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Synthesis of the Methyl Ester of Tritium-labeled AK-toxin I, a Host-specific Toxin Produced by *Alternaria alternata* Japanese Pear Pathotype

Masakazu OKADA, Hisashi MIYAGAWA, and Tamio UENO

Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan

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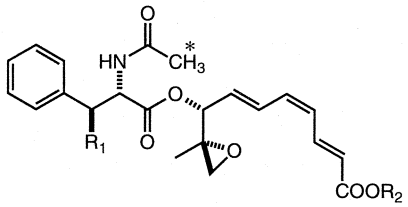
AK-toxin I, a host-specific toxin to Japanese pear (*Pyrus serotina*), was synthesized as its methyl ester from three precursor fragments: conjugated diene-carboxylic acid, chiral epoxyalcohol and β-methylphenylalanine. The epoxyalcohol fragment was derived from D-fructose, in which effective homologation of the hemiacetal carbon to alkyne by using dimethyl 1-diazo-2-oxopropylphosphonate was the key reaction. The diene-carboxylic acid fragment was prepared by repeated Wittig reactions, and was combined with the epoxyalcohol fragment by the Stille reaction. Esterification of the combined product with the stereochemically-pure β-methylphenylalanine fragment afforded the target compound. This method was used to prepare the methyl ester of tritium-labeled AK-toxin I with a specific radioactivity of 213 GBq/mmol.

Key words: AK-toxin; host-specific toxin; Japanese pear; homologation to alkyne; tritium-labeling

The black spot disease to Japanese pear is caused by the fungus *Alternaria alternata* Japanese pear pathotype (formerly named *Alternaria kikuchiana*). This disease occurs in a highly cultivar-dependent manner, and the Nijisseiki and Chojuro cultivars are representative examples of, respectively, susceptible and resistant ones. It has been demonstrated that this disease is caused by phytotoxic metabolites, referred to as AK-toxins, which are produced by the pathogen and belong to a series of pathologically important phytotoxins that are generally termed host-specific toxins.[1] AK-toxins have been shown to consist of two homologs (I and II), and their structures have been elucidated[2,3] as shown in Fig. 1.

Genetic experiments have shown that the sensitivity of a Japanese pear cultivar to AK-toxin is controlled by a single dominant gene.[4] Although biochemical studies[5–8] indicate that a receptor for AK-toxin is present in the cell membrane of susceptible cultivars, little is presently known about the molecular basis for the expression of toxicity.

In searching for the primary site of action of AK-toxin, the use of a radiolabeled ligand represents the method of choice. To this end, several groups have already achieved the total synthesis of AK-toxin II (**2**) or its esters,[9–11] but neither of these methods has been applied to the preparation of the labeled compound. In addition, no attempt has been reported for the synthesis of AK-toxin I, which has higher toxicity and therefore represents a more favorable ligand for the biochemical studies. We report here the synthesis of the methyl ester of naturally-occurring AK-toxin I and its radiolabeling with tritium at the *N*-acetyl methyl group. Although the method is largely modeled on that reported by Ando *et al.* for AK-toxin II,[11] starting from D-fructose, some important modifications were made to improve the efficiency.



1: AK-toxin I: $R_1 = CH_3$, $R_2 = H$
2: AK-toxin II: $R_1 = H$, $R_2 = H$
3: AK-toxin I methyl ester: $R_1 = CH_3$, $R_2 = CH_3$

Fig. 1. Structures of AK-toxins.
The asterisk designates the labeling position for [acetyl-^{3}H]AK-toxin I methyl ester (**3′**).

Materials and Methods

Synthesis of the compounds

General procedures. Melting point (mp) data were determined with a Yanagimoto MP micro-melting point apparatus and are uncorrected. Optical rotation values were measured with JASCO DIP-370 and DIP-1000 spectropolarimeters. NMR spectra were obtained with a Bruker AC-300 instrument (300 MHz) using TMS as an internal standard, and IR spectra were recorded by a Shimadzu IR-420 spectrometer. High-resolution mass spectra (HRMS) were obtained with a JEOL HX211A mass spectrometer by the electron-impact (EI) or chemical-ionization (CI) method. The tritium labeling experiment was carried out at the Radioisotope Research Center of Kyoto University. Radiochemical purity was verified with an Aloka JTC-501 radiochromatogram analyzer. Radioactivity was determined with an Aloka

To whom correspondence should be addressed. Hisashi MIYAGAWA, Fax: +81-75-753-6123; E-mail: miyagawa@kais.kyoto-u.ac.jp

Abbreviations: AIBN, α,α′-azobisisobutyronitrile; DCC, *N*,*N*′-dicyclohexylcarbodiimide; DMF, *N*,*N*-dimethylformamide; Fmoc, 9-fluorenylmethoxycarbonyl; HPLC, high performance liquid chromatography; Ms, methanesulfonyl; PCC, pyridinium chlorochromate; THF, tetrahydrofuran

LSC-1000 liquid scintillation counter, using Aquasol-2 (Packard Instrument Co.) as a scintillator, and corrected for quenching by means of the external standard constant channel ratio method. Silica gel chromatography and silica gel flash chromatography[12] were carried out by using Wakogel C-200 (Wako Pure Chemical Industries) and Silica gel 60, No. 9385 (E. Merck Darmstadt), respectively.

Methyl (E)-4-hydroxy-2-butenoate (**5**). Carbomethoxymethylenetriphenylphosphorane[13] (31.04 g, 92.8 mmol) was added portionwise to a solution of glycolaldehyde dimer **4** (5.31 g, 44.2 mmol) in $CHCl_3$ (300 ml). After the addition was complete, the solution was stirred overnight at room temperature and the solvent was evaporated *in vacuo*. An excess amount of ether was added to the residue, and the precipitate of triphenylphosphine oxide was filtered off. The filtrate was concentrated *in vacuo* and the residual oil was distilled under reduced pressure to give 8.92 g (87% yield) of allyl alcohol **5** as a colorless oil, bp 90–92°C (6 mmHg). NMR δ_H ($CDCl_3$): 2.27 (1H, broad s, -OH), 3.75 (3H, s, CH_3-O), 4.35 (2H, dd, J=3.9, 2.1 Hz, -CH_2-), 6.11 (1H, dt, J=15.7, 2.1 Hz, -CH=), 7.04 (1H, dt, J=15.7, 3.9 Hz, -CH=). IR ν_{max} (film) cm^{-1}: 3420 (O-H), 3020, 2970, 2920, 2860 (C-H), 1720 (O-C=O), 1670 (C=C). HRMS (CI) m/z ($[M+H]^+$): Calcd. for $C_5H_9O_3$: 117.0544, Found: 117.0552.

Methyl (E)-4-oxo-2-butenoate (**6**). In a 1000-ml round-bottomed flask, PCC (24.0 g, 111.4 mmol) was suspended in dry CH_2Cl_2 (400 ml). Allyl alcohol **5** (8.62 g, 74.2 mmol) in dry CH_2Cl_2 (30 ml) was rapidly added, and the solution was stirred at room temperature for 2.5 h. Dry Et_2O (400 ml) was added to the resulting dark brown suspension, which was then stirred for 10 min and filtered. The residue was extracted three times with Et_2O (100 ml) by vigorously shaking. The organic layers were combined, filtered through a layer of anhydrous $MgSO_4$ and concentrated *in vacuo*. The residue was purified by silica gel flash column chromatography (hexane:EtOAc=8:1) to give 7.57 g (89% yield) of aldehyde **6** as a colorless solid, mp 38–39.5°C (lit.[14] mp 38–40°C). NMR δ_H ($CDCl_3$): 3.86 (3H, s, CH_3-O), 6.73 (1H, d, J=16.0 Hz, -CH=), 6.99 (1H, dd, J=16.0, 7.5 Hz, -CH=), 9.78 (1H, d, J=7.5 Hz, -CH=O). IR ν_{max} (film) cm^{-1}: 3050, 3010, 2870 (C-H), 1725 (O-C=O), 1690 (C=O), 1645 (C=C). HRMS (CI) m/z ($[M+H]^+$): Calcd. for $C_5H_7O_3$: 115.0395, Found: 115.0397.

Methyl (2E,4Z)-5-bromo-2,4-pentadienoate (**7a**) *and its (2E,4E) isomer* (**7b**). Bromomethyltriphenylphosphonium bromide[15] (30.5 g, 70.0 mmol) was suspended in dry THF (100 ml) and cooled to −78°C under argon. Potassium *t*-butoxide (7.85 g, 70.0 mmol) was added portionwise to the suspension, and the resulting dark yellow solution was stirred for 30 min, before the dropwise addition of aldehyde **6** (6.95 g, 60.9 mmol) in dry THF (20 ml). After stirring at −78°C for 30 min, the mixture was allowed to warm to room temperature and stirred for an additional 2 h. The mixture was then filtered through a Celite pad, and the filtrate was concentrated *in vacuo*. After the residue had been dissolved in a minimum volume of $CHCl_3$, excess Et_2O was added to the solution, and the resulting precipitate was filtered off. The filtrate was concentrated *in vacuo*, and the black residue was purified by silica gel column chromatography (hexane:EtOAc=50:1→20:1, stepwise) to give 5.07 g (44% yield) of a 5:3 mixture of vinyl bromides **7a** and **7b**.

These two isomers were separated by reversed-phase preparative HPLC (Cosmosil 5C_{18} column, 20 mm I.D. × 250 mm, Nacalai Tesque) using 50% aqueous MeOH as the eluent, whereby vinyl bromide **7a** was eluted faster than **7b**. After removing the methanol from each fraction by evaporating *in vacuo*, each aqueous solution was saturated with NaCl, and extracted with CH_2Cl_2, before the organic extracts were dried over anhydrous $MgSO_4$. Vinyl bromide **7a** was obtained as a pale yellow oil. NMR δ_H ($CDCl_3$): 3.78 (3H, s, CH_3-O), 6.08 (1H, d, J=15.5 Hz, -CH=), 6.59 (1H, d, J=7.3 Hz, -CH=), 6.78 (1H, dd, J=10.7, 7.3 Hz, -CH=), 7.60 (1H, dd, J=15.5, 10.7 Hz, -CH=). IR ν_{max} (film) cm^{-1}: 3100, 3020, 2970 (C-H), 1720 (O-C=O), 1635, 1580 (C=C). HRMS (CI) m/z ($[M+H]^+$): Calcd. for $C_6H_8^{79}BrO_2$: 190.9708, Found: 190.9705. Vinyl bromide **7b** was obtained as a pale yellow solid, mp 43.5–44.5°C. NMR δ_H ($CDCl_3$): 3.76 (3H, s, CH_3-O), 5.93 (1H, d, J=15.3 Hz, -CH=), 6.78 (1H, d, J=13.4 Hz, -CH=), 6.85 (1H, dd, J=13.4, 10.0 Hz, -CH=), 7.18 (1H, dd, J=15.5, 10.0 Hz, -CH=). IR ν_{max} (film) cm^{-1}: 3080, 3020, 2970, 2860 (C-H), 1720 (O-C=O), 1630, 1585 (C=C). HRMS (CI) m/z ($[M+H]^+$): Calcd. for $C_6H_8{}^{79}BrO_2$: 190.9708, Found: 190.9716.

Dimethyl 1-diazo-2-oxopropylphosphonate (**9**). Sodium hydride (60% in oil, 4.00 g, 99.9 mmol), after being washed twice with dry benzene, was suspended in a mixed solvent of dry benzene (270 ml) and dry THF (45 ml), and the suspension was cooled to 0°C. Dimethyl 2-oxopropylphosphonate (15.80 g, 95.1 mmol) in dry benzene (90 ml) was added dropwise to the suspension, and the mixture was stirred at 0°C. After 1 h, *p*-toluenesulfonyl azide[16] (19.70 g, 99.9 mmol) in dry benzene (45 ml) was added dropwise, and the mixture was allowed to warm to room temperature and then stirred for an additional 2 h. The turbid, yellow mixture was filtered through a Celite pad, and the filtrate was concentrated *in vacuo*. The residue was purified by silica gel column chromatography (hexane:EtOAc=1:8) to give 17.84 g (98% yield) of **9** as a yellow oil. NMR δ_H ($CDCl_3$): 2.28 (3H, s, CH_3-CO), 3.85 (3H × 2, d, $J_{H\text{-}P}$=11.9 Hz, CH_3-O). IR ν_{max} (film) cm^{-1}: 3020, 2980, 2870 (C-H), 2150 (C=N=N), 1660 (C=O), 1265 (P=O).

(2S,3R)-2,3-Isopropylidenedioxy-2-methyl-4-pentyn-1-ol (**10**). 2,3-*O*-Isopropylidene-3-*C*-methyl-L-erythrofuranose[11] (**8**; 3.00 g, 17.2 mmol) was dissolved in absolute MeOH (70 ml) and then cooled to 0°C under argon. Dimethyl 1-diazo-2-oxopropylphosphonate (**9**; 6.62 g, 34.4 mmol) and anhydrous K_2CO_3 (6.01 g, 43.5 mmol) were successively added to the solution dropwise and portionwise, respectively. The mixture was allowed to

warm gradually to room temperature and then stirred overnight. The turbid, yellow-greenish mixture was poured into saturated aqueous $NaHCO_3$ and extracted three times with 100-ml portions of CH_2Cl_2. The combined organic layer was washed with brine, dried over anhydrous $MgSO_4$ and concentrated *in vacuo*. The residue was purified by silica gel flash column chromatography ($CHCl_3$:MeOH=80:1) to give 1.83 g (62% yield) of alkyne **10** as a colorless syrup and 1.06 g (35% recovery) of the starting material **8**. Alkyne **10**: $[\alpha]_D^{31}$ +9.5° (*c* 1.08, MeOH). NMR δ_H ($CDCl_3$): 1.39 (3H×2, s, CH_3-), 1.50 (3H, s, CH_3-), 1.85 (1H, broad s, -OH), 2.61 (1H, d, *J*=2.3 Hz, HC≡C), 3.64 (1H, d, *J*=11.5 Hz, $-CH_2-$), 3.68 (1H, d, *J*=11.5 Hz, $-CH_2-$), 4.56 (1H, d, *J*=2.3 Hz, -CH<). IR ν_{max} (film) cm^{-1}: 3470 (O-H), 3300 (C≡C-H), 3000, 2950, 2890 (C-H), 2140 (C≡C). HRMS (CI) *m/z* ($[M+H]^+$): Calcd. for $C_9H_{15}O_3$: 171.1021, Found: 171.1021.

(2S,3R,4E)-2,3-Isopropylidenedioxy-2-methyl-5-(tributyl)stannyl-4-penten-1-ol (**11a**) *and its (4Z) isomer* (**11b**). A mixure of alkyne **10** (1.70 g, 9.99 mmol), tributyltin hydride (3.78 g, 13.0 mmol) and a catalytic amount of AIBN (0.17 g) in a 20-ml round-bottomed flask that was equipped with a condenser was heated at 85°C and stirred for 2 h under argon. The mixture was subjected to silica gel flash column chromatography (hexane:EtOAc=10:1) to give a mixture of vinylstannanes **11a** and **11b** (4.13 g in total, 89% yield). The ratio of **11a** to **11b** was 43:7, based on a ^{1}H-NMR analysis. Vinylstannanes **11a** and **11b** were separated by repeated chromatography, **11a** being eluted first. Vinylstannane **11a** was obtained as a colorless oil. $[\alpha]_D^{31}$ −4.8° (*c* 1.08, MeOH). NMR δ_H ($CDCl_3$): 0.89 (3H×3, t, *J*=7.2 Hz, CH_3-), 0.91 (2H×3, t, *J*=7.2 Hz, $-CH_2$-Sn), 1.24-1.57 (21H, m), 1.81 (1H, dd, *J*=9.0, 4.0 Hz, -OH), 3.29 (1H, dd, *J*=11.0, 9.0 Hz, $-CH_2-O$), 3.54 (1H, dd, *J*=11.0, 4.0 Hz, $-CH_2-O$), 4.33 (1H, dd, *J*=6.5, 1.1 Hz, -CH<), 5.99 (1H, dd, *J*=19.2, 6.5 Hz, -CH=), 6.45 (1H, dd, *J*=19.2, 1.1 Hz, Sn-CH=). IR ν_{max} (film) cm^{-1}: 3500 (O-H), 2970, 2950, 2870 (C-H), 1605 (C=C). HRMS (CI) *m/z* ($[M-H]^-$): Calcd. for $C_{21}H_{41}O_3{}^{120}Sn$: 461.2077, Found: 461.2082. Vinylstannane **11b** was obtained as a colorless oil. $[\alpha]_D^{32}$ +1.5° (*c* 0.95, MeOH). NMR δ_H ($CDCl_3$): 0.89 (3H×3, t, *J*=7.2 Hz, CH_3-), 0.91 (2H×3, t, *J*=7.2 Hz, $-CH_2$-Sn), 1.24-1.57 (21H, m), 1.83 (1H, dd, *J*=8.8, 4.0 Hz, -OH), 3.37 (1H, dd, *J*=11.1, 8.8 Hz, $-CH_2-O$), 3.58 (1H, dd, *J*=11.1, 4.0 Hz, $-CH_2-O$), 4.22 (1H, dd, *J*=7.1, 1.0 Hz, -CH<), 6.27 (1H, dd, *J*=13.1, 1.0 Hz, Sn-CH=), 6.51 (1H, dd, *J*=13.1, 7.1 Hz, -CH=). IR ν_{max} (film) cm^{-1}: 3480 (O-H), 2980, 2940, 2890, 2870 (C-H), 1610 (C=C). HRMS (CI) *m/z* ($[M-H]^-$): Calcd. for $C_{21}H_{41}O_3{}^{120}Sn$: 461.2077, Found: 461.2071.

Methyl (8R,9S,2E,4Z,6E)-10-hydroxy-8,9-isopropylidenedioxy-9-methyl-2,4,6-decatrienoate (**12**). Vinylstannane **11a** (2.82 g, 6.12 mmol) and a catalytic amount of bis(triphenylphosphine)palladium(II) dichloride (688 mg, 0.98 mmol, 16 mol%) were dissolved in dry DMF (75 ml) and the mixture was stirred at room temperature for 15 min under argon. To the resulting dark red solution was added dropwise vinyl bromide **7a** (1.17 g, 6.12 mmol) in dry DMF (5 ml), and the mixture was stirred overnight. The mixture was then poured into Et_2O (300 ml) and the ethereal solution was washed with saturated aqueous NaF (200 ml). The aqueous layer was extracted three times with Et_2O (150 ml×3). The combined organic layer was successively washed with water and brine, dried over anhydrous $MgSO_4$ and concentrated *in vacuo*. The precipitate in the residue was filtered off, and the clear residue was purified by silica gel flash column chromatography (hexane:EtOAc=2:1) to give 1.38 g (80% yield) of triene acetonide **12** as a solid. The concomitant (2*E*,4*E*,6*E*) geometrical isomer (about 10%) was removed by two recrystallizations (EtOAc/hexane), yielding 0.55 g (40% recovery) of pure **12** as a colorless crystal, mp 72-72.5°C. $[\alpha]_D^{32}$ +18.6° (*c* 1.02, MeOH). NMR δ_H ($CDCl_3$): 1.34 (3H, s, CH_3-), 1.44 (3H, s, CH_3-), 1.52 (3H, s, CH_3-), 3.32 (1H, d, *J*=11.1 Hz, $-CH_2-O$), 3.50 (1H, d, *J*=11.1 Hz, $-CH_2-O$), 3.77 (3H, s, CH_3-O), 4.47 (1H, dd, *J*=7.1, 0.8 Hz, -CH<), 5.91 (1H, dd, *J*=15.1, 7.1 Hz, -CH=), 5.94 (1H, d, *J*=15.1 Hz, -CH=), 6.15 (1H, t, *J*=11.3 Hz, -CH=), 6.33 (1H, t, *J*=11.1 Hz, -CH=), 6.96 (1H, dd, *J*=15.1, 11.4 Hz, -CH=), 7.76 (1H, ddd, *J*=15.1, 11.9, 0.8 Hz, -CH=). IR ν_{max} ($CHCl_3$) cm^{-1}: 3500 (O-H), 2930, 2860 (C-H), 1710 (O-C=O), 1625 (C=C). HRMS (EI) *m/z* (M^+): Calcd. for $C_{15}H_{22}O_5$: 282.1467, Found: 282.1474.

Methyl (8R,9S,2E,4Z,6E)- and (8R,9S,2E,4E,6E)-8,9,10-trihydroxy-9-methyl-2,4,6-decatrienoate (**13**). Triene acetonide **12** (1.50 g, 5.31 mmol) was dissolved in a solution of 1% (w/v) of iodine in absolute MeOH (50 ml), and the mixture was stirred at room temperature for about 2 days under argon. Sodium thiosulfate pentahydrate (3.0 g) was added portionwise to the brown mixture, and the resulting colorless suspension was concentrated *in vacuo*. The residue was dissolved in EtOAc (200 ml), and the solution was dried over anhydrous $MgSO_4$, before being concentrated *in vacuo*. The residue was subjected to silica gel flash column chromatography (hexane:EtOAc=1:1→EtOAc), and desired triol **13** was seperated from the unreacted starting material. Recovered **12** was treated again in the same manner as described, and the desired product was separated. The combined products were further purified by silica gel flash column chromatography (EtOAc) to give 1.05 g (82% yield) of triols **13** as a 5:2 mixture of the (2*E*,4*Z*,6*E*) and (2*E*,4*E*,6*E*) isomers as a pale yellow syrup. NMR for the (2*E*,4*Z*,6*E*) isomer δ_H ($CDCl_3$): 1.14 (3H, s, CH_3-), 3.46 (1H, d, *J*=11.3 Hz, $-CH_2-O$), 3.77 (3H, s, CH_3-O), 3.79 (1H, d, *J*=11.3 Hz, $-CH_2-O$), 4.28 (1H, d, *J*=6.0 Hz, -CH<), 5.92 (1H, d, *J*=15.2 Hz, -CH=), 6.00 (1H, dd, *J*=15.2, 6.0 Hz, -CH=), 6.12 (1H, t, *J*=11.3 Hz, -CH=), 6.35 (1H, t, *J*=11.0 Hz, -CH=), 6.95 (1H, dd, *J*=15.1, 11.4 Hz, -CH=), 7.77 (1H, dd, *J*=15.1, 11.9 Hz, -CH=). IR ν_{max} (film) cm^{-1}: 3420 (O-H), 3000, 2970, 2900 (C-H), 1705 (O-C=O), 1625 (C=C). HRMS (CI) *m/z* ($[M+H]^+$): Calcd. for $C_{12}H_{19}O_5$: 243.1232, Found: 243.1237.

Methyl (8R,9S,2E,4Z,6E)- and (8R,9S,2E,4E,6E)-8,9-dihydroxy-10-methanesulfonyloxy-9-methyl-2,4,6-decatrienoate (**14**). A 5:2 mixture of triols **13** (905 mg, 3.66 mmol), which had been dehydrated by azeotropic evaporation with dry benzene, was dissolved in dry CH_2Cl_2 (25 ml) and then cooled to −50°C under argon. After 1.0 M diisopropylethylamine in CH_2Cl_2 (4.03 ml, 4.03 mmol) had been added dropwise to the solution, 1.0 M methanesulfonyl chloride in CH_2Cl_2 (3.84 ml, 3.84 mmol) was slowly added dropwise, and the mixture was stirred for 45 min. The mixture was poured into EtOAc (180 ml) and the EtOAc solution was washed with brine. The organic layer was dried over anhydrous $MgSO_4$ and concentrated *in vacuo*. The residue was purified by silica gel flash column chromatography ($CHCl_3$:MeOH=30:1) to give 888 mg (76% yield) of mesylates **14** as a 5:2 mixture of the (2*E*,4*Z*,6*E*) and (2*E*,4*E*,6*E*) isomers as a pale yellow syrup. NMR for the (2*E*,4*Z*,6*E*) isomer δ_H ($CDCl_3$): 1.26 (3H, s, CH_3–), 3.09 (3H, s, CH_3–SO_2), 3.77 (3H, s, CH_3–O), 4.11 (1H, d, J=10.5 Hz, –CH_2–O), 4.29 (1H, dd, J=6.1, 1.0 Hz, –CH<), 4.38 (1H, d, J=10.5 Hz, –CH_2–O), 5.93 (1H, d, J=15.2 Hz, –CH=), 5.98 (1H, dd, J=15.2, 6.1 Hz, –CH=), 6.14 (1H, t, J=11.3 Hz, –CH=), 6.34 (1H, t, J=11.0 Hz, –CH=), 6.96 (1H, dd, J=15.2, 11.4 Hz, –CH=), 7.76 (1H, ddd, J=15.2, 11.9, 1.0 Hz, –CH=). IR ν_{max} ($CHCl_3$) cm^{-1}: 3570 (O–H), 2880 (C–H), 1710 (O–C=O), 1625 (C=C), 1365 (O=S=O). HRMS (CI) m/z ($[M+H]^+$): Calcd. for $C_{13}H_{21}O_7S$: 321.1025, Found: 321.1013.

Methyl (8R,9S,2E,4Z,6E)-9,10-epoxy-8-hydroxy-9-methyl-2,4,6-decatrienoate (**15a**) *and (2E,4E,6E) isomer* (**15b**). A 5:2 mixture of mesylates **14** (905 mg, 3.66 mmol), which had been dehydrated by azeotropic evaporation with dry benzene, was dissolved in dry THF (15 ml) and cooled to 0°C under argon. To the solution was added dropwise 1.0 N *t*-BuOK in *t*-BuOH (2.98 ml, 2.98 mmol), and the resulting mixture was stirred for 10 min. The black mixture was poured into a suspension of ice and CH_2Cl_2, and a small amount of brine was added to the resulting emulsion to make the separation easier. The aqueous layer was extracted five times with 100-ml portions of CH_2Cl_2. The combined organic layer was washed with brine, dried over anhydrous $MgSO_4$ and concentrated *in vacuo*. The residue was purified by silica gel flash column chromatography (hexane:EtOAc=3:2) to give 433 mg (68% yield) of a 5:2 mixture of epoxyalcohols **15a** and **15b** as a pale yellow syrup.

These two isomers were separated by reversed-phase preparative HPLC (Cosmosil 5C_{18}-AR column, 20 mm I.D. × 250 mm, Nacalai tesque) using 35% aqueous MeOH as the eluent. Epoxyalcohol **15b** eluted faster than **15a**. After removing the methanol by evaporation *in vacuo*, each aqueous solution was extracted with CH_2Cl_2. The organic extracts were washed with brine, dried over anhydrous $MgSO_4$ and concentrated *in vacuo*. Epoxyalcohol **15a** (38 mg): $[\alpha]_D^{20}$ +5.3° (*c* 1.02, CH_2Cl_2), lit.[9] $[\alpha]_D^{25}$ +6.1° (*c* 1.15, CH_2Cl_2). NMR δ_H ($CDCl_3$): 1.38 (3H, s, CH_3–), 2.64 (1H, d, J=4.6 Hz, –CH_2–), 2.93 (1H, d, J=4.6 Hz, –CH_2–), 3.77 (3H, s, CH_3–O), 4.27 (1H, d, J=6.6 Hz, –CH<), 5.84 (1H, dd, J=15.1, 6.6 Hz, –CH=), 5.93 (1H, d, J=15.1 Hz, –CH=), 6.14 (1H, t, J=11.3 Hz, –CH=), 6.33 (1H, t, J=11.1 Hz, –CH=), 6.95 (1H, dd, J=15.1, 11.4 Hz, –CH=), 7.76 (1H, dd, J=15.1, 11.9 Hz, –CH=). IR ν_{max} (film) cm^{-1}: 3450 (O–H), 3010, 2970 (C–H), 1710 (O–C=O), 1625, 1580 (C=C). The NMR spectrum for epoxyalcohol **15b** was obtained for a crude sample. NMR δ_H ($CDCl_3$): 1.37 (3H, s, CH_3–), 2.63 (1H, d, J=4.6 Hz, –CH_2–), 2.92 (1H, d, J=4.6 Hz, –CH_2–), 3.75 (3H, s, CH_3–O), 4.23 (1H, d, J=6.7 Hz, –CH<), 5.85 (1H, dd, J=14.5, 6.7 Hz, –CH=), 5.91 (1H, d, J=15.5 Hz, –CH=), 6.35 (1H, dd, J=14.5, 11.2 Hz, –CH=), 6.46 (1H, dd, J=14.3, 11.2 Hz, –CH=), 6.56 (1H, dd, J=14.4, 10.7 Hz, –CH=), 7.31 (1H, dd, J=15.2, 11.1 Hz, –CH=).

N-(9-Fluorenylmethoxycarbonyl)-(2S,3S)-erythro-L-β-methylphenylalanine (**16**). (2*S*,3*S*)-erythro-L-β-Methylphenylalanine[17] (300 mg, 1.67 mmol) was dissolved in 10% (w/v) aqueous Na_2CO_3 (1.95 ml, 1.84 mmol). *N*-(9-Fluorenylmethoxycarbonyl)succinimide (621 mg, 1.84 mmol) in dioxane (2 ml) was added dropwise to the solution, and the mixture was stirred overnight at room temperature. The mixture was diluted with water (8 ml) and washed twice with EtOAc (5 ml). The aqueous layer was poured into EtOAc (15 ml), acidified with hydrochloric acid and then separated. The aqueous layer was further extracted three times with EtOAc (15 ml). The combined organic layer was successively washed twice with 1N HCl (15 ml), twice with water (15 ml) and once with brine (15 ml), and then dried over anhydrous $MgSO_4$. After evaporation of the solvent *in vacuo*, 637 mg (95% yield) of Fmoc-erythro-L-β-MePhe **16** was obtained as a viscous syrup. Crystalization from EtOAc/hexane gave colorless, fibrous crystals, mp 157.5–159°C. $[\alpha]_D^{33}$ +20.1° (*c* 1.03, $CHCl_3$). NMR δ_H ($CDCl_3$): 1.38 (3H, d, J=7.0 Hz, CH_3–), 3.45 (1H, dq, $J \approx 6$ Hz, –CH<), 4.20 (1H, t, J=6.7 Hz, fluorenyl –CH<), 4.35 (1H, dd, J=10.5, 6.7 Hz, fluorenyl –CH_2–), 4.46 (1H, dd, J=10.5, 7.3 Hz, fluorenyl –CH_2–), 4.62 (1H, dd, J=9.0, 5.2 Hz, –CH<), 4.98 (1H, d, J=9.0 Hz, >NH), 7.17 (2H, d, J=7.0 Hz, Ar–H), 7.25–7.33 (7H, m, Ar–H), 7.50–7.56 (2H, m, Ar–H), 7.77 (2H, d, J=7.5 Hz, Ar–H). IR ν_{max} (nujol) cm^{-1}: 3400 (N–H), 3150 (O–H), 1720, 1685 (O–C=O, N–C=O), 1605 (C=C). HRMS (EI) m/z (M^+): Calcd. for $C_{25}H_{23}NO_4$: 401.1627, Found: 401.1624.

Methyl (8R,9S,2'S,3'S,2E,4Z,6E)-9,10-epoxy-8-[2'-(9-fluorenylmethoxycarbamino)-3'-phenylbutanoyloxy]-9-methyl-2,4,6-decatrienoate (**17**). Epoxyalcohol **15a** (40.0 mg, 171 μmol) was dissolved in dry EtOAc (1 ml), and the solution was stirred at room temperature. Fmoc-*erythro*-L-β-MePhe **16** (86 mg, 214 μmol), DCC (71 mg, 342 μmol) and a catalytic amount of 4-pyrrolidinopyridine (2.5 mg, 17 μmol) were successively added portionwise to the solution and the mixture was stirred for an additional 2 h. The precipitate of dicyclohexylurea was filtered off, and the filtrate was concentrated *in vacuo*. The residue was purified by silica gel flash

column chromatography (hexane:EtOAc=4:1→3:1, stepwise) to give 99.8 mg (96% yield) of Fmoc derivative **17** as an amorphous solid. $[\alpha]_D^{29}$ +48.4° (*c* 1.02, $CHCl_3$). NMR δ_H ($CDCl_3$): 1.26 (3H, s, CH_3–), 1.36 (3H, d, *J*=7.0 Hz, CH_3–), 2.58 (1H, d, *J*=4.7 Hz, $-CH_2$–), 2.71 (1H, d, *J*=4.7 Hz, $-CH_2$–), 3.40 (1H, dq, *J*≈6 Hz, –CH<), 3.75 (3H, s, CH_3–O), 4.20 (1H, t, *J*=6.9 Hz, fluorenyl –CH<), 4.33 (1H, dd, *J*=10.4, 6.9 Hz, fluorenyl $-CH_2$–), 4.45 (1H, dd, *J*=10.4, 7.3 Hz, fluorenyl $-CH_2$–), 4.65 (1H, dd, *J*=9.0, 5.2 Hz, –CH<), 5.08 (1H, d, *J*=9.0 Hz, >NH), 5.27 (1H, d, *J*=7.8 Hz, –CH<), 5.77 (1H, dd, *J*=15.0, 7.8 Hz, –CH=), 5.93 (1H, d, *J*=15.1 Hz, –CH=), 6.17 (1H, t, *J*≈11 Hz, –CH=), 6.28 (1H, t, *J*≈11 Hz, –CH=), 6.90 (1H, dd, *J*=15.1, 11.3 Hz, –CH=), 7.13 (2H, d, *J*=6.6 Hz, Ar-H), 7.20–7.42 (7H, m, Ar-H), 7.51–7.56 (2H, m, Ar-H), 7.70 (1H, dd, *J*=15.0, 11.5 Hz, –CH=), 7.76 (2H, d, *J*=7.5 Hz, Ar-H). IR ν_{max} ($CHCl_3$) cm^{-1}: 3420 (N-H), 1715 (O-C=O), 1620, 1600 (C=C). HRMS (CI) *m/z* ($[M+H]^+$): Calcd. for $C_{37}H_{38}NO_7$: 608.2648, Found: 608.2656.

Methyl (8R,9S,2′S,3′S,2E,4Z,6E)-8-(2′-amino-3′-phenylbutanoyloxy)-9,10-epoxy-9-methyl-2,4,6-decatrienoate (**18**). To Fmoc derivative **17** (71.3 mg, 117 μmol) in a 10-ml round-bottomed flask was added 5% (w/v) piperidine in dry DMF (3 ml), and the mixture was stirred at room temperature for 5 min. The volatile components were evaporated *in vacuo*, and the residue was purified by silica gel flash column chromatography (hexane:EtOAc=1:2) to give 23.5 mg (52% yield) of amine precursor **18** as a yellow syrup. $[\alpha]_D^{26}$ +83.3° (*c* 1.17, $CHCl_3$). NMR δ_H ($CDCl_3$): 1.30 (3H, s, CH_3–), 1.36 (3H, d, *J*=7.1 Hz, CH_3–), 2.61 (1H, d, *J*=4.7 Hz, $-CH_2$–), 2.78 (1H, d, *J*=4.7 Hz, $-CH_2$–), 3.13 (1H, dq, *J*≈7 Hz Hz, –CH<), 3.63 (1H, d, *J*=6.8 Hz, –CH<), 3.76 (3H, s, CH_3–O), 5.33 (1H, d, *J*=7.8 Hz, –CH<), 5.81 (1H, dd, *J*=15.2, 7.8 Hz, –CH=), 5.94 (1H, d, *J*=15.2 Hz, –CH=), 6.18 (1H, t, *J*=11.3 Hz, –CH=), 6.31 (1H, t, *J*=11.0 Hz, –CH=), 6.93 (1H, dd, *J*=15.2, 11.3 Hz, –CH=), 7.17–7.32 (5H, m, Ar-H), 7.72 (1H, dd, *J*=15.2, 11.7 Hz, –CH=). IR ν_{max} ($CHCl_3$) cm^{-1}: 2910 (C-H), 1705 (O-C=O), 1620, 1600 (C=C). HRMS (CI) *m/z* ($[M+H]^+$): Calcd. for $C_{22}H_{28}NO_5$: 386.1967, Found: 386.1959.

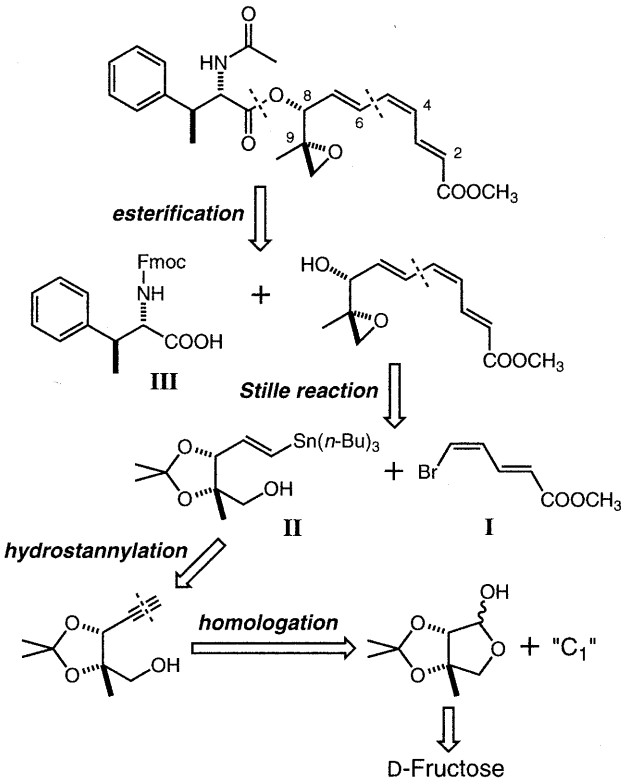
AK-toxin I methyl ester: methyl (8R,9S,2′S,3′S,2E,4Z,6E)-8-(2′-acetamino-3′-phenylbutanoyloxy)-9,10-epoxy-9-methyl-2,4,6-decatrienoate (**3**). To amine precursor **18** (20.1 mg, 52.1 μmol) in a 10-ml round-bottomed flask was added 1.0 M Ac_2O in dry pyridine (0.11 ml, 110 μmol), and the mixture was stirred at room temperature for 2 h under argon. The mixture was then concentrated *in vacuo*, and the residue was diluted with EtOAc (10 ml). The EtOAc solution was successively washed with 5% aqueous citric acid (5 ml), 5% aqueous Na_2CO_3 (5 ml) and brine (5 ml), and then dried over anhydrous $MgSO_4$ and concentrated *in vacuo*. The residue was purified by silica gel flash column chromatography (hexane:EtOAc=2:3) to give 15.8 mg (71% yield) of AK-toxin I methyl ester **3** as a pale yellow syrup. $[\alpha]_D^{31}$ +76.2° (*c* 0.77, $CHCl_3$). NMR δ_H ($CDCl_3$): 1.30 (3H, s, CH_3–), 1.35 (3H, d, *J*=7.1 Hz, CH_3–), 1.98 (3H, s, CH_3–CO), 2.60 (1H, d, *J*=4.7 Hz, $-CH_2$–), 2.72 (1H, d, *J*=4.7 Hz, $-CH_2$–), 3.36 (1H, dq, *J*≈7 Hz, –CH<), 3.76 (3H, s, CH_3–O), 4.86 (1H, dd, *J*=8.2, 5.8 Hz, –CH<), 5.27 (1H, d, *J*=7.8 Hz, –CH<), 5.68 (1H, broad d, *J*=8.2 Hz, >NH), 5.79 (1H, dd, *J*=15.2, 7.8 Hz, –CH=), 5.95 (1H, d, *J*=15.2 Hz, –CH=), 6.20 (1H, t, *J*=11.2 Hz, –CH=), 6.31 (1H, t, *J*=11.1 Hz, –CH=), 6.90 (1H, dd, *J*=15.2, 11.2 Hz, –CH=), 7.12–7.33 (5H, m, Ar-H), 7.71 (1H, dd, *J*=15.2, 11.7 Hz, –CH=). IR ν_{max} ($CHCl_3$) cm^{-1}: 3420 (N-H), 1730, 1710 (O-C=O), 1675 (N-C=O), 1620, 1600 (C=C). HRMS (CI) *m/z* ($[M+H]^+$): Calcd. for $C_{24}H_{30}NO_6$: 428.2073, Found: 428.2065.

[Acetyl-^{3}H]AK-toxin I methyl ester (**3′**). The reaction was carried out in a glove box ventilated by air suction. A cold trap, which was cooled to −78°C, was placed between the outlet of the glove box and the pump. To a pyridine solution of [^{3}H]acetic anhydride (924.2 MBq, 426 GBq/mmol) was added amine precursor **18** (1.83 mg, 4.75 μmol) in dry pyridine (*ca.* 100 μl), and the mixture was shaken. The mixture was then allowed to stand at room temperature for 3 h, before being concentrated *in vacuo*. The residue was diluted with EtOAc (5 ml) and then successively washed with 5% (w/v) aqueous citric acid, 5% (w/v) aqueous Na_2CO_3 and brine (2.5 ml each). After drying over anhydrous $MgSO_4$ and removing the insoluble material by filtration through a pad of cotton, the product was purified by silica gel column chromatography (hexane:EtOAc=2:3) to give [acetyl-^{3}H]AK-toxin I methyl ester **3′** (213 GBq/mmol) in radiochemically pure form. The total radioactivity was determined to be 168.8 MBq, and the radiochemical yield was 37%.

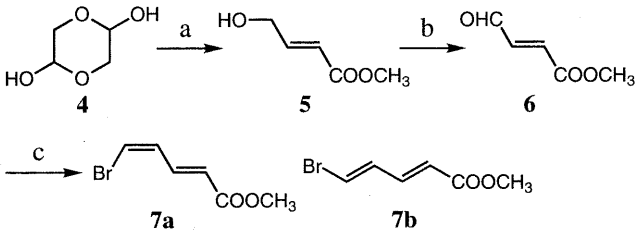
Bioassay. Branches of Japanese pear (*Pyrus serotina*) *cvs.* Nijisseiki and Hosui, representing, respectively, a susceptible and resistant cultivar, were collected at the Experimental Farm of Kyoto University. After storing at 4°C for at least 40 days,[18] the lower end of the branch cutting was soaked in tap water to induce sprouting. The newly produced leaves (3–4 weeks old) were subjected to the toxicity assay described in the literature.[3]

Results and Discussion

The target molecule was divided into three fragments for synthetic convenience: a conjugated alkene fragment (**I**), a chiral allyl alcohol fragment (**II**) and a β-methylphenylalanine fragment (**III**). Accordingly, the the strategy for the synthesis is shown in Scheme 1. Of these steps, the construction of fragment **II** is particularly important, because the absolute configurations of 8*R*,9*S* in this fragment have been shown to be essential for the host-specific toxicity.[9,19] In a previous synthesis of AK-toxin II, Ando *et al.* skilfully constructed this fragment by using 2,3-*O*-isopropylidene-3-*C*-methyl-L-erythrofuranose (**8**),[11] which contains the same structural features as those of C_8–C_{10} of AK-toxins, as the chiral source which is readily available in a large scale from D-



Scheme 1. Retrosynthesis of AK-toxin I Methyl Ester.



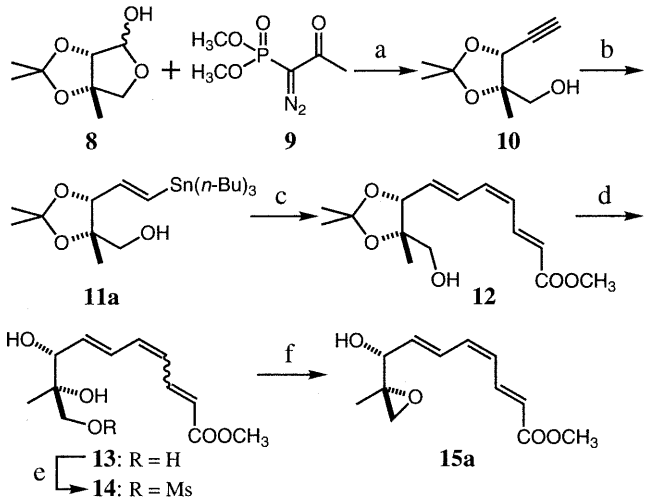
Scheme 2. Synthesis of the Conjugated Alkene Fragment.
Reagents and conditions: (a) $Ph_3P{=}CHCOOCH_3$, $CHCl_3$, r.t., 87%; (b): PCC, CH_2Cl_2, r.t., 89%; (c) $(Ph_3PCH_2Br)^+Br^-$, *t*-BuOK, THF, −78°C, 44%.

fructose. In their work, however, the pathway for the subsequent construction of fragment **II** from fragment **I** appears inefficient and would benefit from improvement. In this respect, the method of Crombie *et al.*,[10] in which the formation of the 2*E*,4*Z*,6*E* triene structure was effectively achieved by using acetylene hydrostannylation and Pd-mediated coupling with vinyl halide (Stille reaction),[20,21] has some advantages. Therefore, we attempted to improve the preparation of the epoxytrienecarboxylic acid moiety of AK-toxin by combining these two synthetic approaches. This acid moiety was then combined with stereochemically-pure (2*S*,3*S*)-*erythro*-L-*β*-methylphenylalanine (fragment **III**) which had been prepared by the procedure of Li *et al.*[17]

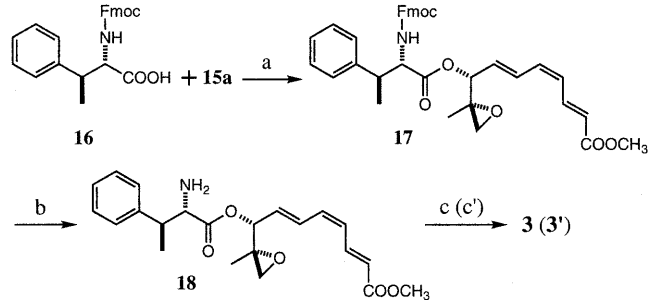
The preparation of fragment **I** is outlined in Scheme 2. The *trans* double bond of C_2–C_3 of **3** was constructed *via* a Wittig olefination of glycolaldehyde dimer **4** with carbomethoxymethylenetriphenylphosphorane in $CHCl_3$.[14] The reaction proceeded with high selectivity to almost exclusively give *trans* allyl alcohol **5** in an 87% yield, the configuration of which was confirmed by the ^{1}H-NMR coupling constant between H_2 and H_3 (16.0 Hz). PCC oxidation[14,22] in CH_2Cl_2 afforded *trans* aldehyde **6** in an 89% yield as the sole product. The *cis* double bond of C_4–C_5 was then constructed by the Wittig olefination of aldehyde **6** with bromomethyltriphenylphosphonium bromide in THF at −78°C, using *t*-BuOK as a base.[10,23] The reaction proceeded *cis* preferentially to give a 5:3 mixture of (2*E*,4*Z*) vinyl bromide **7a** and (2*E*,4*E*) isomer **7b** in a 44% yield. The configurations of the C_4–C_5 double bond of **7a** and **7b** were determined according to the coupling constants of 7.3 Hz and 13.4 Hz, respectively. These two isomers were separated by reversed-phase HPLC, and desired **7a** was obtained in a pure form.

Fragment **II** was synthesized from 2,3-*O*-isopropylidene-3-*C*-methyl-L-erythrofuranose (**8**), which had been derived from D-fructose in five steps according to the method of Ando *et al.*[11] Attempts to convert **8** to alkyne **10** by a combination of Wittig olefination and a subsequent elimination reaction were unsuccessful. For example, a four-step procedure consisting of the olefination of **8** with methylenetriphenylphosphorane, benzoylation of the hydroxy group, addition of Br_2 to the double bond and elimination with *t*-BuOK failed to give the desired alkyne. Instead, the conversion was accomplished in one step by using dimethyl 1-diazo-2-oxopropylphosphonate (**9**) which has been reported to be effective for the homologation of aliphatic[24,25] and aromatic[26] aldehydes under relatively mild conditions. Namely, treating **8** with **2** equivalents of **9**, which had been prepared by diazo-transfer from *p*-toluenesulfonyl azide to dimethyl 2-oxopropylphosphonate,[27] in the presence of anhydrous K_2CO_3 in MeOH gave **10** in a 62% yield. This yield could not be significantly improved, even when 4 equivalents of **9** was used. However, since unreacted starting material **8** could be easily recovered chromatographically from the reaction mixture, the practical yield could be raised by repeating the reaction. Treatment of **10** with *n*-Bu_3SnH in the presence of a catalytic amount of AIBN[28] afforded *trans* vinylstannane **11a** along with its *cis* isomer **11b** in a ratio of 43:7, which were readily separated from one another by silica gel column chromatography. Two fragments **7a** and **11a** were then combined by the Stille reaction. By using bis(triphenylphosphine)palladium(II) dichloride as a catalyst, the reaction proceeded smoothly in DMF to give triene acetonide **12** in an 80% yield. However, because of the concomitant isomerization of *cis* double bond C_4–C_5, the product was contaminated with about 10% of the undesired (2*E*,4*E*,6*E*) isomer, which could be removed by repeated recrystallization.

Deprotection of the diol in **12** was carried out under neutral conditions at ambient temperature, using 1% I_2 in MeOH,[29] in order to prevent isomerization of the conjugated triene system. Although a preliminary experiment with a small amount (50 μmol) of **12** gave triol **13** almost quantitatively with only slight isomerization of



Scheme 3. Synthesis of the Chiral Epoxyalcohol Fragment.
Reagents and conditions: (a) K_2CO_3, MeOH, 0°C→r.t., 62%; (b) *n*-Bu_3SnH, AIBN, 85°C, 89%, *E*:*Z*=43:7; (c) **7a**, cat. $(Ph_3P)_2PdCl_2$, DMF, r.t., 80%; (d) I_2, MeOH, r.t., 82%; (e) MsCl, *i*-Pr_2NEt, CH_2Cl_2, −50°C, 76%; (f) *t*-BuOK, THF, 0°C, 68%.



Scheme 4. Synthesis of AK-toxin I Methyl Ester.
Reagents and conditions: (a) DCC, 4-pyrrolidinopyridine, EtOAc, r.t., 96%; (b) piperidine, DMF, r.t., 52%; (c) Ac_2O, pyridine, r.t., 71%; (c′) [^{3}H]Ac_2O, pyridine, r.t., 37%.

the double bond (less than 5%), a scaled-up reaction using 5 mmol of **12** unexpectedly proceeded very sluggishly, and substantial isomerization was observed. After recovering the unreacted material and repeating the deprotection process in the same manner, triol **13** was obtained in an 82% yield as a 5:2 mixture of the (2*E*,4*Z*,6*E*) and (2*E*,4*E*,6*E*) isomers. The triol was then selectively mesylated at its primary hydroxyl group with methanesulfonyl chloride and diisopropylethylamine in CH_2Cl_2 at −50°C to give monomesylate **14** in a 76% yield as a mixture of isomers with respect to its triene structure. Epoxide formation was effected by treating **14** with *t*-BuOK in a 68% yield, and, at this stage, the mixture of geometrical isomers could be readily separated by reversed-phase HPLC to afford desired epoxyalcohol **15** (Scheme 3).

Epoxyalcohol **15** was then condensed with β-methylphenylalanine fragment **III**. (2*S*,3*S*)-*erythro*-L-β-Methylphenylalanine was prepared by the stereoselective introduction of an amino group into (±)-3-phenylbutanoic acid according to the procedure described by Li *et al.*[17] To avoid racemization during the condensation process, the amino acid was converted to *N*-Fmoc[30] derivative **16** which was then condensed with epoxyalcohol **15** using DCC and a catalytic amount of 4-pyrrolidinopyridine[31] in EtOAc, to give **17** in a 96% yield. After removing the Fmoc group by treating with 5% piperidine in DMF in a 52% yield, resulting amine **18** was acetylated with Ac_2O in pyridine to give AK-toxin I methyl ester (**3**) in a 71% yield (Scheme 4). Although obtained **3** was found to contain about 10% of the (2*E*,4*E*,6*E*) isomer as the result of isomerization during the amino acid-deprotection and/or acetylation procedures, the isomer is unlikely to have a negative effect on necrosis induction since the (2*E*,4*E*,6*E*) isomer of AK-toxin II methyl ester has the same activity as AK-toxin II methyl ester.[9] The compound **3** induced clear necrosis on the leaves of susceptible cultivar Nijisseiki at 1×10^{-6} M, while no necrosis was induced on the leaves of resistant cultivar Hosui even at 1×10^{-3} M. The threshold concentration inducing necrosis was 1×10^{-7} M. Although the necrotic activity of **3** is about 20 times as low as that of natural AK-toxin I,[3] compound **3** is toxic enough to be used for biochemical experiments.

By using [^{3}H]Ac_2O in this final acetylation reaction, [acetyl-^{3}H] AK-toxin I methyl ester (**3′**) with a specific activity of 213 GBq/mmol was successfully prepared in a 37% radiochemical yield. Biochemical studies to probe the binding site(s) of AK-toxin in the tissues of Japanese pear are now in progress by using this radiolabeled ligand.

Acknowledgments

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