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Synthesis of Glucose−Fipronil Conjugate and Its Phloem Mobility

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ABSTRACT: Phloem-mobile insecticides are preferred to achieve economically useful activity. However, only a few phloem-mobile synthetic insecticides are available. One approach to converting nonmobile insecticides into phloem-mobile types is introducing sugar to the parent compound. To test whether the addition of a glucose group to a non-phloem-mobile insecticide enables conversion into phloem-mobile, *N*-[3-cyano-1-[2,6-dichloro-4-(trifluoromethyl)phenyl]-4-[(trifluoromethyl)sulfinyl]-1*H*-pyrazol-5-yl]-1-(β-D-glucopyranosyl)-1*H*-1,2,3-triazole-4-methanamine (GTF) was prepared through click chemistry. A phloem-mobility test in *Ricinus communis* L. seedlings confirmed that GTF was mobile in the sieve tubes. Although GTF exhibited lower insecticidal activity against the third-instar larvae of *Pzlutella xylostella* than fipronil did, it can be reconverted into fipronil in adult plants of castor bean, thereby offsetting the decrease of insecticidal activity. Therefore, the presence of a glucose core confers phloem mobility to fipronil.

KEYWORDS: glucose, fipronil, phloem mobility, click chemistry

INTRODUCTION

The redistribution of insecticides to newly emerging tissues is enhanced by phloem mobility because these tissues are connected to the plant vascular system and supplied with nutrients from the phloem stream.[1] Controlling sucking insects, such as aphids, is difficult using non-phloem-mobile insecticides because these insecticides cannot be delivered to the site where pests cause the most feeding damage. This is especially obvious when the aphids are settled on roots or inside leaf deformations and galls.[2] Therefore, phloem-mobile insecticides are preferred for pest control efficacy. However, only a few phloem-mobile synthetic insecticides and even fewer bidirectional transported (phloem and xylem mobility) pesticides are available. Only spirotetramat, a newly developed insecticide, can currently be bidirectionally transported into plants.[3] Developing a novel insecticide with phloem-mobile properties is costly and difficult. An alternative efficient strategy is to add a carboxyl group, an amino acid, or a sugar to the parent compounds of existing non-phloem-mobile insecticides to make them cell permeant. For example, the addition of a glycinyl group into fipronil resulted in higher phloem mobility than possessed by fipronil alone in soybean.[4]

D-Glucose can be taken up by plant cells, tissues, or organs. Recent molecular techniques have revealed increasing numbers of hexose transporters that may mediate glucose transportation within plant cells.[5−7] 2-[*N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxyglucose, a conjugate that contains a D-glucose group and a 7-nitrobenz-2-oxa-1,3-diazole (NBD) moiety, was developed in 1996 and has been successfully applied in various studies,[8,9] Its transport is mediated via a plant plasmalemma-bound hexose transporter.[10] Research on its uptake has shown that it has transport properties similar to those of glucose.[11] The uptake activity of salicylic acid−glucose conjugates in tobacco cells was investigated by Dean et al.[12] The vacuolar uptake of glucose-conjugated chlorsulfuron was studied by Bartholomew et al.[13] The phloem-mobile attributes of hydroxymethyloxamyl glucurzonide

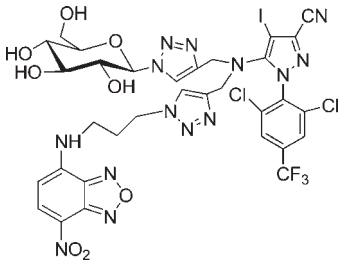

Figure 1. Chemical structure of IPGN.

(JR522) were described by Hsu et al.[14] These previous studies have indicated that glucose moieties can also guide its conjugates into plant cells, tissues, or organs, which led us to consider whether glucose-conjugated insecticides can endow non-phloem-mobile insecticides with phloem-mobile properties.

N-{3-Cyano-1-[2,6-dichloro-4-(trifluoromethyl)phenyl]-4-iodo-1*H*-pyrazol-5-yl}-*N*-{[1-(β-D-glucopyranosyl)-1*H*-1,2,3-triazole-4-yl]-methyl}-*N*-{[1-((*N*-(7-nitrobenz-2-oxa-1,3-diazole-4-amine))-propyl)-1*H*-1,2,3-triazole-4-yl]methyl}amine (IPGN, Figure 1) was synthesized in our previous work. The uptake experiments on tobacco cells proved that the glucose moiety can guide IPGN into tobacco cells via hexose transporters.[15] This result led us to further hypothesize that the addition of a glucose group to a non-phloem-mobile phenylpyrazole insecticide can enable the conversion of non-phloem-mobile insecticides into phloem-mobile types. Fipronil is a commercially successful phenylpyrazole insecticide. Although the xylem transport property of this insecticide has been demonstrated in sunflower plants,[16] its phloem transport property has not been reported to date. As part of our

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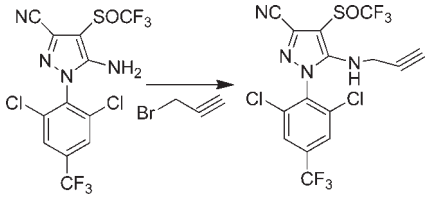
ongoing research program aimed at the synthesis of glucose and phenylpyrazole insecticides for phloem-mobility evaluation in plant tissues, we report herein the click chemistry-based[17] synthesis of a novel conjugate containing glucose and fipronil (GTF) and the investigation of its phloem mobility. The present paper includes also bioactivity assessments of GTF and a test on the reconversion of GTF into fipronil in adult castor bean plants.

MATERIALS AND METHODS

General Information for Synthesis. Reagents and anhydrous solvents were used as purchased without further purification. Melting points were determined on an XT4A digital micro melting point apparatus; data were left uncorrected. NMR spectra were obtained on a Bruker AV-600 instrument. Chemical shifts were expressed in parts per million with TMS as internal standard. The mass spectra (MS) of new compounds were obtained by a Finnigan MAT-95 mass spectrometer with electron impact (EI) ionization or by a Waters ZQ 4000 with electron-spray ionization (ESI). Data were reported as *m*/*z*. Analytical thin layer chromatography (TLC) was performed on silica gel GF254. Silica gel was used for column chromatography.

Plant Materials. Castor bean seeds (*Ricinus communis* L.) were obtained from the Agricultural Science Academy of Zibo Shandong China and grown as previously described.[18] Six days after sowing, average-sized seedlings were selected for the experiments.

Scheme 1. Reagents and Conditions: KOH, Acetone, Room Temperature, 15 h



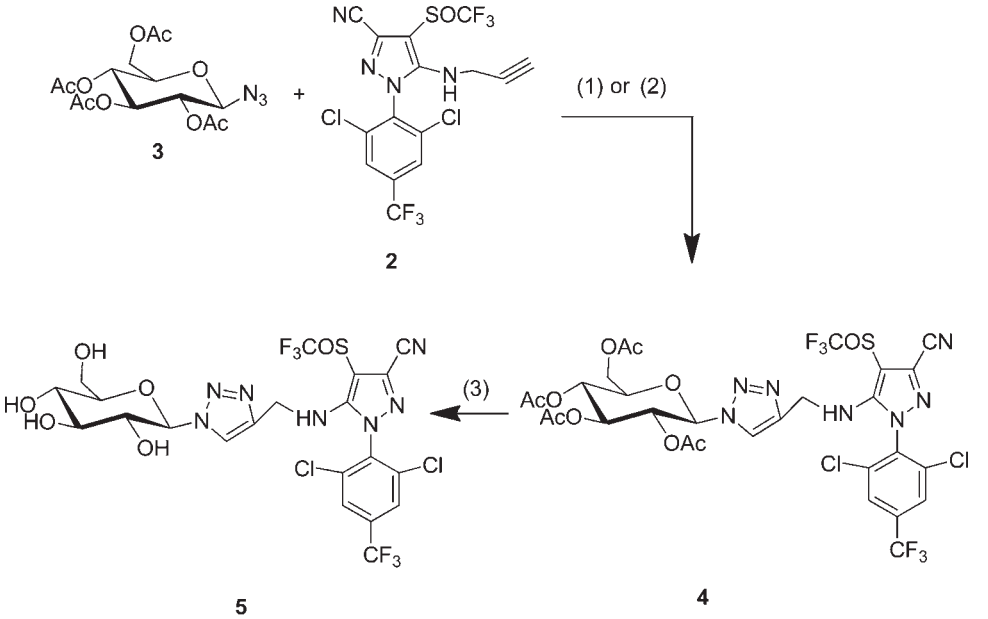
The adult plants of castor bean were obtained according to the previously described procedures.[19] Briefly, 6-day-old seedlings were grown in half-strength Hoagland's solution for 3−4 weeks until they possessed four true leaves, when the cotyledons and primary leaves were removed. The plants were used in the tests 2 days later.

Insects. Larvae of *Plutella xylostella* and their feed were obtained from Guangzhou Biological Control Station and raised with feed at 26 ± 2 °C and 70% relative humidity under a photoperiod of 16:8 h (light/dark).

Synthesis of 5-(2-Propyn-1-ylamino)-1-[2,6-dichloro-4-(trifluoromethyl)phenyl]-4-[(trifluoromethyl)sulfinyl]-1*H*-pyrazole-3-carbonitrile (2) (Scheme 1). Potassium hydroxide (370 mg, 6.6 mmol) was added to the solution of **1** (1.34 g, 3 mmol) and propargyl bromide (3.3 mmol) in dry acetone (15 mL). The mixture was then stirred at room temperature for 15 h. The reaction mixture was quenched by adding ice−water, and the resultant mixture was extracted with ethyl acetate (15 mL × 3). The combined organic layers were washed with aqueous sodium hydrogen carbonate and brine, dried with sodium sulfate, filtered, and evaporated in vacuo. The residues were purified by column chromatography to obtain the desired product **2** as a yellow solid in 61% yield, which was crystallized from ethyl acetate/hexane to provide a white solid: mp 187−189 °C; ^{1}H ($CDCl_3$, 600 MHz), δ 7.80 (s, 2H), 5.90 (br s, 1H) 3.68 (m, 2H), 2.25 (t, J = 2.4 Hz, 1H); ^{13}C ($CDCl_3$, 150 MHz), δ 151.2, 136.6, 136.5, 135.6, 134.8 (q, J = 34.5 Hz), 128.5, 126.6, 126.3, 126.2 (q, J = 335 Hz), 124.1, 122.6 (q, J = 274 Hz), 121.8, 111.0, 96.1, 77.1, 74.0, 34.3; EI-MS, *m*/*z* 474 $[M]^+$

Synthesis of *N*-[3-Cyano-1-[2,6-dichloro-4-(trifluoromethyl)-phenyl]-4-[(trifluoromethyl)sulfinyl]-1*H*-pyrazol-5-yl]-1-(2,3,4,-6-tetra-*O*-acetyl-β-D-glucopyranosyl)-1*H*-1,2,3-triazole-4-methanamine (4) (Scheme 2). Compound **2** (474 mg, 1 mmol) was added to a vigorously stirred suspension of 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl azide **3** (373 mg, 1 mmol) in 3 mL of *tert*-butyl alcohol. The reaction was initiated by the addition of a solution of $CuSO_4 \cdot 5H_2O$ (100 mg, 0.4 mmol) and sodium ascorbate (173 mg, 0.8 mmol) in distilled water (3 mL). The deep yellow suspension was stirred vigorously at 60 °C for 3 h. Distilled water (10 mL) was added, and the aqueous layer was extracted with chloroform (10 mL × 3). The combined organic extracts were washed with aqueous sodium

Scheme 2. Reagents and Conditions: (1) $CuSO_4 \cdot 5H_2O$ (0.4 equiv), Sodium Ascorbate (0.8 equiv), 1:1 t-BuOH/H_2O, 60 °C, 3 h; (2) CuI/DIPEA, Acetonitrile, Room Temperature, 20 min; (3) 0.05 M NaOMe/MeOH, Room Temperature, 30 min



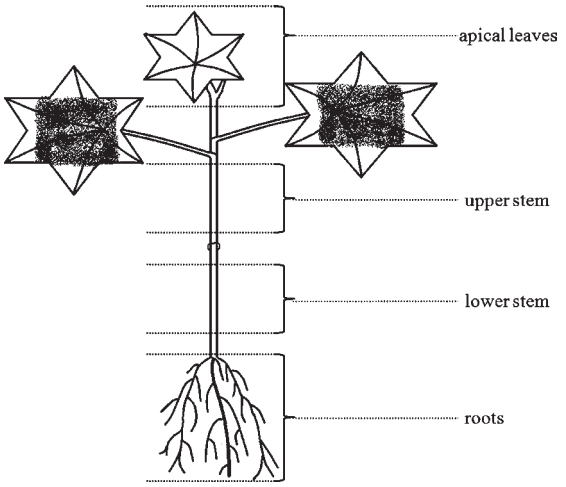


Figure 2. Diagrammatic representation of the various sections of *R. communis* plant individually analyzed following the application of compounds to the two mature leaves (black area).

hydrogen carbonate and brine, dried with sodium sulfate, filtered, and evaporated in vacuo. The residues were purified by column chromatography to achieve the desired product **4** as a white solid in 95% yield: mp 135−137 °C; ^{1}H ($CDCl_3$, 600 MHz), δ 7.76, 7.72 (2s, 2H), 7.68 (s, 1H), 6.05 (br s, 1H), 5.79 (d, J = 9.0 Hz, 1H), 5.39 (t, J = 9.6 Hz, 1H), 5.34 (t, J = 9.6 Hz, 1H), 5.20 (t, J = 9.0 Hz, 1H), 4.37 (m, 1H), 4.27 (m, 1H), 4.17 (d, J = 12.6 Hz, 2H), 3.98 (ddd, J = 1.8, 4.8, 10.2 Hz, 1H), 2.08, 2.07, 2.02, 1.85 (4s, 12H); ^{13}C ($CDCl_3$, 150 MHz), δ 170.3, 169.8, 169.3, 168.9, 151.5, 144.0, 136.3, 136.2, 134.9 (q, J = 34.5 Hz), 127.0, 126.6, 126.4, 126.3 (q, J = 335 Hz), 124.2, 122.6 (q, J = 270 Hz), 120.6, 120.3, 110.1, 95.7, 85.7, 75.2, 72.3, 70.1, 67.5, 61.4, 40.4, 20.6, 20.5, 20.4, 20.1.; ESI-MS, m/z 848 $[M + 1]^+$, 870 $[M + Na]^+$.

Synthesis of *N*-[3-Cyano-1-[2,6-dichloro-4-(trifluoromethyl)-phenyl]-4-[(trifluoromethyl)sulfinyl]-1*H*-pyrazol-5-yl]-1-(β-D-glucopyranosyl)-1*H*-1,2,3-triazole-4-methanamine (5) (Scheme 2). Compound **4** (164 mg, 0.2 mmol) was added to a solution of sodium methoxide in dry methanol (0.05 M, 3 mL). The resultant solution was stirred for 30 min at room termperature. The mixture was neutralized with Amberlite IR 120 (H^+) resin and filtered, and the filtrate was then evaporated. The residues were purified by column chromatography to obtain the desired product **5** as a white solid in 87% yield: mp 204−206 °C; ^{1}H (MeOD, 600 MHz), δ 8.05 (s, 2H), 8.04 (s, 1H), 5.56 (d, J = 9.0 Hz, 1H), 4.60 (dd, J = 4.2 Hz, J = 10.2 Hz, 1H), 4.55 (br s, 1H), 4.45 (dd, J = 1.8 Hz, J = 10.2 Hz, 1H), 3.84−3.88 (m, 2H), 3.68−3.71 (m, 1H), 3.52−3.56 (m, 2H), 3.45 (m, 1H); ^{13}C (MeOD, 150 MHz), δ 152.0, 145.2, 137.9, 136.1, 135.8 (q, J = 34.5 Hz), 128.1, 128.0, 127.9, 126.3 (q, J = 335 Hz), 125.9, 123.6, 122.7 (q, J = 270 Hz), 112.1, 97.7, 89.5, 81.1, 78.6, 74.0, 70.9, 62.4, 41.1; ESI-MS, m/z 680 $[M + 1]^+$, 702 $[M + Na]^+$.

Membrane Potential Measurements. Plasma membrane potential was measured using flow cytometric analysis of the protoplasts of *R. communis* cotyledons; bis(1,3-dibutylbarbituric acid)trimethine oxonol [$DiBAC_4(3)$] was selected as the fluorescent membrane potential indicator dye. Initially, the cotyledons (4 g) of castor bean seedlings were used to prepare the protoplasts. Protoplast isolation was performed according to a previously described method.[20] The protoplasts were obtained by digesting cell walls with 20 mL of digestion solution containing 1.0% cellulase, 0.2% pectinase, 0.6 M mannitol, 0.25 mM $MgCl_2$, 0.5 mM $CaCl_2$, and 20 mM MES/NaOH (pH 5.8) for 4 h at 28 °C. At the end of digestion, the protoplasts were suspended in 30 mL of buffer solution containing 0.6 M mannitol, 20 mM MES/NaOH (pH 5.8), 0.25 mM $MgCl_2$, and 0.5 mM $CaCl_2$. Then, GTF or fipronil was added to the

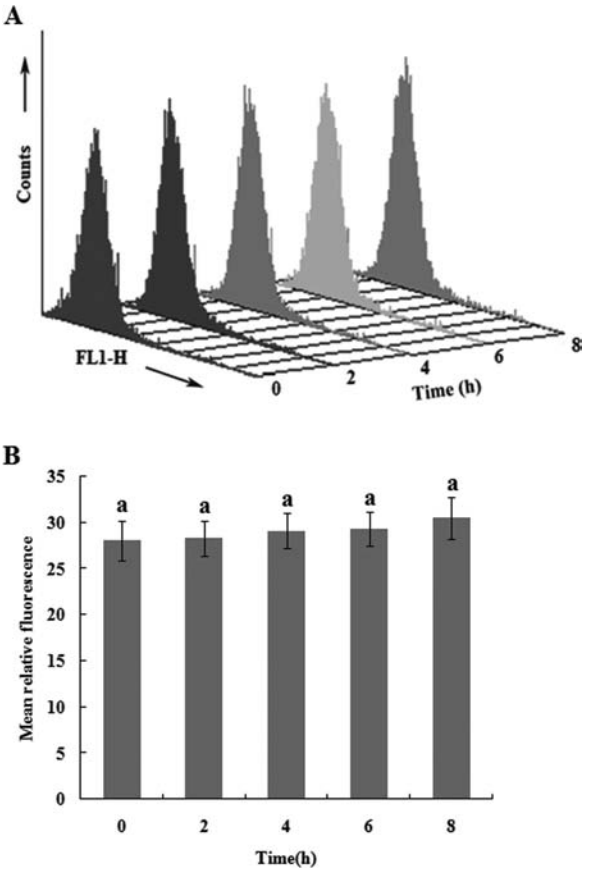
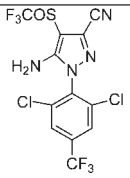
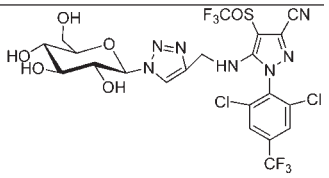


Figure 3. Time course of plasma membrane potential in the protoplasts of *R. communis* cotyledons: (A) fluorescent intensity histogram marked $DiBAC_4(3)$ after addition of GTF, control (0 h); (B) relative fluorescence after addition of GTF, control (0 h). The protoplasts were suspended in a buffered solution at pH 5.8 with or without (control) GTF (200 μM) for 8 h. The protoplasts were analyzed by flow cytometry for $DiBAC_4(3)$ fluorescence. The data (mean ± SE, n = 3) within a column topped by the same letter are not significantly different as shown by Duncan's multiple-range test ($P > 0.05$).

protoplast suspension (10 mL each, including the blank control) at 200 μM concentration. The protoplast suspension was then gently and constantly oscillated on a reciprocal shaker at 28 °C. Samples (1 mL) from the total protoplast suspension (10 mL) were obtained every 2 h during an 8 h period and incubated with 2 μM $DiBAC_4(3)$ for 30 min at 28 °C prior to flow cytometric analysis. Fluorescence was measured with a Becton Dickinson and FACSort equipped with CellQuest software (Becton Dickinson). To determine membrane potential, the $DiBAC_4(3)$ fluorescence of 20000 viable cells was measured on FL1 (excitation at 488 nm; emission at 530 nm). The percentage of cells with increased $DiBAC_4(3)$ fluorescence was determined by gating on the fresh, viable population of cells. Cells with $DiBAC_4(3)$ fluorescence greater than that of the fresh population were quantified for each treatment. Duncan's multiple-range test at a 5% probability level was used to determine statistical differences among treatments.

Sap Collection from *R. communis* L. Seedlings and Analysis. The phloem sap was collected from the upper part of the hypocotyl according to previously described methods,[18,21,22] The endosperm of seedlings was carefully removed without bending or crushing the cotyledons. These latter organs were incubated in buffer solution containing 20 mM MES (pH 5.5), 0.25 mM $MgCl_2$, and 0.5 mM $CaCl_2$ supplemented with 200 μM GTF or 200 μM fipronil. After 1 h of incubation, the hypocotyl

Table 1. Chemical Structures and Physicochemical Descriptor [Molecular Mass (MW), Log *D*, Polar Surface Area (PSA), Number of Hydrogen Bond Donors (HBD), and Number of Hydrogen Bond Acceptors (HBA)] of Fipronil and GTF[a]

Feature	fipronil	GTF
Structural formula		
MW (D)	437	680
Log D	3.98 ±1.52	2.80 ±1.59
PSA (Å^2)	103.91	210.78
HBD	2	5
HBA	5	13

[a] All parameters were computed using ACD Log D suite version 12.02 software.

was severed in the hook region for phloem exudation, and the collected sap was stored in ice until analysis.

The phloem sap was analyzed by HPLC [HP 1100 (Agilent Co.) system equipped with quaternary pump, autosampler, and UV−visible detector] after dilution with pure water (phloem sap/pure water, 1:5, v/v). Separations were made with a C_{18} reversed-phase column (5 μm, 250 × 4.6 mm i.d., Agilent Co.) at 25 °C. The mobile solvent system consisted of acetonitrile and water (50:50, v/v) at a flow rate of 1.0 mL min^{-1}. The detector wavelength was 210 nm for GTF and fipronil. An external calibration method was used to determine the concentrations of GTF and fipronil. A series of standard solutions of GTF and fipronil (0.5, 1, 5, 10, 25, 50, and 100 μM) for linearities were prepared in methanol. The linear equation of GTF was $y = 20.36x - 0.52$ ($R^2 = 0.9997$) and that for fipronil was $y = 20.05x + 3.06$ ($R^2 = 0.9999$).

Identification of GTF in phloem sap was conducted with an HPLC−mass spectrometer (MS) (LCQ-DECA, Finnigan) using atmospheric pressure chemical ionization (APCI). The LC system consisted of a P9000 pump, an AS3000 autosampler, and a UV6000LP detector (set at 210 nm). The operating conditions of the HPLC were the same as described above. The instrument parameters used for MS system were as follows: capillary temperature, 200 °C; spray voltage, 4.5 kV; spray current, 5 μA; positive ion mode; scan range, 100−1000 amu in 1 s.

Insecticidal Activity of GTF against *P. xylostella*. Assessments of the GTF and fipronil bioactivities on the third-instar larvae of *P. xylostella* were performed according to the previously described procedures[4] by the leaf disk-dipping assay. Briefly, GTF and fipronil were dissolved in acetone and suspended in distilled water containing Tween 80 (0.1%). Leaf disks (5.5 cm diameter) were dipped in each test solution for 30 s and allowed to dry for 2 h. The treated leaf disks were placed into Petri dishes (9 cm diameter). Ten *P. xylostella* larvae were then introduced into each dish. Distilled water containing acetone (10%) and Tween 80 (0.1%) solutions, but not the tested compound, was used as the control. Petri dishes were incubated at 26 ± 2 °C and 70% relative humidity under a photoperiod of 16:8 h (light/dark). All treatments were repeated five times. Mortalities were determined 24 h post treatment.

Assay for GTF Reconversion to Fipronil in Adult Plants of Castor Bean. Foliar-applied chemical was conducted according to the described procedures of Smith and Milburn.[23] Briefly, 100 mm^2 of the adaxial surface of two fully expanded leaves was gently abraded with a slurry of fine Celite 545 (USA) silica particles, followed by washing with distilled water, and then blotted dry with absorbent tissue. Then, 200 μL

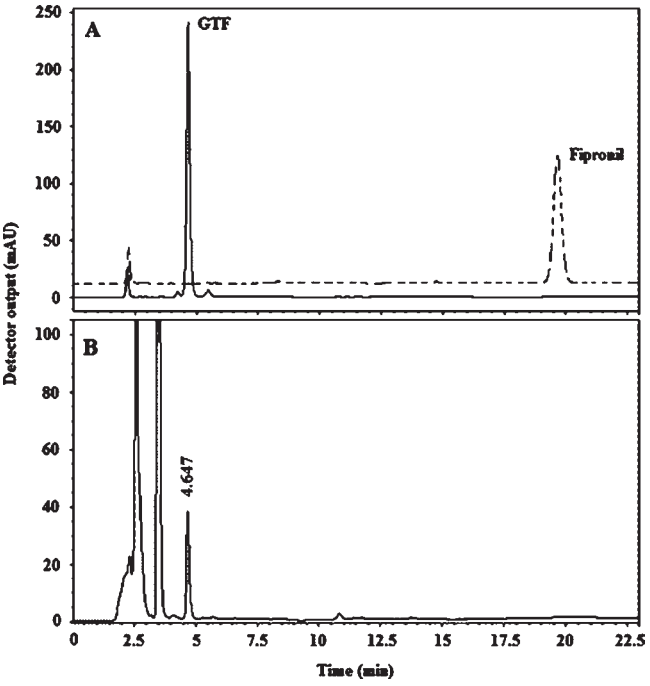


Figure 4. HPLC chromatograms of the standard solution and *R. communis* phloem sap: (A) standard solution, retention time (RT) of GTF (RT 4.64 min) and fipronil (RT 19.61 min); (B) treated set, cotyledons incubated in buffer solution containing GTF (RT 4.65) at 200 μM.

of 200 μM GTF aqueous solution containing Tween 80 (0.1%) and acetone (10%) was then applied to the abraded area of each leaf, and the aqueous solution containing Tween 80 (0.1%) and acetone (10%) but not GTF was used as the control. The plants were grown for 48 and 72 h after the application of GTF. The plants (three replicates) were then sectioned into apical leaves, upper stem, lower stem, and roots (Figure 2). Samples were stored at −15 °C until they could be analyzed.

Plant samples (2 g) were macerated with portions of acetone in a glass mortar and pestle, transferred to volumetric flasks (50 mL), and ultrasonically treated for 15 min with 10 mL of acetone. The extracts were filtered, and filtrate residues were reextracted twice. The combined extracts were rotary-evaporated to dryness at a bath temperature not exceeding 40 °C, and the residues were transferred in 2 mL of methanol to a solid-phase extraction (SPE) cartridge (AccuBond C18 SPE, Agilent Technologies). The SPE columns were eluted in another 2 mL methanol, the collected solution was evaporated to a small volume (0.5 mL) under nitrogen at room temperature, and the resulting solutions were used for HPLC analysis using the aforementioned system.

The efficiencies of these extraction procedures, assessed by adding known amounts of fipronil to untreated plant tissues, were >90%. All results were corrected for the appropriate efficiency of recovery.

Identification of fipronil in the plant samples was performed using an HPLC-MS (LCQ^{DECA}, Finnigan) using the system above but with negative ion mode.

Physicochemical Properties. The physicochemical properties [molecular mass (MW), log *D*, polar surface area (PSA), number of hydrogen bond donors (HBD), and number of hydrogen bond acceptors(HBA)] of fipronil and GTF were predicted using ACD LogD suite version 12.02 software.

RESULTS AND DISCUSSION

Synthesis and Characterization. To prepare a conjugate containing both fipronil and glucose moieties via click chemistry,

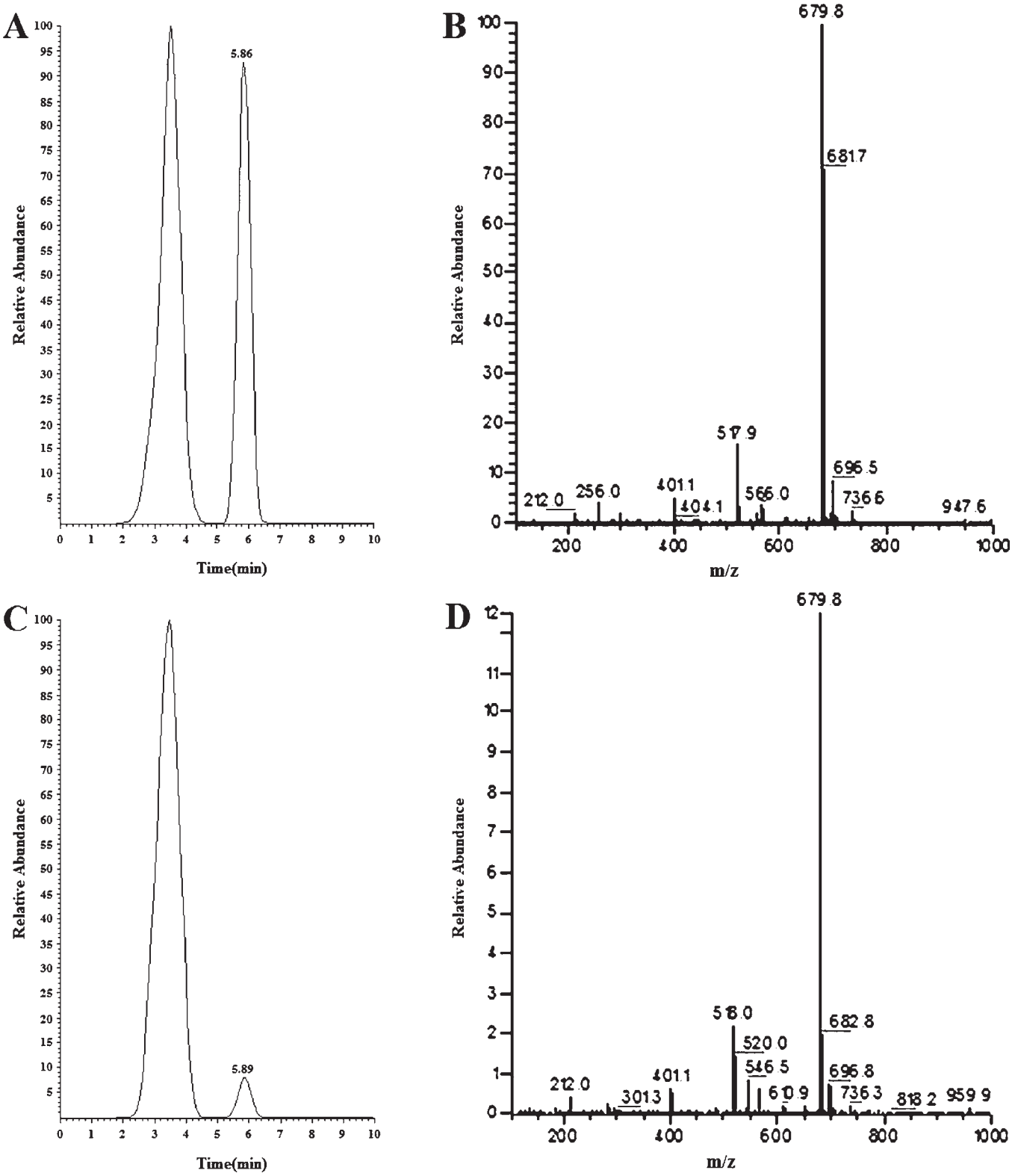


Figure 5. LC-MS analysis of GTF in *R. communis* phloem sap when the cotyledons were incubated in buffer solution containing 200 μM GTF: (A) standard solution of GTF; (B) positive ion APCI mass spectrum of 5.86 min peak in panel A showing $[M + H]^-$ ion at m/z 680; (C) phloem extraction solution of *R. communis* seedlings; (D) positive ion APCI mass spectrum of 5.89 min peak in panel C confirming the expected $[M + H]^-$ ion at m/z 680 corresponding to GTF.

an azide group was introduced into β-D-glucopyranose pentaacetate, whereas propargyl was introduced into fipronil as a coupling partner. As outlined in Scheme 1, propargyl bromide was reacted with fipronil in the presence of potassium hydroxide in acetone at room temperature for 15 h to produce intermediate **2** as a yellow solid in 61% yield. Two widely used click reaction systems,

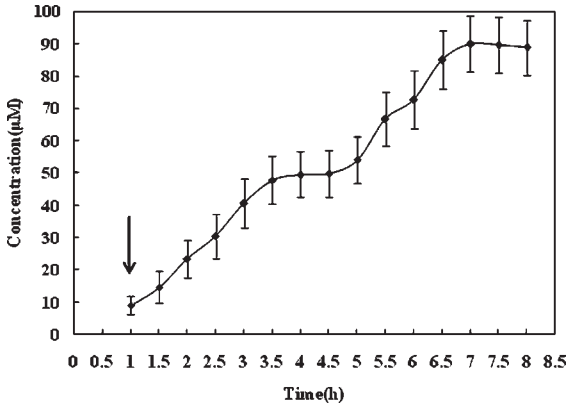


Figure 6. Time course of GTF concentration in phloem sap of *R. communis*. The cotyledons were incubated in a buffered solution containing 200 μM GTF at pH 5.0. The hook was severed at time 1 h (arrow), and then the sap was collected every 0.5 h during 8 h (mean of 10 plants ± SE, n = 5).

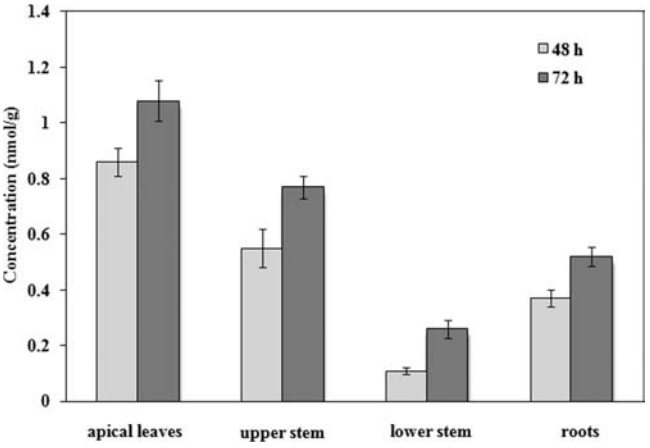


Figure 7. Concentration of fipronil detected in adult castor bean plants 48 and 72 h post application of 200 μL of GTF solution (2 mM) on each of the two mature leaves (mean ± SE, n = 3).

$CuSO_4 \cdot 5H_2O$/ascorbate and CuI/DIPEA,[24,25] were explored to prepare conjugate **4** (Scheme 2). However, only the $CuSO_4 \cdot 5H_2O$/ascorbate system gave a satisfactory yield of 95%. After the condensation of **2** with **3**, the coupling product was deprotected by treatment with 0.05 M solution of sodium methoxide in methanol. Conjugate **5** (GTF) containing glucose and fipronil was obtained in 87% yield.

The structures of **2**, **4**, and **5** were confirmed by ^{1}H, ^{13}C NMR, and ESI-MS spectra (EI-MS spectra except **3**). In the ^{1}H NMR spectra of **3**, the peaks of alkyne, methylene, and aryl protons were found at 2.25, 3.68, and 7.80 ppm, respectively. The peak area ratio among them was 2:2:1, which coincides well with the expected values of compound **3** containing one propargyl moiety. The characteristic signals of the terminal alkyne carbon atoms at 77.1 and 74.0 ppm could be observed in the ^{13}C NMR spectra of compound **2**. Compared with compound **2**, conjugate **4** exhibited the characteristic signals of 1,2,3-triazole and sugar rings, as well as the acetyl groups in its ^{1}H and ^{13}C NMR spectra. The removal of acetyl groups from conjugate **4** was confirmed by the ^{1}H and ^{13}C NMR spectra of **5**. The characteristic peaks at 1.8−2.0 ppm in the ^{1}H NMR spectra and the peaks at 20 and 169−170 ppm in the ^{13}C NMR spectra disappeared after deprotection. The mass spectra of the products exhibited excellent correlation with the calculated molecular mass.

Systemicity of GTF. The castor bean system is widely employed to evaluate phloem mobility of nutrients as well as xenobiotics,[18,26] However, this model is reliable only if the xenobiotics tested are nonphytotoxic in short-term experiments, particularly if they do not depolarize the transmembrane potential difference (PD). The values of the transmembrane PD are good indicators of the activity of plasma membrane proton-pumping ATPase, which energizes the secondary translocation of nutrients and maintains acidic molecule influx through the lipid bilayer,[18,27]

Flow cytometry is a technique for measuring the properties of cells or cellular particles in liquid suspension. It is widely used to measure membrane potential in animal cells; most developed flow cytometry methods in animal cells have been adapted for application to plant cells.[28] Petit[29] used this technique to measure membrane potential in plant mitochondria. $DiBAC_4(3)$ is a commonly used indicator dye in the membrane potential measurements of animal cells. The suitability of $DiBAC_4(3)$ for plant cell membrane potential measurement was demonstrated by Konrad and Hedrich.[30] In the current work, the plasma membrane potential was measured by flow cytometric analysis of the protoplasts of *Ricinus* cotyledons, with $DiBAC_4(3)$ as the fluorescent indicator dye for membrane potential. Figure 3 shows that, compared with the control, the relative fluorescence data were not significantly different during the treatment period. Results indicate that GTF has no depolarizing effect on the transmembrane PD, similar to fipronil (data not shown). Therefore, the *Ricinus* system is suitable for testing the phloem systemicity of GTF.

When *Ricinus* cotyledons were incubated with 200 μM fipronil for 8 h, the insecticide was not detected in the phloem sap. By contrast, when the cotyledons were incubated in GTF solution at the same concentration, the conjugate was clearly observed in the phloem sap. Fipronil cannot be found in the phloem sap due to its high log D value (log D = 3.98 ± 1.52, Table 1). GTF in the phloem sap was further identified by HPLC-MS (Figure 5). In the latter experiment, the parent molecule fipronil was not detected in the phloem sap (Figure 4), indicating that GTF was relatively stable in the sieve element−companion cell complex during the 8 h test period. Given that *Ricinus* is a symplastic−apoplastic loader, three pathways for external GTF into the sieve tubes of the *Ricinus* cotyledons should be considered. GTF may be loaded into the sieve tubes after passage through the cell wall space (direct apoplasmic loading), from the apoplasm after transit through mesophyll cells (indirect apoplasmic loading), or via plasmodesmata after uptake by mesophyll cells (symplasmic loading). Time course experiments have indicated that GTF concentration in the phloem sap increased for 3.5 h before reaching the first plateau and reached the second plateau at 7 h (Figure 6). Its concentration in the phloem sap was approximately 0.25- and 0.45-fold than those of the incubated solution buffered at 3.5 and 7 h, respectively.

The addition of a carboxyl group, an amino acid, or a sugar to the parent compound is an efficient strategy for converting a non-mobile crop protection product into a phloem-mobile type. In the last two approaches, the uptake and translocation of conjugates may involve two components of specific active carrier-mediated systems and passive transport. For example, 2,4D–Lys uptake was proved to involve an active carrier-mediated system aside from diffusion.[21] The authors' previous study has also indicated that an active transport process was involved in the uptake of IPNG into tobacco cells and that the glucose group in this molecule played a

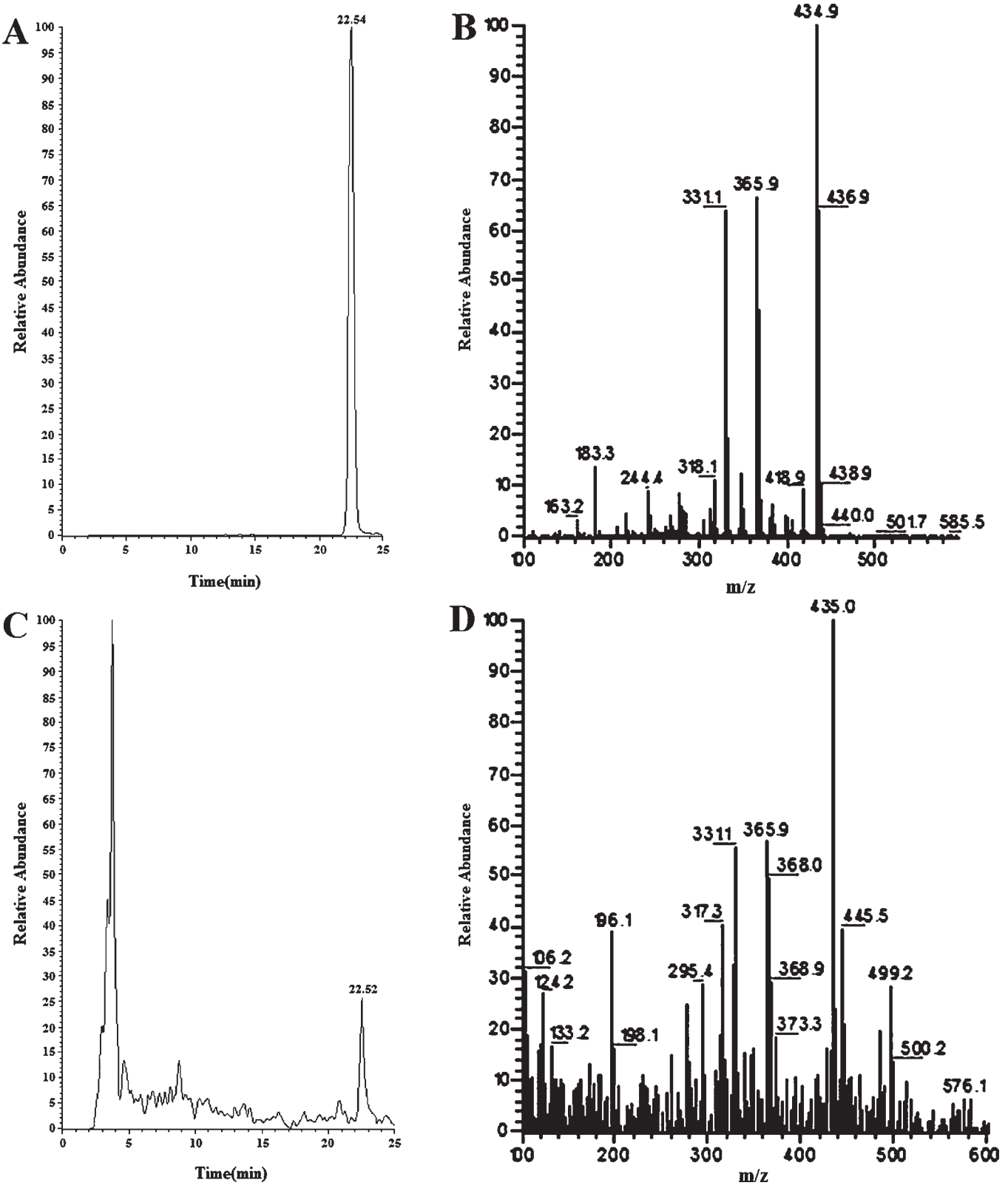


Figure 8. LC-MS analysis of fipronil in castor bean plants after GTF application: (A) standard solution of fipronil; (B) negative ion APCI mass spectrum of 22.54 min peak in panel A showing $[M - H]^-$ ion at m/z 435; (C) plant sample extracting solution of *R. communis*; (D) negative ion APCI mass spectrum of 22.52 min peak in panel C confirming the expected $[M - H]^-$ ion at m/z 435 corresponding to fipronil.

key role in translocating the molecule into the cells.[15] The "Rule of Five" widely adopted within the pharmaceutical industry[31] can also be used for the prediction of diffusion of endogenous molecules or xenobiotics through the plant membranes.[32] According to this rule,

passive absorption of small molecules is more likely when their molecular mass (MW), polar surface area (PSA), number of hydrogen bond donors (HBD), and number of hydrogen bond acceptors (HBA) are less than 500 Da, 60 Å^2, 5, and 10, respectively. Taking into account the physicochemical properties (MW = 680 Da, PSA = 211 Å^2, HBA = 13) of GTF in Table 1, the diffusion of this conjugate through the membranes must be very low. Therefore, a carrier system is likely involved in the long-distance transport of GTF.

Insecticidal Activity. The insecticidal activity of GTF against *P. xylostella* was evaluated and compared with that of fipronil over a wide range of concentrations. GTF exhibited low insecticidal activity against the third-instar larvae of *P. xylostella* with a 24 h LC_{50} value (95% confidence limits) of 167.28 mg L^{-1} (124.71−224.38 mg L^{-1}). This LC_{50} value is significantly higher than that of fipronil [21.39 mg L^{-1} (16.32−28.03 mg L^{-1})]. The addition of the glucose group to fipronil resulted in insecticidal activity lower than that of fipronil alone. As noted by Hsu et al.,[14] specific structural modifications that confer phloem mobility to pesticides usually have the unintended consequence of suppressing biological activity. This phenomenon was also observed in the present work. The addition of the glucose group into fipronil led to enhanced phloem systemicity, but decreased biological activity.

GTF Reconversion into the Parent Molecule in the Plant. Given the aforementioned approaches to transformation into phloem-mobile insecticides, the conjugated molecule should exhibit the same (or similar) insecticidal activity, or it can be reconverted to the parent molecule in the plants. The conjugate of hydroxymethyloxamyl glucuronide is a concrete case of site-specific release to the parent molecule in plants.[14] Because GTF exhibits considerably lower insecticidal activity than did fipronil, the possibility of GTF reconversion into fipronil in plants determines the success of the strategy proposed in the current work. A test on GTF reconversion into fipronil in adult plants of castor bean was conducted. As shown in Figure 7, fipronil was detected in all harvested sections (apical leaves, upper stem, lower stem, and roots) 48 and 72 h post treatment, further identified by HPLC-MS (Figure 8). GTF was cleaved to release the parent compound in the target tissues. Given that GTF was cleaved to release the nonsystemic parent compound, the distribution of fipronil in the entire plant is the result of the long-distance transport of the conjugate. This long-distance transport with a concentration in the roots higher than in the "lower stem" is completely incompatible with a diffusion mechanism within the donor leaf veins. Because GTF is nonionizable at biological pH values, its diffusion through membranes must occur in both directions; this diffusion must dramatically restrict the symplastic transport of GTF within the plant. These data clearly indicate that an active carrier system is involved in the phloem loading of GTF and its long-distance transport.

In summary, a conjugate containing both fipronil and glucose moieties was synthesized via click chemistry with good overall yield. The phloem-mobility tests in which the *Ricinus* system was used have demonstrated that the conjugate moves in the phloem without being degraded during an 8 h test period in detectable amounts. This phenomenon indicates that the conjugate was relatively stable in the sieve tubes that facilitate the export from leaf tissues and long-distance transport within the plant. Therefore, the addition of a glucose core into fipronil promotes phloem mobility. In addition, the existing two GTF uptake plateaus in the phloem sap and long-distance transport to the sinks indicate that the phloem loading mechanism may involve active carrier-mediated systems, a result that requires further study. Although GTF exhibited lower insecticidal activity than did fipronil, the degradation test showed that GTF was reconverted into fipronil in adult castor bean plants when a foliar-applied chemical was performed. This specific degradation property enables GTF to maintain the insecticidal activity when it is applied to plants, although this conclusion requires further confirmation. Hence, the addition of a glucose core is a reasonable and feasible approach to conferring phloem mobility to fipronil. The current paper offered a supplementary strategy for the efforts in converting non-phloem-mobile insecticidal products into phloem-mobile types.

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ABBREVIATIONS USED

GTF, *N*-[3-cyano-1-[2,6-dichloro-4-(trifluoromethyl)phenyl]-4-[(trifluoromethyl)sulfinyl]-1*H*-pyrazol-5-yl]-1-(β-D-glucopyranosyl)-1*H*-1,2,3-triazole-4-methanamine; NBD, 7-nitrobenz-2-oxa-1,3-diazole; IPGN, *N*-{3-cyano-1-[2,6-dichloro-4-(trifluoromethyl)phenyl]-4-iodo-1*H*-pyrazol-5-yl}-*N*-{[1-(β-D-glucopyranosyl)-1*H*-1,2,3-triazole-4-yl]methyl}-*N*-{[1-((*N*-(7-nitrobenz-2-oxa-1,3-diazole-4-amine))propyl)-1*H*-1,2,3-triazole-4-yl]methyl}amine; $DiBAC_4(3)$, bis(1,3-dibutylbarbituric acid)trimethine oxonol; SPE, solid-phase extraction; DIPEA, *N*,*N*-diisopropylethylamine; LC_{50}, median lethal concentration.

REFERENCES

(1) Lichtner, F. Phloem mobility of crop protection products. *Aust. J. Plant Physiol.* **2000**, *27*, 609–614.

(2) Dedryver, C. A.; Le, R. A.; Fabre, F. A. The conflicting relationships between aphids and men: a review of aphid damage and control strategies. *C. R. Biol.* **2010**, *333*, 539–553.

(3) Bruck, E.; Elbert, A.; Fischer, R.; Krueger, S.; Kuhnhold, J.; Klueken, A. M.; Nauen, R.; Niebes, J. F.; Reckmann, U.; Schnorbach, H. J. Movento®, an innovative ambimobile insecticide for sucking insect pest control in agriculture: biological profile and field performance. *Crop Prot.* **2009**, *28*, 838–844.

(4) Jiang, D. X.; Lu, X. L.; Hu, S.; Zhang, X. B.; Xu, H. H. A new derivative of fipronil: effect of adding a glycinyl group to the 5-amine of pyrazole on phloem mobility and insecticidal activity. *Pestic. Biochem. Phys.* **2009**, *95*, 126–130.

(5) Schmidt, S.; Joost, H. G.; Schürmann, A. GLUT8, the enigmatic intracellular hexose transporter. *Am. J. Physiol.—Endocrinol. Metab.* **2009**, *296*, E614–E618.

(6) Cheeseman, C. GLUT7: a new intestinal facilitated hexose transporter. *Am. J. Physiol.—Endocrinol. Metab.* **2008**, *295*, E238–E241.

(7) Chikamori, M.; Fukushima, K. A new hexose transporter from *Cryptococcus neoformans*: molecular cloning and structural and functional characterization. *Fungal Genet. Biol.* **2005**, *42*, 646–655.

(8) Zou, C.; Wang, Y.; Shen, Z. 2-NBDG as a fluorescent indicator for direct glucose uptake measurement. *J. Biochem. Biophys. Methods* **2005**, *64*, 207–215.

(9) Oh, K. B.; Matsuoka, H. Rapid viability assessment of yeast cells using vital staining with 2-NBDG, a fluorescent derivative of glucose. *Int. J. Food Microbiol.* **2002**, *76*, 47–53.

(10) Yoshioka, K.; Takahashi, H.; Homma, T.; Saito, M.; Oh, K. B.; Nemoto, Y.; Matsuoka, H. A novel fluorescent derivative of glucose applicable to the assessment of glucose uptake activity of *Escherichia coli*. *Biochim. Biophys. Acta—Gen. Subj.* **1996**, *1289*, 5–9.

(11) Etxeberria, E.; González, P.; Tomlinson, P.; Pozueta-Romero, J. Existence of two parallel mechanisms for glucose uptake in heterotrophic plant cells. *J. Exp. Bot.* **2005**, *56*, 1905–1912.

(12) Dean, J. V.; Mohammed, L. A.; Fitzpatrick, T. The formation, vacuolar localization, and tonoplast transport of salicylic acid glucose conjugates in tobacco cell suspension cultures. *Planta* **2005**, *221*, 287–296.

(13) Bartholomew, D. M.; Van Dyk, D. E.; Lau, S. M. C.; O'Keefe, D. P.; Rea, P. A.; Viitanen, P. V. Alternate energy-dependent pathways for the vacuolar uptake of glucose and glutathione conjugates. *Plant Physiol.* **2002**, *130*, 1562–1572.

(14) Hsu, F. C.; Sun, K.; Kleier, D. A.; Fielding, M. J. Phloem mobility of xenobiotics VI. A phloem-mobile pro-nematicide based on oxamyl exhibiting root-specific activation in transgenic tobacco. *Pestic. Sci.* **1995**, *44*, 9–19.

(15) Hu, A. L.; Yang, W.; Xu, H. H. Novel fluorescent conjugate containing glucose and NBD and its carrier-mediated uptake by tobacco cells. *J. Photochem. Photobiol. B—Biol.* **2010**, *101*, 215–223.

(16) Aajoud, A.; Raveton, M.; Aouadi, H.; Tissut, M.; Ravanel, P. Uptake and xylem transport of fipronil in sunflower. *J. Agric. Food Chem.* **2006**, *54*, 5055–5060.

(17) Kolb, H. C.; Finn, M. G.; Sharpless, K. B. Click chemistry: diverse chemical function from a few good reactions. *Angew. Chem., Int. Ed.* **2001**, *40*, 2004–2021.

(18) Chollet, J. F.; Rocher, F.; Jousse, C.; Delétage Grandon, C.; Bashiardes, G.; Bonnemain, J. L. Synthesis and phloem mobility of acidic derivatives of the fungicide fenpiclonil. *Pest Manag. Sci.* **2004**, *60*, 1063–1072.

(19) Bromilow, R. H.; Rigitano, R. L. O.; Briggs, G. G.; Chamberlain, K. Phloem translocation of non-ionised chemicals in *Ricinus communis*. *Pestic. Sci.* **1987**, *19*, 85–99.

(20) Denis, M. H.; Delrot, S. Carrier-mediated uptake of glyphosate in broad bean (*Vicia faba*) via a phosphate transporter. *Physiol. Plant.* **1993**, *87*, 569–575.

(21) Delétage-Grandon, C.; Chollet, J. F.; Faucher, M.; Rocher, F.; Komor, E.; Bonnemain, J. L. Carrier-mediated uptake and phloem systemy of a 350-Dalton chlorinated xenobiotic with an α-amino acid function. *Plant Physiol.* **2001**, *125*, 1620–1632.

(22) Kallarackal, J.; Orlich, G.; Schobert, C.; Komor, E. Sucrose transport into the phloem of *Ricinus communis* L. seedlings as measured by the analysis of sieve-tube sap. *Planta* **1989**, *177*, 327–335.

(23) Smith, J. A. C.; Milburn, J. A. Phloem transport, solute flux and the kinetics of sap exudation in *Ricinus communis* L. *Planta* **1980**, *148*, 35–41.

(24) Wilkinson, B. L.; Bornaghi, L. F.; Poulsen, S. A.; Houston, T. A. Synthetic utility of glycosyl triazoles in carbohydrate chemistry. *Tetrahedron* **2006**, *62*, 8115–8125.

(25) Dedola, S.; Nepogodiev, S. A.; Field, R. A. Recent applications of the CuI-catalyzed Huisgen azide-alkyne 1,3-dipolar cycloaddition reaction in carbohydrate chemistry. *Org. Biomol. Chem.* **2007**, *5*, 1006–1017.

(26) Antognoni, F.; Fornalè, S.; Grimmer, C.; Komor, E.; Bagni, N. Long-distance translocation of polyamines in phloem and xylem of *Ricinus communis* L. plants. *Planta* **1998**, *204*, 520–527.

(27) Chollet, J. F.; Rocher, F.; Jousse, C.; Delétage Grandon, C.; Bashiardes, G.; Bonnemain, J. L. Acidic derivatives of the fungicide fenpiclonil: effect of adding a methyl group to the N-substituted chain on systemicity and fungicidal activity. *Pest Manag. Sci.* **2005**, *61*, 377–382.

(28) Yanpaisan, W.; King, N. J. C.; Doran, P. M. Flow cytometry of plant cells with applications in large-scale bioprocessing. *Biotechnol. Adv.* **1999**, *17*, 3–27.

(29) Petit, P. X. Flow cytometric analysis of rhodamine 123 fluorescence during modulation of the membrane potential in plant mitochondria. *Plant Physiol.* **1992**, *98*, 279–286.

(30) Konrad, K. R.; Hedrich, R. The use of voltage-sensitive dyes to monitor signal-induced changes in membrane potential-ABA triggered membrane depolarization in guard cells. *Plant J.* **2008**, *55*, 161–173.

(31) Bhal, S. K.; Kassam, K.; Peirson, I. G.; Pearl, G. M. The Rule of Five revisited: applying log D in place of log P in drug-likeness filters. *Mol. Pharmaceutics* **2007**, *4*, 556–560.

(32) Rocher, F.; Chollet, J. F.; Legros, S.; Jousse, C.; Lemoine, R.; Faucher, M.; Bush, D. R.; Bonnemain, J. L. Salicylic acid transport in *Ricinus communis* involves a pH-dependent carrier system in addition to diffusion. *Plant Physiol.* **2009**, *150*, 2081–2091.