JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

Cite This: J. Agric. Food Chem. XXXX, XXX, XXX−XXX

pubs.acs.org/JAFC

Metabolism of the Strobilurin Fungicide Mandestrobin in Wheat

Daisuke Ando,*[,†] Takuo Fujisawa,[†] and Toshiyuki Katagi[‡]

[†]Environmental Health Science Laboratory, Sumitomo Chemical Co., Ltd., 4-2-1, Takarazuka, Hyogo 665-8555, Japan

[‡]Bioscience Research Laboratory, Sumitomo Chemical Co., Ltd., 3-1-98, Kasugade-naka 3-chome, Konohana-ku, Osaka-city, Osaka 554-8558, Japan

ABSTRACT: The metabolic fate of a new fungicide, mandestrobin, labeled with ^{14}C at the phenoxy or benzyl ring was examined in wheat after a single spray application at 300 g/ha. Mandestrobin penetrated into foliage over time, with both radiolabels showing similar ^{14}C distribution in wheat, and 2.8−3.3% of the total radioactive residue remained on the surface of straw at the final harvest. In foliage, mandestrobin primarily underwent mono-oxidation at the phenoxy ring to produce 4-hydroxy or 2-/5-hydroxymethyl derivatives, followed by their subsequent formation of malonylglucose conjugates. In grain, the cleavage of its benzyl phenyl ether bond was the major metabolic reaction, releasing the corresponding alcohol derivative, while the counterpart 2,5-dimethylphenol was not detected. The constant *RS* enantiomeric ratio of mandestrobin showed its enantioselective metabolism to be unlikely on/in wheat.

KEYWORDS: *mandestrobin, wheat metabolism, malonylglucose conjugate, enzymatic hydrolysis*

INTRODUCTION

Mandestrobin [**1**, (*RS*)-2-methoxy-*N*-methyl-2-[α-(2,5-xylyloxy)-*o*-tolyl]acetamide] is a new fungicide developed by Sumitomo Chemical Co., Ltd., possessing a 2-methoxy-*N*-methylacetamide substructure as a unique toxophore that is different from other strobilurin fungicides on the market and shows cross-resistant character with them. The fungicide was registered in various countries (e.g., Japan, South Korea, European Union, Canada, Australia, and United States) with the aim to control Sclerotinia rot, fruit tree scab, and wide varieties of crop diseases by acting on mitochondrial respiratory chain complex III as a quinone outside inhibitor (QoI).[1] **1** has an asymmetric center at the 2-carbon of the acetamide moiety and is manufactured as a racemate and, thus, can be categorized as a chiral pesticide. For these groups, less or no bioactivity has often been identified on either one of the enantiomers. In the case of **1**, the *R* isomer possesses superior fungicidal activity to that of the antipode.[1,2]

Incidentally, it is essential to examine and clarify the metabolic fate of a synthetic pesticide in crop(s) to evaluate its toxicological risks for consumers who intake both the pesticide and its metabolites/degradates from agricultural commodities. Several research articles on metabolic profiles of strobilurin fungicides in crops have been published. For instance, azoxystrobin, which was discovered at an early stage of the synthetic strobilurin fungicide development history and has an enol ether/ester toxophore, like natural strobilurin A, is known to undergo cleavages of the ester/ether linkage and the pyrimidine ring as major metabolic reactions combined with sunlight-driven *E*/*Z* isomerization at the olefin bond on the plant surface.[3,4] Basically, similar profiles have been reported for kresoxim-methyl and trifloxystrobin, which have an oxime ether moiety as an alternative to the enol function.[4] Unlike these precedent strobilurins, **1**, which does not possess enol/oxime ether, ester, or olefin functions, may have the potential to undergo a dissimilar metabolic pathway.

In this study, we investigated the metabolic behavior of **1** in wheat sprayed once at the representative agricultural application rate of 300 g/ha, using two different [^{14}C]**1** separately radiolabeled at the phenoxy and benzyl rings to clarify the fate of individual substructures as a general approach for the safety evaluation of pesticides. The study revealed the distribution of ^{14}C and metabolites in wheat at a series of growth stages, i.e., forage, hay, straw, and grain. Due to the presence of the chiral center, **1** potentially undergoes isomerization or enantioselective degradation/fractionation on and in plants either by abiotic and/or biotic reactions.[5−7] To confirm such possibility, we also quantitated the *RS* enantiomeric composition of **1** after foliar application on wheat.

MATERIALS AND METHODS

Chemicals. Two kinds of ^{14}C-radiolabeled **1** were prepared in our laboratory. The compound was uniformly radiolabeled at the dimethylphenoxy and benzyl rings, abbreviated as [Ph-^{14}C]**1** [specific radioactivity 120 mCi/mmol (4.44 GBq/mmol)] and [Bz-^{14}C]**1** [123 mCi/mmol (4.55 GBq/mmol)], respectively. The radiochemical purity of each ^{14}C compound exceeded 98.9% in reversed-phase high-performance liquid chromatography (HPLC) analysis. The *RS* isomeric ratio was confirmed to be 50:50 by the chiral HPLC analysis. Nonradiolabeled authentic standards of **1** (*R*:*S*, 50:50) and its metabolites were prepared in our laboratory. The structures of the following compounds are given in Figure 1: (*RS*)-2-[2-(2-hydroxymethyl-5-methylphenoxymethyl)phenyl]-2-methoxy-*N*-methylacetamide, **2**; (*RS*)-2-[2-(4-hydroxy-2,5-dimethylphenoxymethyl)phenyl]-2-methoxy-*N*-methylacetamide, **3**; (*RS*)-2-[2-(5-hydroxymethyl-2-methylphenoxymethyl)phenyl]-2-methoxy-*N*-methylacetamide, **4**; (*RS*)-3-{2-[1-methoxy-1-(*N*-methylcarbamoyl)methyl]benzyloxy}-4-methylbenzoic acid, **5**; (*RS*)-2-hydroxy-*N*-methyl-2-[α-(2,5-xylyloxy)-

Received: July 11, 2018
Revised: September 9, 2018
Accepted: September 12, 2018
Published: September 12, 2018

DOI: 10.1021/acs.jafc.8b03639
J. Agric. Food Chem. XXXX, XXX, XXX−XXX

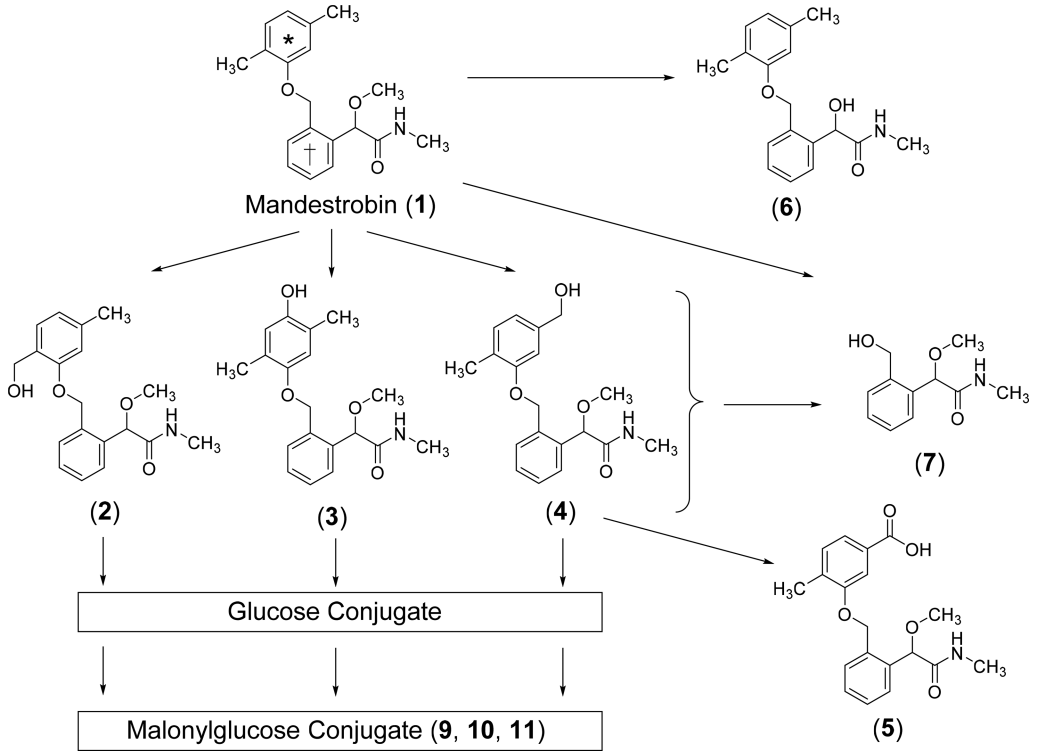

Figure 1. Proposed metabolic pathway of mandestrobin (**1**) on/in wheat plant after the foliar application. The symbols * and † in **1** indicate the radiolabeled positions of [Ph-^{14}C]**1** and [Bz-^{14}C]**1**, respectively.

o-tolyl]acetamide, **6**; (*RS*)-2-(2-hydroxymethylphenyl)-2-methoxy-*N*-methylacetamide, **7**. Compounds **6** and **7** were prepared according to the previous methods.[1,8] Other compounds were synthesized in our laboratory as follows with structural confirmation by high-resolution mass spectrometry (HRMS) in electron spray ionization (ESI) mode and ^{1}H-nuclear magnetic resonance (^{1}H NMR) analyses. In addition, 2,5-dimethylphenol (**8**) was purchased from Sigma-Aldrich Co. (St Louis, MO). The chemical purity of each standard was >98%. All the reagents and solvents used in this experiment were of analytical grade. The enzymes *β*-glucosidase (EC 3.2.1.21., from almond, 37 units/mg) and Driselase (from *Basidiomycetes* sp., cellulase >100 units/g, laminarinase >10 units/g, xylanase >3 units/g) were purchased from Oriental Yeast Co. Ltd. (Tokyo, Japan) and Sigma-Aldrich Co. (Tokyo, Japan), respectively. All other chemicals were of a reagent grade and obtained from the commercial suppliers.

Compound ***2***. To 7 (1.50 g, 7.17 mmol) dissolved in tetrahydrofuran (THF, 30 mL) were added 4-methylsalicylic acid methyl ester (1.43 g, 8.60 mmol, Tokyo Chemical Industry, Tokyo, Japan), triphenylphosphine (2.26 g, 8.60 mmol, Tokyo Chemical Industry, Tokyo, Japan), and diisopropyl azodicarboxylate (1.74 g, 8.60 mmol, Tokyo Chemical Industry, Tokyo, Japan), and the mixture reacted under nitrogen atmosphere for 4 h with stirring at room temperature. The reaction mixture was evaporated and purified by silica gel column chromatography using toluene:acetone (2:1, v:v) to obtain the methyl ester of **2** (2.43 g, yield 94.8%, white solid). To 1.57 g (4.40 mmol) of the methyl ester product dissolved in dichloromethane (CH_2Cl_2, 20 mL) was added diisobutylaluminum hydride (1 mol/L in CH_2Cl_2, 13.2 mL, 13.2 mmol, Tokyo Chemical Industry, Tokyo, Japan) dropwise under nitrogen atmosphere at −78 °C with stirring, and the mixture reacted for 3 h. The reaction mixture was then poured into 2 M HCl with ice, and then extracted using CH_2Cl_2, dried with anhydrous magnesium sulfate, and purified by silica gel column using hexane/ethyl acetate (2:1, v:v) to obtain **2** (692 mg, yield 47.7%, white solid). HRMS (ESI+): *m*/*z* 352.1511 $[M + Na]^+$, −2.384 ppm error for $C_{19}H_{23}O_4NNa$. ^{1}H NMR (400 MHz, $CDCl_3$): *δ* 2.36 (3H, s, Ph−CH_3), 2.78 (3H, d, *J* = 4.8 Hz, CONHCH_3), 3.34 (3H, s, OCH_3), 3.83 (3H, s, COOCH_3), 4.54 (1H, d, *J* = 12.4 Hz, CH_2OH), 4.65 (1H, d, *J* = 12.4 Hz, CH_2OH), 4.97 (1H, s, −C*H*(OCH$_3$)CO−), 5.11 (1H, d, *J* = 11.2 Hz, −CH_2O−), 5.37 (1H, d, *J* = 11.2 Hz, −CH_2O−), 6.76−7.47 (8H, m, CON*H*CH$_3$, Ph).

Compound ***3***. *p*-Xyloquinone (5.00 g, 36.7 mmol, Tokyo Chemical Industry, Tokyo, Japan) dissolved in 70 mL of diethyl ether was partitioned with 35 mL of sodium hydrosulfate (Tokyo Chemical Industry, Tokyo, Japan), washed with water, and dried using anhydrous sodium sulfate to obtain *p*-xylohydroquinone (3.63 g, yield 71.6%, pale yellow powder). To the THF solution dissolving the hydroquinone (1.09 g, 7.90 mmol) and **7** (1.57 g, 7.50 mmol) were added triphenylphosphine (1.97 g, 7.50 mmol) and diisopropyl azodicarboxylate (1.52 g, 7.50 mmmol), and the mixure reacted under nitrogen atmosphere for 18 h at room temperature with stirring. The reaction mixture was then evaporated and purified by silica gel column using hexane:ethyl acetate (2:1, v:v) to obtain **3** (550 mg, yield 22.3%, white solid). HRMS (ESI+): *m*/*z* 330.1690 $[M + Na]^+$, −2.952 ppm error for $C_{19}H_{24}O_4N$. ^{1}H NMR (400 MHz, $CDCl_3$): *δ* 2.15 (3H, s, hydroquinone-CH_3), 2.19 (3H, s, hydroquinone-CH_3), 2.83 (3H, d, *J* = 4.8 Hz, CONHCH_3), 3.33−3.35 (3H, m, OCH_3), 3.83 (3H, s, COOCH_3), 4.72 (1H, s, O*H*), 4.99 (1H, d, *J* = 11.6 Hz, CH_2OH), 5.04 (1H, s, −C*H*(OCH$_3$)CO−), 5.38 (1H, d, *J* = 11.6 Hz, CH_2OH), 6.58 (1H, s, hydroquinone ring), 6.71 (1H, s, hydroquinone ring), 6.82−6.83 (1H, br, −CON*H*CH$_3$), 7.26−7.52 (4H, m, Ph).

Compound ***4***. To the ethanol solution (30 mL) dissolving 3-hydroxy-*p*-toluic acid (20 g, 131 mmol, Tokyo Chemical Industry, Tokyo, Japan) was added 0.71 mL of concentrated sulfuric acid, and the mixture refluxed for 13 h. After evaporation, 50 mL of water was added to the reaction mixture, the pH was adjusted to 7 using sodium carbonate, and then the mixture was extracted with *tert*-buthyl methyl ether. The organic solution was then dried using anhydrous sodium sulfate and evaporated to obtain 3-hydroxy-*p*-toluic acid ethyl ester (21.8 g, yield 92.2%, pale yellow liquid). A 1.55 g portion of 3-hydroxy-*p*-toluic acid ethyl ester (8.60 mmol) and 1.50 g of **7** (7.17 mmol) were dissolved in 30 mL of THF. To the solution were added triphenylphosphine (2.26 g, 8.60 mmol) and diisopropyl azodicarboxylate (1.74 g, 8.60 mmol) and, the mixture was reacted under nitrogen

DOI: 10.1021/acs.jafc.8b03639
J. Agric. Food Chem. XXXX, XXX, XXX−XXX

atmosphere for 3 h at room temperature with stirring. The reaction mixture was then evaporated and purified by silica gel column using hexane:ethyl acetate (3:2, v:v) to obtain the ethyl ester of **5** (2.03 g, yield 76.1%, white solid). To the ethyl ester of **5** (1.89 g, 5.10 mmol) dissolved in CH_2Cl_2 (25 mL) was added diisobutylaluminum hydride (1 mol/L in CH_2Cl_2, 15.3 mL, 15.3 mmol) dropwise at −78 °C with stirring under a nitrogen atmosphere, and the mixture reacted for 3 h. The reaction mixture was poured into 2 M HCl with ice and was then extracted using CH_2Cl_2, dried with anhydrous magnesium sulfate, and purified by silica gel column using hexane/ethyl acetate (2:1, v:v) to obtain **4** (940 mg, yield 56.0%, white solid). HRMS (ESI+): m/z 330.1695 $[M + H]^+$, −1.498 ppm error for $C_{19}H_{24}O_4N$. ^1H NMR (400 MHz, $CDCl_3$): δ 2.25 (3H, s, Ph−CH_3), 2.83 (3H, d, J = 4.8 Hz, CONHCH_3), 3.35 (3H, s, OCH_3), 4.61 (1H, brs, CH_2OH), 4.99 (1H, s, −CH(OCH$_3$)CO−), 5.17 (1H, d, J = 12.4 Hz, −CH_2O−), 5.45 (1H, d, J = 12.4 Hz, −CH_2O−), 6.82−7.53 (8H, m, CONHCH$_3$, Ph).

Compound ***5***. To the ethyl ester of **5** (1.30 g, 3.50 mmol) dissolved in ethanol (50 mL) was added 10 mL of 30% NaOH, and the mixture reacted for 1.5 h at room temperature with stirring. The reaction solution was washed with diethyl ether, and the aqueous solution was pH-adjusted to 2 using concentrated HCl. The aqueous solution was extracted with ethyl acetate, and then, the organic layer was dried using anhydrous sodium sulfate and evaporated to obtain **5** (1.17 g, yield 97.1%, white solid). HRMS (ESI−): m/z 342.1341 $[M - H]^-$, 1.435 ppm error for $C_{19}H_{20}O_5N$. ^1H NMR (400 MHz, $CDCl_3$) δ 2.27 (3H, s, Ph−CH_3), 2.59 (3H, d, J = 4.8 Hz, CONHCH_3), 3.30 (3H, s, OCH_3), 5.00 (1H, s, −CH(OCH$_3$)CO−), 5.25 (1H, d, J = 12.2 Hz, −CH_2O−), 5.45 (1H, d, J = 12.2 Hz, −CH_2O−), 6.83 (1H, br m, CONHCH$_3$), 7.28−8.12 (1H, m, CONHCH$_3$, *Ph*).

Chromatography. The reversed-phase HPLC system to analyze the test substance and its metabolites consisted of a series 1200 LC module (Agilent, CA) or a L-7000 module (Hitachi, Tokyo, Japan). The column used was a 5 μm, 150 × 6 mm, SUMIPAX ODS A-212 (Sumika Chemical Analytical Service, Ltd., Osaka, Japan). The following gradient system consisting of 0.1% formic acid (solvent A) and acetonitrile (solvent B) was employed at a flow rate of 1 mL/min: 0−2 min, 5% B; 10 min, 30% B; 30 min, 35% B; 40−45 min, 0% B. The typical retention times of **1** and other synthetic standards were 42.2 min (**1**), 33.4 min (**2**), 36.4 min (**3**), 35.8 min (**4**), 37.5 min (**5**), 39.2 min (**6**), 17.1 min (**7**), and 25.7 min (**8**). In addition, three unknown metabolites with no corresponding reference standards were detected at 22.0 min (**9**, **10**) and 26.5 min (**11**); thus, another HPLC condition was developed for the separation, quantitation, and isolation of the coeluted peaks **9** and **10**: a 4 μm, 4.6 mm i.d. × 30 cm, Synergi Polar-RP column (Phenomenex Inc.) was applied with isocratic elution [acetonitrile with 0.1% formic acid:0.1% formic acid, 25:75 (v:v)]. The chiral analysis for the *R* and *S* isomers of **1** was conducted with a LC-module Series 1100 (Hewlett-Packard, Waldbronn, Germany) using a 5 μm, 150 × 4.6 mm Chiralpak AD RH column (Daicel Chemical Industries, Tokyo, Japan) with an isocratic eluent of acetonitrile:water, 1:1 (v:v), at a flow rate 1 mL/min (retention times: *R* isomer/5.6 min, *S* isomer/7.1 min). The radioactivity in the column effluent was measured using a Radiomatic 525TR or 625TR (PerkinElmer Co., Ltd.) flow scintillation analyzer with Ultima-Flo A or M (PerkinElmer, Co., Ltd.) as the scintillator. The detection limit of the HPLC analyses was 150 dpm (2.5 Bq).

Thin-layer chromatography (TLC) analysis was carried out for an analytical purpose using 20 × 20 cm, 0.25 mm thickness LK5DF thin-layer silica gel plates (Whatman). The nonradiolabeled reference standards were detected by exposing the chromatoplates to ultraviolet light for direct visualization. The radioactivity in each spot on the plate was detected by a AR2000 imaging scanner (Bioscan Inc.) The solvent systems for TLC were as follows: chloroform:methanol, 9:1 (v:v) [R_f values of 0.68 (**1**), 0.63 (**2**), 0.62 (**3**), 0.60 (**4**), 0.21 (**5**), 0.65 (**6**), 0.66 (**7**)]; ethyl acetate:methanol:acetic acid, 18:2:1 (v:v:v) [R_f values of 0.65 (**1**), 0.58 (**2**), 0.65 (**3**), 0.50 (**4**), 0.20 (**5**), 0.56 (**7**)]; hexane:2-propanol, 5:2 (v:v) [R_f values 0.56 (**1**), 0.43 (**2**), 0.49 (**3**), 0.33 (**4**), 0.25 (**5**), 0.50 (**7**)].

Radioanalysis. Radioactivity in the plant rinsates and extracts was determined by liquid scintillation counting (LSC) with a model 2900TR spectrometer (Packard Instrument Co.) or model LS6500 instrument (Beckman Coulter Inc.) after mixing each aliquot with Emulsifier Scintillator Plus or Ultima Gold (PerkinElmer Co., Ltd.). The detection limit of LSC analysis was 40 dpm (0.67 Bq). The postextracted bound residues of plants were combusted using a model OX-500 or OX-700 biological oxidizer with oxidizer LSC cocktail (RJ Harvey Instrument Co.). The radioactivity therein was quantitated by LSC. The efficiency of combustion was determined to be greater than 95.0%.

Spectroscopy. Liquid chromatography−electrospray ionization mass spectrometry (LC−ESI-MS) analysis was conducted to characterize metabolites of **1** using a TQD tandem quadruple spectrometer equipped with an Acquity UPLC (ultraperformance liquid chromatograph) and an Acquity photodiode array detector (Waters Co.). The following parameters controlled by MassLynx software (version 4.1, Waters Co.) were used for the typical analysis: source temperature, 150 °C; desolvation temperature, 450 °C; capillary voltage, 3.2 kV; cone voltage, 10−40 V; collision energy, 5−20 V. The HPLC column eluent was diverted in the ratio 4:1 and introduced to the mass spectrometer and radiodetector, respectively. For the confirmation of synthetic standards, HRMS was obtained using a Q-Exactive Focus (Thermo Fisher Scientific Inc.) mass spectrometer with infusion injection. The analytical parameters at the mass module controlled by the Xcalibur software (version 2.2) are as follows: sweep gas flow, 10; source temperature, 100 °C; desolvation temperature, 350 °C; capillary voltage, 3.5 kV, cone voltage, 10−40 eV. In addition, ^1H NMR spectra of the standards were recorded in $CDCl_3$ using a Unity-400 spectrometer at 400 MHz (Varian Inc.) with tetramethylsilane as an internal standard.

Plant Materials and Maintenance. The wheat "Promontory" was purchased from Granite Seed (Lehi, UT). The soil used for the wheat cultivation was a combination of local natural loamy sand from Rochester, MA, and a commercial potting soil (Metro Mix 360, SunGro Horticulture Distribution, Inc.) blended at a 9:1 ratio. The loamy sand was collected from a fallow field where no pesticide application was recorded in the previous 3 years. The soil used in the study had no detectable ^{14}C-radioactivity exceeding the minimum quantifiable level. The pH of the soil mixture was adjusted from 4.5 to 6.2 by the addition of powdered limestone. The characterization of the soil was as follows: soil texture, sand 71%, silt 22%, clay 7%; soil classification, sandy loam; organic carbon content, 8.3%; pH 6.2; moisture, 29%. Wheat seeds (approximately 30 seeds per pot) were planted in each of the 27 pots (11-in. diameter). Wheat plants were grown in a greenhouse located at Smithers Viscient (Wareham, MA) with the light cycle of 16 h day/8 h night. The temperature in the greenhouse was set at 28 °C day/21 °C night, and continuously monitored/recorded using a thermometer. The relative humidity in the greenhouse ranged from 19−85% for the duration of the study. The pots were watered with either well water or a dilute liquid fertilizer as necessary until harvest. The plants were fertilized with Peters 20−20−20 at a concentration of 200 mg/L.

Spray Application and Sampling. The spray application was conducted with 25% suspension concentrate (SC) formulation at the application rate of 300 g/ha. A 4.6 mg portion of each radiolabeled compound **1** was individually mixed with 13.7 mg of the nonradiolabeled one for isotopic dilution (specific radioactivity of 200 kdpm/mg, 3.33 kBq/mg). Then, the blank formulation was added to the mixture, along with water for concentration adjustment, to prepare 25% SC spray solution. The final concentration of **1** in the spray solution was 0.3 mg/mL. The formulation was sprayed onto the wheat at the stem elongation stage (grown 37 days after seeding) using a hand pump sprayer from the top of plants. After the application, the radioactivity remaining in the bottle was recovered by rinsing with acetonitrile and the rinsate was assayed by LSC. After 7, 14, and 104 d, forage, hay, and straw/grain were harvested by cutting them individually at approximately 2−4 cm above the soil surface using pruning scissors. Plant samples were removed from any adhering soil by gentle shaking or brushing, and the total weight of

DOI: 10.1021/acs.jafc.8b03639
J. Agric. Food Chem. XXXX, XXX, XXX−XXX

Table 1. ^{14}C Distribution and Metabolites on/in Foliage

	percent of the total radioactive residue[a] (% TRR)					
	forage		hay		straw	
	[Ph-^{14}C]	[Bz-^{14}C]	[Ph-^{14}C]	[Bz-^{14}C]	[Ph-^{14}C]	[Bz-^{14}C]
surface rinses	**41.0**	**33.9**	**23.3**	**19.1**	**3.9**	**2.8**
1 (mandestrobin)	38.5	30.3	21.0	16.4	0.3	0.5
2	ND	ND	0.3	ND	1.1	0.4
3	ND	ND	ND	ND	ND	0.1
4	ND	ND	ND	ND	0.4	0.2
5	ND	ND	ND	ND	0.2	0.2
6	0.3	0.3	0.5	0.2	0.1	<0.1
7	NA	0.7	NA	ND	NA	0.5
9	0.3	0.2	0.2	0.3	0.11[c]	ND
10	0.1	0.2	0.2	0.1		
11	ND	0.2	ND	ND	ND	<0.1
others	1.8	2.0	1.1	2.1	1.7	0.9
extractables[b]	**53.2**	**60.6**	**65.8**	**72.8**	**58.5**	**64.7**
1 (mandestrobin)	12.5	29.6	5.3	6.3	1.1	1.5
2	ND	0.2	1.0	0.9	8.4	6.0
3	ND	ND	ND	ND	1.3	1.4
4	ND	ND	ND	ND	1.7	2.7
5	ND	ND	ND	ND	2.7	4.4
6	ND	2.5	0.2	0.6	0.6	0.4
7	NA	2.5	NA	1.5	NA	11.3
9	10.3	5.3	11.0	12.3	3.9[c]	2.8[c]
10	3.3	5.2	12.9	5.4		
11	6.1	4.1	6.3	6.9	1.2	ND
others	21.0[d]	11.2[e]	29.1[f]	38.9[g]	37.6[h]	34.2[I]
unextractables	**5.8**	**5.5**	**10.9**[j]	**8.1**[j]	**37.6**[j]	**32.5**[j]
total	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**

[a]ND: not detected. NA: not applicable. [b]The sum of acetone/water and acetone/HCl extracts. [c]Compounds **9** and **10** were not separated/quantitated using the secondary HPLC system. [d]Consists of more than seven components, each below 3.0% TRR. [e]Consists of more than five components, each below 3.3% TRR. [f]Consists of more than eight components, each below 2.0% TRR. [g]Consists of more than eight components, each below 4.4% TRR. [h]Consists of more than eight components, each below 7.3% TRR. [I]Consists of more than seven components, each below 4.0% TRR. [j]Further fractionated by harsh extractions.

each plant sample was recorded. The surface of each foliage was individually rinsed using acetonitrile, pulverized with dry ice, and then stored frozen until further extraction.

Extraction and Analysis. Each 10−30 g of pulverized plant was placed into a plastic bottle and extracted with acetone:water (4:1, v:v, 10 mL/g plant) using a model 133 tissue homogenizer (Bio Spec Products Inc.) at high speed for 1 min. The sample was centrifuged at 3000 rpm for 10 min to separate the extract from the plant solids. The supernatant was removed and the extraction was repeated twice in the same manner, and then the supernatants were combined. The remaining plant matrices were successively extracted with acetone:-water:concentrated HCl (80:20:1, v:v:v, 10 mL/g plant) using the above procedures. The radioactivity in each extract was quantitated by LSC in duplicate and analyzed by HPLC. The extraction and LSC/HPLC analysis of each plant sample were performed within 1 month after the harvest. The unextractable residues were allowed to dry and then subjected to combustion analysis. The unextractable residues of hay were further characterized by Driselase treatment [0.5−1.0 g of residues and 40 mg of enzyme dissolved in 10 mL of 0.1 M sodium acetate buffer (pH 4.5) were incubated at 38 °C, overnight] and 0.1 M HCl and 0.1 M NaOH extractions (40 °C, overnight). For straw, the unextractable residues were sequentially extracted using 0.1 M HCl (40 °C, overnight), 6 M HCl (80 °C, 4 h), 0.1 M NaOH (40 °C, overnight), and 6 M NaOH (80 °C, 4 h). The final ^{14}C-bound residues in hay and straw were determined by combustion analysis.

Metabolite Identification and Characterization. Identification of each metabolite was basically conducted by HPLC and TLC cochromatographies with nonradiolabeled reference standards. To verify whether unknown metabolites **9**−**11** are conjugate or not, enzymatic and alkaline hydrolysis was representatively conducted using the [Ph-^{14}C] hay extract. For the confirmation of glucose conjugate, approximately 200 kdpm (3.33 kBq) of the hay extract or 50 kdpm (833 Bq) of each HPLC-isolated unknown fraction was evaporated to dryness and dissolved in 1 mL of 10 mM acetate buffer at pH 5.0. To the mixture, 3 mg of β-glucosidase was added, mixed well, and incubated in a BR-180LF BioShaker (TAITEC Co. Ltd.) at 37 °C and 100 rpm. Aliquots of the mixture were sequentially sampled and analyzed by HPLC. For malonylglucose conjugates, the dried extract or each isolate was separately subjected to alkaline hydrolysis by adding 1 mL of 0.03 M NaOH as pretreatment prior to the enzymatic reaction. The mixture was reacted at room temperature for 0.5 h and neutralized using 1 M HCl, and the reaction mixture was subjected to HPLC and LC−MS analyses. Successively, 1 mL of 10 mM acetate buffer and 3 mg of β-glucosidase were added to the neutralized mixture, and the reaction was periodically monitored by HPLC.

Estimation of Chemical Property. The *n*-octanol/water partition coefficient (K_{ow}) of **7** was predicted using the Estimation Program Interface (EPI) Suite from the U.S. EPA (version 4.11).[8]

DOI: 10.1021/acs.jafc.8b03639
J. Agric. Food Chem. XXXX, XXX, XXX−XXX

RESULTS AND DISCUSSION

Distribution of Radioactivity in Wheat. The actual application ratios of [^{14}C]**1**, determined from the ^{14}C that remained in the postspraying bottle, were 303 and 306 g/ha for phenoxy and benzyl label treatments, respectively. The recovered radioactivities in forage, hay, straw, and grain for Ph/Bz label were (%/%) 83.8/93.0, 90.3/96.2, 23.7/30.4, and 0.006/0.008 of the corresponding sprayed radioactivities, respectively. Since the recovery of the applied radioactivities largely decreased at the final harvest (straw and grain), it is considered that **1** underwent photodegradation on the plant surface and became mineralized/volatilized. Such a tendency was previously confirmed in the photodegradation study of **1**.[9] The distribution of ^{14}C and constituents on/in foliage (forage, hay, and straw), summarized in Table 1, was generally similar between the two radiolabels. The total radioactive residue (TRR) in foliage was calculated as the sum of ^{14}C in the surface rinse, extractable, and unextractable fraction. For foliage, the recovered radioactivity by the surface rinse decreased along with the cultivation period to 33.9−41.0% TRR (3.542−4.570 ppm) in forage and 2.8−3.9% TRR (0.069−0.070 ppm) in straw. In the surface rinses, unaltered **1** was consistently the main component that remained in forage and hay at 30.3−38.5% TRR (3.163−4.289 ppm) and 16.4−21.0% TRR (1.302−1.478 ppm), respectively, and then became as minor as 0.3−0.5% TRR (0.005−0.012 ppm) in straw. As minor products, **2**, **3**, **4**, **5**, **6**, and **7** were detected at their maximum of 1.1% TRR (0.020 ppm, straw), 0.1% TRR (0.003 ppm, straw), 0.4% TRR (0.007 ppm, straw), 0.2% TRR (0.004 ppm, straw), 0.5% TRR (0.031 ppm, hay), and 0.7% TRR (0.072 ppm, forage), respectively. Three polar unknowns, **9**−**11**, which were later characterized as malonylglucose conjugates of **2**−**4**, were also observed, each at below 0.4% TRR. Several other ^{14}C minor constituents also existed as minors, each amounting to less than 2.1% TRR for both radiolabels. With respect to the acetone/water and acetone/HCl extracts, the radioactivities therein were 50.0−58.6 and 2.0−3.2% TRR (forage), 61.1−70.6 and 2.2−4.7% TRR (hay), and 41.1−46.7 and 17.4−18.0% TRR (straw), respectively. In the sum of extracts, i.e., acetone/water and acetone/HCl, **1** declined from 12.5−29.6% TRR (13.950−3.091 ppm, forage) to 1.1−1.5% TRR (0.020−0.037 ppm, straw) along with the plant growth. The relevant metabolites observed in the extracts were **9**, **10**, and **11**, which reached their maximum values in hay as 11.0−12.3% TRR (0.688−1.108 ppm), 5.4−12.9% TRR (0.484−0.803 ppm), and 6.3−6.9% TRR (0.390−0.619 ppm), respectively. The maximum residual levels of **2**, **3**, and **4** were 6.0−8.4% TRR (0.151−0.156 ppm, straw), 1.3−1.4% TRR (0.023−0.035 ppm, straw), and 1.7−2.7% TRR (0.032−0.069 ppm, straw), respectively. The benzyl-label-specific degradate **7** amounted to the maximum of 11.3% TRR (0.281 ppm, straw), while the structural counterpart **8** unique to the phenyl label was not detected in any amount. There were no other ^{14}C components exceeding 7.3% TRR in the foliage extracts for both radiolabels. The unextractable residues of forage, hay, and straw increased from 5.5−5.8% TRR (0.569−0.642 ppm, forage) to 32.5−37.6% TRR (0.696−0.810 ppm, straw), in parallel with the ^{14}C decrease on the plant surface. There was a further attempt to characterize the unextractable residues of hay and straw by Driselase treatment and successive harsh extractions using HCl and NaOH solutions. By these procedures, the unextractable residues of hay released 1.5−3.5% TRR (0.091−0.319 ppm, Driselase), 1.4−1.5% TRR (0.090−0.133 ppm, 0.1 M HCl), and 2.3−5.8% TRR (0.208−0.362 ppm, 0.1 M NaOH), and finally, 0.4−1.3% TRR (0.037−0.081 ppm) remained as the bound residue for both radiolabels. From straw, the radioactivity recovered by acidic solvents (0.1 and 6.0 M HCl) was less than 5.9% TRR (<0.123 ppm). Successive extractions using alkaline (0.1 and 6.0 M NaOH) released more radioactivity (10.3−16.4% TRR, 0.210−0.304 ppm), but these fractions contained large amounts of matrices, which were unable to be analyzed chromatographically. The final radioactivity in bound residue of straw accounted for 0.6−1.1% TRR (0.014−0.020 ppm).

The hydroxylated products **2**−**4**, carboxylated **5**, and demethylated **6** were considered to be typical metabolites generated by enzymatic function(s) at inner plant tissues of phase I detoxification processes of cytochrome P450 and/or other oxygenases, and the malonylglucose conjugates **9**−**11** were regarded as phase II conjugation products of glucosyl/malonyl transferases.[10,11] In the wheat surface rinse fractions aimed to analyze ^{14}C-residues on the plant surface where such phase I/II enzymes would hardly be accessible, not only **1** but also small amounts of **2**−**6** and the malonylglucose conjugates **9**−**11** were detected. Although epiphytic microbes present on the plant surface may have contributed to generate **2**−**6**,[12] the components in the rinse were regarded as the portions eluted from inner plant tissues during the surface wash, since the majority of these products were detected in the wheat extract, and the same theory could be applied for the existence of malonylglucose conjugates on the surface. This assumption is supported by the study of Myung et al.[13] They investigated the structural change of the epicuticular wax layer after washing the surface of wheat leaf using various organic solvents and showed that acetonitrile somewhat deteriorates the wax composition, although it was the most mild solvent they examined. With respect to **7**, it is known as the major photodegradate rapidly generated via photoinduced homolytic bond cleavage at the benzyl ether bond,[2,9,14] which is the reaction that could proceed on the wheat surface by sunlight. However, especially in straw, the degradate dominantly distributed inside the plant. Taking into account the hydrophilic nature of **7** (log K_{ow} −0.26; estimated using EPI Suite), which was generally considered unable to penetrate the hydrophobic embedding epicuticular wax,[15,16] it was assumed to be produced mainly by enzymatic reaction(s) in wheat. The minor contribution to the photodegradation may be due to the sunlight screening effect by the morphological complexity of the leaf surface and the epicuticular wax layer, which mostly consists of aliphatic hydrocarbons but also UV-absorptive unsaturated fatty acids, aldehydes/ketones, flavonoids, etc., preventing photolysis from proceeding as **1** diffused within the layer.[17]

With respect to grain, the distribution of radioactivity and ^{14}C constituents is summarized in Table 2. The TRR in grain was calculated as the sum of ^{14}C in the extractable and unextractable fractions because grain was extracted without a surface rinse since it did not receive any direct ^{14}C spray due to the application timing prior to grain setting. The TRR of benzyl labeled specimen showed approximately 7 times higher ^{14}C residues than that of phenoxy label (phenoxy, 0.012 ppm; benzyl, 0.089 ppm). The radioactivities in acetone/water and acetone/HCl extracts were combined and subjected to HPLC/TLC analyses due to the low residual level. For the phenoxy label, 67.0% TRR (0.008 ppm) was extractable. No radioactive components matched with the reference standards, but

DOI: 10.1021/acs.jafc.8b03639
J. Agric. Food Chem. XXXX, XXX, XXX−XXX

Table 2. ^{14}C Distribution and Metabolites in Grain

	percent of the total radioactive residue[a] (% TRR)	
	[Ph-^{14}C]	[Bz-^{14}C]
extractables[b]	**67.0**	**72.7**
1 (mandestrobin)	ND	ND
2	ND	3.1
7	NA	60.6
9/**10**[c]	ND	1.3
others	67.0[d]	7.7[e]
unextractables	33.0	27.3
total	100.00	100.00

[a]ND: not detected. NA: not applicable. [b]The sum of acetone/water and acetone/HCl extracts. [c]Not separated/quantitated using the secondary HPLC system. [d]Consists of more than three components, each below 25.6% TRR. [e]Consists of core than five components, each below 4.3% TRR.

multiple constituents were detected in which the maximum single component amounted to 25.6% TRR (0.003 ppm). These products were not characterized further due to their extremely low radioactivity. For the benzyl label, 72.7% TRR (0.065 ppm) was extractable. The metabolites detected therein were **2**, **7**, and **9**/**10**, which amounted to 3.1% TRR (0.003 ppm), 60.6% TRR (0.054 ppm), and 1.3% TRR (0.001 ppm), respectively. Similarly with foliage, **8** was not detected. Several unknown ^{14}C constituents were observed, but they were not further characterized due to their low TRR concentrations, <0.01 ppm. The unextractable residue accounted for 33.0 and 27.3% TRR, which was equivalent to 0.004 and 0.024 ppm for phenoxy and benzyl labels, respectively.

Both in wheat foliage and grain, **8** or its particular derivatives, as a possible counterpart of **7**, was not detected on/in wheat, which could be explained by their biotic (enzymatic) and abiotic (photolytic) processes. For biotic processes, the metabolism of dimethylphenols has been extensively studied in bacteria.[18−21] It was shown that the metabolism of **8** proceeds via oxidation of the methyl group at the 5-position and the phenyl ring at the 4-position to form 4-methylgentisic acid, which successively undergoes benzene ring cleavage to produce various minor organic acids. In addition, phenolic compounds widely distributed as plant natural constituents in various higher plants are known to be incorporated into lignin in cell wall and anthocyanin in vacuole under the shikimate pathway.[22] For the abiotic process, by employing electron spin resonance (ESR) and radical trapping detection techniques, Adachi et al.[9] succeeded in detecting the benzyl radical form of **7**, whereas the counterpart phenoxy radical of **8** was undetectable. The authors concluded that the phenoxy radical was unstable and rapidly decomposed to multiple components and was mineralized via successive radical chain reactions interacting with other molecules or via self-decomposition. Thus, in summary, **8** is considered to be rapidly degraded into multiple degradates and incorporated into plant natural components once generated on/in a plant. Such explanation could be supported by the results in which foliage and grain of the phenoxy label treatment overall showed higher rates of multiple degradates and unextractables than the benzyl label.

***RS* Enantiomeric Ratio of 1 in Wheat.** The *RS* isomeric ratio of **1** remaining in the rinsates and extracts of forage and hay was investigated by the chiral HPLC analysis. As representative, the chiral HPLC chromatogram of **1** in [Ph-^{14}C] straw is shown in Figure 2. The *RS* ratio ranged

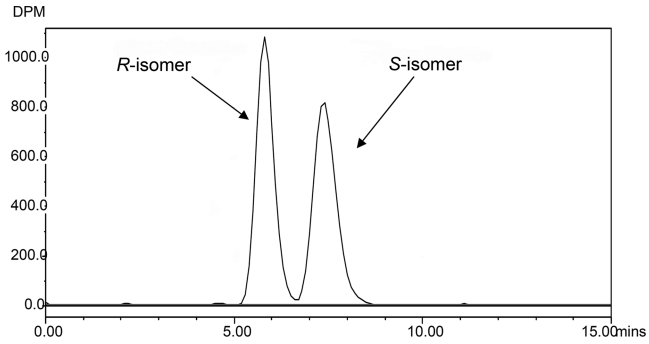

Figure 2. Chiral HPLC chromatogram of [Ph-^{14}C] straw extract.

from *R*:*S* = 50.1:49.9 to 50.4:49.6 in the surface rinsates and from 50.2:49.8 to 44.1:55.9 in the extracts, mostly remaining as racemate. The results indicated that *RS* isomerization at the 2-carbon of acetamide as well as enantioselective degradation were insignificant at both surface and inner portions of wheat plant. Although the possibility of enantiomerization cannot be concluded unless the behavior of each isomer is clarified, the results are important evidence to support that the fungicidal-active *R* isomer would not predominantly convert to less effective *S* isomer and vice versa in crops after agricultural use.

Characterization of the Metabolites 9−11. The unknown metabolites **9**−**11** detected as major components in foliage extracts were further characterized in detail using the hay extract treated with [Ph-^{14}C]**1**. When a portion of the original extract containing the unknowns was subjected to the enzymatic hydrolysis using *β*-glucosidase, **9**−**11** were slowly (deglycosylation not completed by 7 days) transformed to the corresponding aglycons, **2**−**4**, showing their possible identities as glucose conjugates (Figure 3A,B). By the LC−MS analysis of each unknown, the following ions were obtained in the negative ion mode ($C_{28}H_{35}NO_{12}$, M_w 577): *m*/*z* 690 [M + CF_3COO]$^-$, 576 [M − H]$^-$, 532 [M − COOH]$^-$. As representative, the mass spectrum of **9** is shown in Figure 4A. From these results, the structures of **9**, **10**, and **11** were proposed as malonylglucose conjugates of **2**, **3**, and **4**, respectively, and these metabolites were likely generated by malonylation, which is a well-known reaction in plants, for example, to stabilize labile biomolecules[23] and to enhance solubility of xenobiotics to transport them into the vacuole for detoxification.[24] As another hydrolysis experiment, the alkaline pretreatment was introduced prior to the enzymatic digestion in an attempt to release possible endogenous malonyl moieties. By the pretreatment, individual malonylglucose conjugates in the hay extract were immediately converted to the corresponding new single peaks on the HPLC chromatogram, each of which eluted at approximately 3−4 min earlier retention times within 0.5 h (Figure 3C), and successive *β*-glucosidase hydrolysis released each aglycon by 3 h (Figure 3D,E). The postalkaline treatment products of **9**−**11** were also confirmed by LC−MS analysis. The following molecular mass and characteristic fragments were detected in the positive ion mode, revealing the glucose conjugate structures ($C_{25}H_{33}NO_9$, M_w 491): *m*/*z* 514 [M + Na]$^+$, 492 [M + H]$^+$, 330 [M −

DOI: 10.1021/acs.jafc.8b03639
J. Agric. Food Chem. XXXX, XXX, XXX−XXX

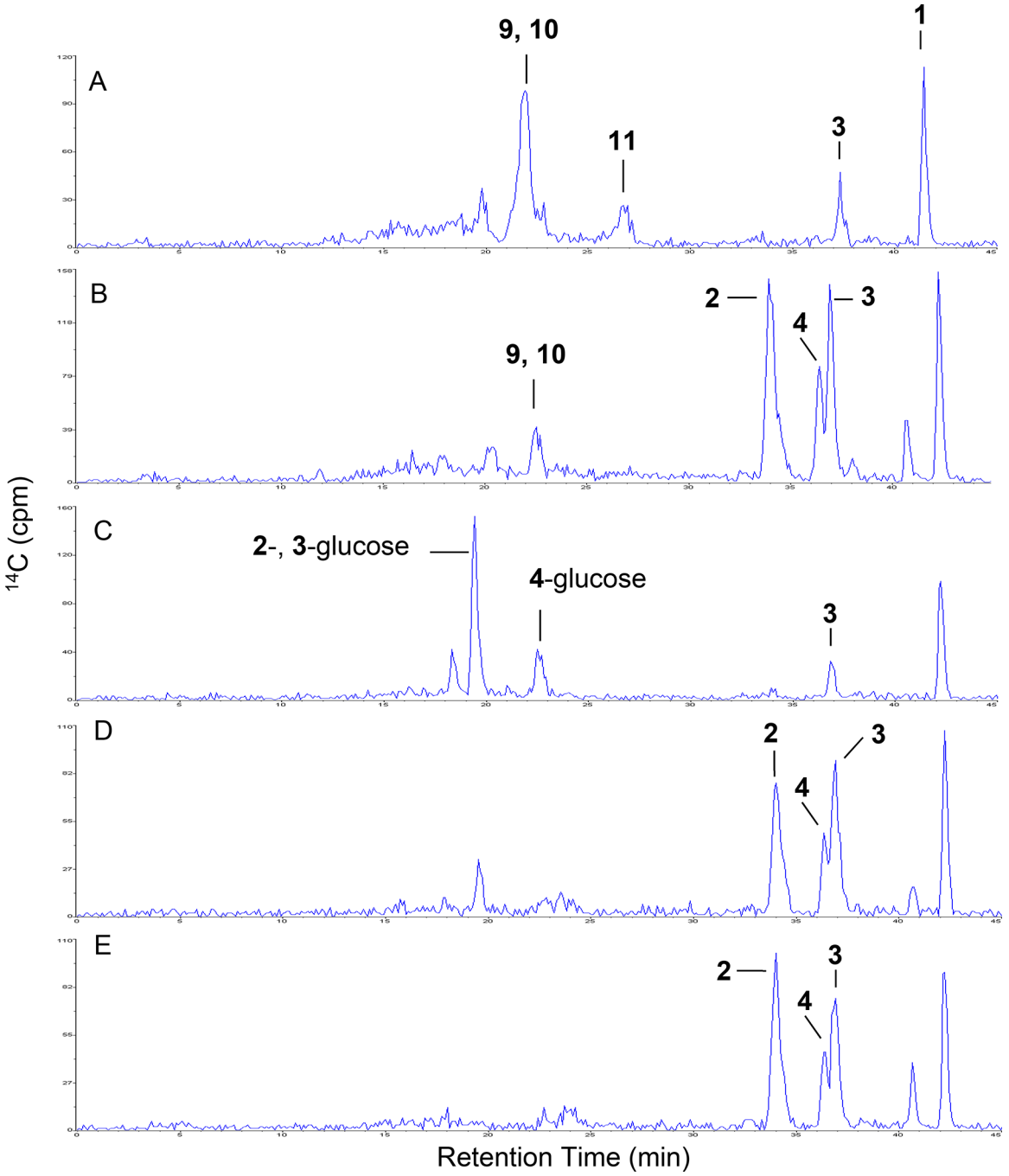

Figure 3. Hydrolytic behavior of [Ph-^{14}C] hay extract analyzed by HPLC. (A) original whole extract, (B) 7 days after β-glucosidase treatment, (C) 0.5 h after alkaline treatment; (D) 1 h after β-glucosidase treatment following 0.5 h of alkaline hydrolysis, (E) 3 h after β-glucosidase treatment following 0.5 h of alkaline hydrolysis.

glucose + OH + H]$^+$, 312 [M − glucose + H]$^+$, 192 [M − glucose − dimethylphenol + H]$^+$. Figure 4B is shown as the representative of the demalonyl conjugate of **9**. These results demonstrated the elimination of the malonyl group from the glucose by the alkaline treatment while each glycosidic linkage remained intact. As the results, **9**, **10**, and **11** were fully characterized as **2**-, **3**-, and **4**-malonyl glucosides, respectively.

Similar to our finding, the poor reactivity of malonylglucose conjugates against β-glucosidase is known for a wide varieties of substrates, both natural products (e.g., isoflavones) and xenobiotics.[25−27] The β-glucosidase from almond applied in this study is categorized in the glycoside hydrolase (GH) family GH 1,[28] and many β-glucosidases from GH 1, GH 3, etc., possess a similar reactive domain with highly conserved catalytic amino acid residues.[29−33] The binding of the glucose moiety at subsite −1 is considered to be strongly controlled by hydrogen bonds formed between the hydroxyl groups of the glucose moieties at the 2-, 3-, 4-, and 6-positions, where malonyl moiety bounds, and amino acid residues.[29,30,34−39] Such interactions are also believed to stabilize the deglycosylation transition state[29−37] and enable the key glutamine residue(s), or others, to function as catalytic acid/base and nucleophile.[29,31,35,38] Overall, on the basis of the critical importance of hydrogen bonds formed at glucose OH groups,

DOI: 10.1021/acs.jafc.8b03639
J. Agric. Food Chem. XXXX, XXX, XXX−XXX

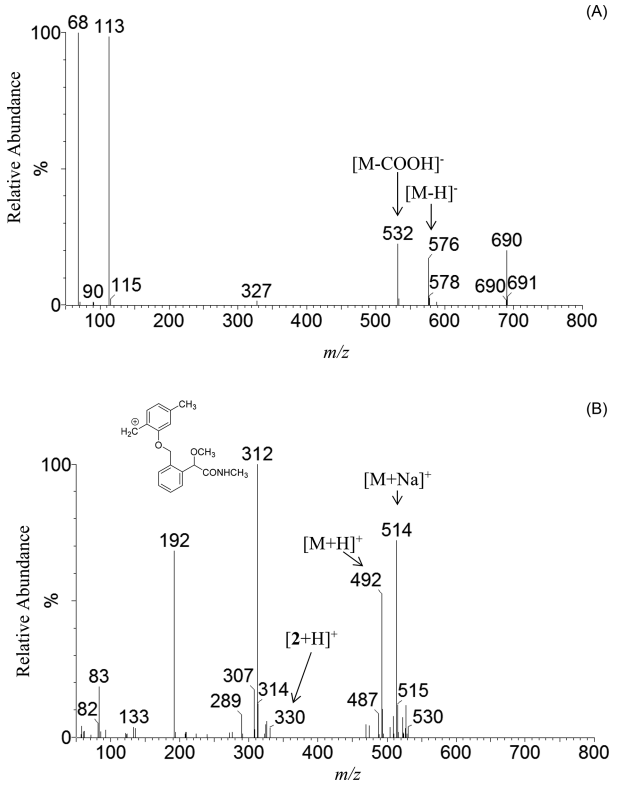

Figure 4. Mass spectrum of (A) malonylglucoside and (B) demalonylated glucoside of **2**.

including 6-OH, it is speculated that the decreased deglycosylation efficiency observed for malonylglucose can be attributed to the malonyl additive causing unfavorable electrostatic repulsion or steric hindrance in forming proper hydrogen bond interactions.

In conclusion, the metabolic pathway of **1** in wheat is proposed in Figure 1. Compound **1** penetrated into wheat tissue along with the growth period and underwent extensive metabolism via phase I and II oxidation followed by incorporation into plant components. The *RS* isomerization or enantioselective degradation was considered unlikely in the wheat metabolism.

AUTHOR INFORMATION

Corresponding Author

*Tel: +81-797-74-2116. Fax: +81-797-74-2132. E-mail: andod@sc.sumitomo-chem.co.jp.

ORCID

Daisuke Ando: 0000-0003-2133-2437

Notes

The authors declare no competing financial interest.

REFERENCES

(1) Hirotomi, D.; Ueda, N.; Kiguchi, S.; Hirota, M.; Iwashita, K.; Kodaka, R. Research and development of a novel fungicide 'Mandestrobin'. *Sumitomo Kagaku* **2016**, 1−16.

(2) European Food Safety Authority (EFSA). Conclusion on the peer review of the pesticide risk assessment of the active substance mandestrobin. *EFSA Journal* **2015**, *13*, 1−72.

(3) Joseph, I. S. R. Metabolism of azoxystrobin in plants and animals. In *Pesticide Chemistry and Bioscience: The Food Environment Challenge*; Brooks, G. T., Roberts, T. R., Eds.; The Royal Society of Chemistry: Cambridge, U.K., 1999; pp 265−278.

(4) Balba, H. Review of strobilurin fungicide chemicals. *J. Environ. Sci. Health, Part B* **2007**, *42*, 441−451.

(5) Ma, Y.; Gan, J.; Liu, W. Chiral pesticides and environmental safety. In *Chiral Pesticides: Stereoselectivity and Its Consequence*. American Chemical Society: Washington, DC, 2011; pp 97−106.

(6) Perez de Albuquerque, N. C.; Carrão, D. B.; Habenschus, M. D.; Moraes de Oliveira, A. R. Metabolism studies of chiral pesticides. *J. Pharm. Biomed. Anal.* **2018**, *147*, 89−109.

(7) Katagi, T. Isomerization of chiral pesticides in the environment. *J. Pestic. Sci.* **2012**, *37*, 1−14.

(8) *Estimation Programs Interface Suite for Microsoft Windows*, v. 4.11; United States Environmental Protection Agency: Washington, DC, 2017; https://www.epa.gov/tsca-screening-tools/epi-suitetm-estimation-program-interface (accessed Nov 5, 2017).

(9) Adachi, T.; Suzuki, Y.; Nishiyama, M.; Kodaka, R.; Fujisawa, T.; Katagi, T. Photodegradation of strobilurin fungicide mandestrobin in water. *J. Agric. Food Chem.* **2018**, *66*, 8514−8521.

(10) Siminszky, B. Plant cytochrome P450-mediated herbicide metabolism. *Phytochem. Rev.* **2006**, *5*, 445−458.

(11) Sandermann, H., Jr. Higher plant metabolism of xenobiotics: the "green liver"concept. *Pharmacogenetics* **1994**, *4*, 225−241.

(12) Van Eerd, L. L.; Hoagland, R. E.; Zablotowicz, R. M.; Hall, J. C. Pesticide metabolism in plants and microorganisms. *Weed Sci.* **2003**, *51*, 472−495.

(13) Myung, K.; Parobek, P. A.; Godbey, A. J.; Bowling, J. A.; Pence, E. H. Interaction of organic solvents with the epicuticular wax layer of wheat leaves. *J. Agric. Food Chem.* **2013**, *61*, 8737−8742.

(14) Haga, N.; Takayanagi, H. Mechanism of photochemical rearrangement of diphenyl ethers. *J. Org. Chem.* **1996**, *61*, 735−745.

(15) Dettenmaier, E. M.; Doucette, W. J.; Bugbee, B. Chemical hydrophobicity and uptake by plant roots. *Environ. Sci. Technol.* **2009**, *43*, 324−329.

(16) Trapp, S. Fruit tree model for uptake of organic compounds from soil and air. *SAR QSAR. Environ. Res.* **2007**, *18*, 367−387.

(17) Katagi, T. Photodegradation of pesticides on plant and soil surfaces. *Rev. Environ. Contam. Toxicol.* **2004**, *182*, 1−189.

(18) Poh, C.; Bayly, R. Evidence for isofunctional enzymes used in m-cresol and 2,5-xylenol degradation via the gentisate pathway in *Pseudomonas alcaligenes*. *J. Bacteriol.* **1980**, *143*, 59−69.

(19) Hopper, D.; Chapman, P. Gentisic acid and its 3- and 4-methyl-substituted homologues as intermediates in the bacterial degradation of m-cresol, 3,5-xylenol and 2,5-xylenol. *Biochem. J.* **1971**, *122*, 19−28.

(20) Hopper, D.; Kemp, P. Regulation of enzymes of the 3,5-xylenol-degradative pathway in *Pseudomonas putida*: evidence for a plasmid. *J. Bacteriol.* **1980**, *142*, 21−26.

(21) Ewers, J.; Rubio, A. M.; Knackmuss, H. J.; Freier-Schroder, D. Bacterial metabolism of 2,6-xylenol. *Appl. Environ. Microbiol.* **1989**, *55*, 2904−2908.

(22) Mohr, H.; Schopfer, P. *Pflanzen Physiologie*, 4th ed., Springer, 1992.

(23) Heller, W.; Forkmann, G. Biosynthesis of flavonoids. In *The Flavonoids*; Harborne, J. B., Ed.; Chapman & Hall: London, U.K., 1994.

(24) Taguchi, G.; Ubukata, T.; Nozue, H.; Kobayashi, Y.; Yamamoto, H.; Hayashida, N.; Takahi, M. Malonylation is a key reaction in the metabolism of xenobiotic. *Plant J.* **2010**, *63*, 1031−1041.

(25) Schmitt, R.; Kaul, J.; Trenck, T. V. D.; Schaller, E.; Sandermann, H. *β*-D-glucosyl and *O*-malonyl-*β*-D-glucosyl conjugate of pentachlorophenol in soybean and wheat: identification and enzymatic synthesis. *Pestic. Biochem. Physiol.* **1985**, *24*, 77−85.

(26) Petroutsos, D.; Katapodis, P.; Samiotaki, M.; Panayotou, G.; Kekos, D. Detoxification of 2,4-dichlorophenol by de marine microalga *Tetraselmis marina*. *Phytochemistry* **2008**, *69*, 707−714.

(27) Ismail, B.; Hayes, K. *β*-glucosidase activity toward different glycosidic forms of isoflavones. *J. Agric. Food Chem.* **2005**, *53*, 4918−4924.

DOI: 10.1021/acs.jafc.8b03639
J. Agric. Food Chem. XXXX, XXX, XXX−XXX

(28) He, S.; Withers, S. G. Assignment of sweet almond β-glucosidase as a family 1 glycosidase and identification of its active site nucleophile. *J. Biol. Chem.* **1997**, *272*, 24864−24867.
(29) Marana, S. R. Molecular basis of substrate specificity in family 1 glycoside hydrolases. *IUBMB Life* **2006**, *58*, 63−73.
(30) Ketudat Cairns, J. R.; Esen, A. β-glucosidase. *Cell. Mol. Life Sci.* **2010**, *67*, 3389−3405.
(31) Rye, C. S.; Withers, S. G. Glycosidase mechanisms. *Curr. Opin. Chem. Biol.* **2000**, *4*, 573−580.
(32) Henrissat, B.; Callebaut, I.; Fabrega, S.; Lehn, P.; Mornon, J.-P.; Davies, G. Conserved catalytic machinery and the prediction of a common fold for several families of glycosyl hydrolases. *Proc. Natl. Acad. Sci. U. S. A.* **1995**, *92*, 7090−7094.
(33) Jenkins, J.; Lo Leggio, L.; Harris, G.; Pickersgill, R. β-Glucosidase, β-galactosidase, family A cellulases, family F xylanases and two barley glycanases form a superfamily of enzymes wit 8-fold β/α architecture and with two conserved glutamates near the carboxyterminal ends of β-strands four and seven. *FEBS Lett.* **1995**, *362*, 281−285.
(34) Vasella, A.; Davies, J. G.; Bohm, J. Glycosidase mechanisms. *Curr. Opin. Chem. Biol.* **2002**, *6*, 619−629.
(35) Sansenya, S.; Opassiri, R.; Kuaprasert, B.; Chen, C. J.; Ketudat Cairns, J. R. K. The crystal structure of rice (*Oryza sativa L.*) Os4BGlu12, an oligosaccharide and tuberonic acid glucoside-hydrolyzing β-glucosidase with significant thioglucohydrolase activity. *Arch. Biochem. Biophys.* **2011**, *510*, 62−72.
(36) Cicek, M.; Blanchard, D.; Bevan, D. R.; Esen, A. The aglycone specificity-determining sites are different in 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA)-glucosidase (maize beta-glucosidase) and dhurrinase (sorghum beta-glucosidase). *J. Biol. Chem.* **2000**, *275*, 20002−20011.
(37) Verdoucq, L.; Moriniere, J.; Bevan, D. R.; Esen, A.; Vasella, A.; Henrissat, B.; Czjze, M. Structural determinants of substrate specificity in family 1 β -glucosidases: novel insights from the crystal structure of sorghum dhurrinase-1, a plant β-glucosidase with strict specificity, in complex with its natural substrate. *J. Biol. Chem.* **2004**, *279*, 31796−31803.
(38) Zechel, D. L.; Boraston, A. B.; Gloster, T. M.; Boraston, C. M.; Macdonald, J. M.; Tilbrook, D. M. G.; Stick, R. V.; Davies, G. J. Iminosugar glycosidase inhibitors: structural and thermodynamic dissection of the binding of isofagomine and 1-deoxynojirimycin to β-glucosidases. *J. Am. Chem. Soc.* **2003**, *125*, 14313−14323.
(39) Matsuzawa, T.; Jo, T.; Uchiyama, T.; Manninen, J. A.; Arakawa, T.; Miyazaki, K.; Fushinobu, S.; Yaoi, K. Crystal structure and identification of a key amino acid for glucose tolerance, substrate specificity and transglycosylation activity of metagenomic β-glucosidase Td2F2. *FEBS J.* **2016**, *283*, 2340−2353.

DOI: 10.1021/acs.jafc.8b03639
J. Agric. Food Chem. XXXX, XXX, XXX−XXX