves and stems had turned yellow. At this point, different doses of elicitors were added to the culture flasks as described above. The cultured material in each flask was then filtered through qualitative filter paper after elicitor treatment for one week. The filtrates were extracted with an equal volume of EtOAc (×3), and the *in vitro* cultured plantlets were extracted with MeOH (×3). According to the results of LCMS, the amount of DAs in the liquid medium was up to 85% of the total DAs in both the medium and the plantlets. Thus, only the DA content of the medium was measured in this study.

Feeding L-[2-^{13}C,^{15}N]Serine to Chitosan-Treated Cultures. *In vitro* cultured plantlets (0.5 g) grown for four weeks were cut and precultured for two weeks as described above. L-[2-^{13}C,^{15}N]Serine (100 mg/L) and chitosan (200 mg/L) were added to the culture system. As a control, only chitosan (200 mg/L) was added to the culture medium. All flasks were incubated in the dark at 25 °C with shaking at 120 rpm. After one week, the culture materials were filtered through qualitative filter paper. The filtrates were extracted exhaustively with EtOAc (×3),

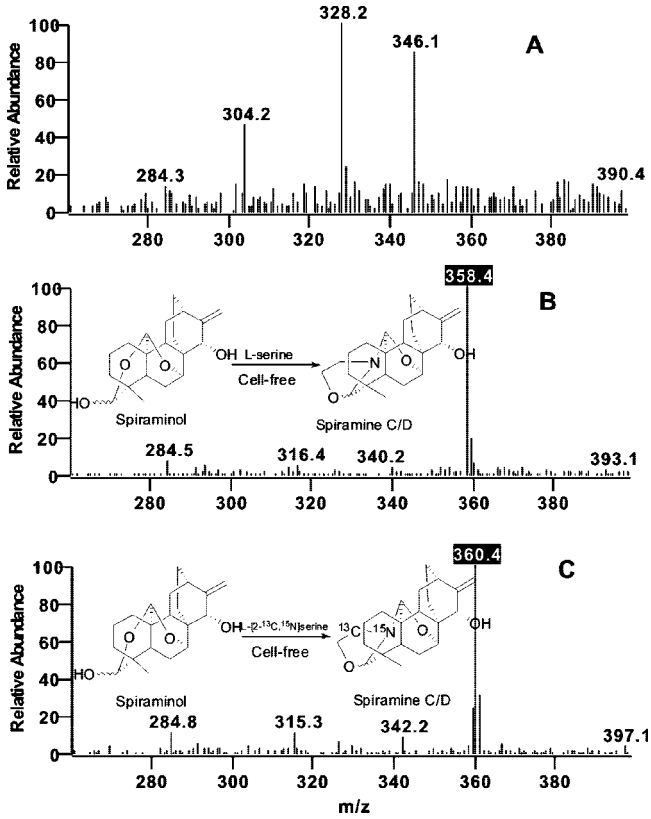

Figure 5. MS profiles of cell-free reaction extracts. (A) MS profile of cell-free blank control extract; (B) MS profile of cell-free system fed L-serine and spiraminol extract; (C) MS profile of cell-free system fed L-[2-^{13}C,^{15}N]serine and spiraminol extract.

followed by the removal of the solvent under vacuum. The residues were dissolved in MeOH and transferred into a 2 mL volumetric flask for LCMS analysis.

Tandem MS to Identify Labeled Products. The products of feeding L-[2-^{13}C,^{15}N]serine were different from those of the control based on LCMS analysis. Therefore, initial isolation was carried out to separate the products. The extracts (59 mg) were purified by medium-pressure chromatography on an RP-18 silica gel (24 g) column, eluted with H_2O containing increasing amounts of MeOH. According to the mass spectra (the quasimolecular ion peaks of the target products were at *m/z* 358, 360, 400, and 402), the target product was combined into fraction sp-1, which was eluted with 30% (v/v) MeOH. Fraction sp-1 was subjected to medium-pressure chromatography on an RP-18 (24 g) column, eluted with H_2O containing 0.1% Et_3N and increasing amounts of MeOH. The target products were eluted with 90% MeOH containing 0.1% Et_3N, then combined into fraction sp-1-1. This fraction was separated chromatographically on Sephadex LH-20 (30 g) by elution with MeOH to obtain the mixture sp-11 (1 mg), which was used for tandem MS analysis. The labeled products were identified by comparing the split modes of the tandem MS with those of standard spiramines A/B and C/D (purity ≥99%).

Transformation of L-[2-^{13}C,^{15}N]Serine in the Cell-Free System to Confirm the Precursor. For the cell-free extracts, *in vitro* cultured plantlets (10 g) grown for four weeks were ground to a fine powder in liquid N_2 and extracted at 4 °C with 10 mL of extraction buffer (50 mM Tris-HCl [pH 8.0], 100 mM NaCl, 10 mM $MgCl_2$, 1 mM DDT, 0.5 mM EDTA). The extracts were centrifuged (3000*g* for 20 min at 4 °C) to remove the cell debris, and the supernatant was centrifuged again (30000*g* for 30 min at 4 °C) to isolate the supernatant. The supernatant was dialyzed against buffer (50 mM Tris-HCl [pH 8.0], 100 mM NaCl, 10 mM $MgCl_2$, 1 mM DDT, 0.5 mM EDTA) for 56 h at 4 °C to obtain the cell-free extract (about 15 mL). Three types of control experiments were used. One was a blank control, and two were assayed for crude enzyme activity with different substrates: 10 mM spiraminol and 10 mM L-[2-^{13}C,^{15}N]serine added to 0.2 mL of cell-free extract or 10 mM spiraminol and 10 mM L-serine added to 0.2 mL of cell-free extract. All reactions were incubated in a 37 °C water bath for 5 h. Each reaction was terminated and extracted with *n*-butanol (2 × 100.0 μL). The *n*-butanol phase was collected by centrifugation and used directly for LCMS analysis. The extracts of the reactions were analyzed by LCMS as described above. To achieve precise LCMS spectra, we omitted the preparative column from the LCMS system. All reactions were repeated twice for confirmation.

Acknowledgment. This work was supported by the National Natural Science Foundation of China (30070087).

Supporting Information Available: Methods for tandem MS and quantitative analyses. This material is available free of charge via the Internet at http://pubs.acs.org.

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NP800657J