Bioorganic & Medicinal Chemistry 19 (2011) 2392–2396

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Contribution of cinnamic acid analogues in rosmarinic acid to inhibition of snake venom induced hemorrhage

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ARTICLE INFO

Article history:
Received 7 December 2010
Revised 8 February 2011
Accepted 9 February 2011
Available online 13 February 2011

Keywords:
Rosmarinic acid
Cinnamic acid
Snake venom
Anti-hemorrhage
Protobothrops flavoviridis

ABSTRACT

In our previous paper, we reported that rosmarinic acid (**1**) of *Argusia argentea* could neutralize snake venom induced hemorrhagic action. Rosmarinic acid (**1**) consists of two phenylpropanoids: caffeic acid (**2**) and 3-(3,4-dihydroxyphenyl)lactic acid (**3**). In this study, we investigated the structural requirements necessary for inhibition of snake venom activity through the use of compounds, which are structurally related to rosmarinic acid (**1**). By examining anti-hemorrhagic activity of cinnamic acid analogs against *Protobothrops flavoviridis* (Habu) venom, it was revealed that the presence of the *E*-enoic acid moiety (–CH=CH–COOH) was critical. Furthermore, among the compound tested, it was concluded that rosmarinic acid (**1**) (IC_{50} 0.15 μM) was the most potent inhibitor against the venom.

1. Introduction

Snakebites are a serious medical problem in many regions of the world, especially in tropical and subtropical countries. Snake venoms are complex mixtures of biologically active proteins, peptides, metal ions and organic compounds[1,2] possessing a variety of biological activities. Envenomations due to snakebites are commonly treated by parenteral administration of horse or sheep-derived polyclonal antivenom aimed at the neutralization of toxins. However, antivenom does not provide sufficient protection against venom induced hemorrhage, necrosis, nephrotoxicity and, furthermore, can often cause hypersensitiviy reactions.[3,4] It has been known for many years that animal sera and many plants can be used to neutralize snake venom, and, consequently, several reports and reviews of their constituents with respect to antivenom activity have been published. However, to the best of our knowledge, no report on the structural requirements to exhibit antivenom activity has been published.

Hemorrhage is one of the most conspicuous consequences of snake envenoming, and it is sometimes lethal. Unfortunately, the only clinical treatment is antiserum against snake venoms. Snakebites often occur outdoors, far from medical institutions. Because of this, drugs to treat snakebites must be transported to remote locations ahead of time, requiring them to be stable against light, oxygen, and other forms of decomposition without the aid of refrigeration or a special container.

In our previous study, we reported on the anti-hemorrhagic activity of rosmarinic acid (**1**) from the methanolic extract of *Argusia argentea* against crude venoms as well as purified toxins.[5] This compound (**1**) consists of two similar phenylpropanoids: caffeic acid (**2**) and 3-(3,4-dihydroxyphenyl)lactic acid (**3**). Both of these compounds possess a 3,4-dihydroxyphenyl group in the molecule which we speculated to be a key moiety in the molecule for the exhibition of antivenom activity. In this report, we demonstrate the structural requirements of rosmarinic acid (**1**) for the inhibition of hemorrhage induced by the hemorrhagic snake venom of *Protobothrops flavoviridis* (Habu) through the use of cinnamic acid analogues.

2. Results and discussion

2.1. Separation and anti-hemorrhagic activity of *E*- and *Z*-isomers of rosmarinic acid (1) and contribution of the double bond to the activity

Prior to the investigation of rosmarinic acid analogue activities, we tried to separate the *E*- and *Z*-isomers of rosmarinic acid (**1**). Due to the precise nature of NMR analysis, the spectrum showed signals correspond to (*E*)- and (*Z*)-rosmarinic acid (**1a** and **1b**, respectively). Based on the integration values of signals derived from olefinic protons on the ^{1}H NMR spectra, the ratio of each isomer was revealed to be approximately 9:1. These isomers were separated by using HPLC equipped with COSMOSIL Cholester column, and fractionated the isomers. Unfortunately, the *Z*-isomer (**1b**) was quite unstable and thus difficult to isolate as a single iso-

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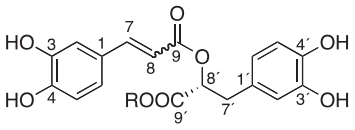
doi:10.1016/j.bmc.2011.02.013

mer. The *Z*-isomer (**1b**) was immediately converted to the *E*-isomer (**1a**), even under dark or cold conditions. Conversely, the *E*-isomer proved to be stable. Accordingly, only the *E*-isomer (**1a**) gave an exact antivenom activity that was significantly stronger (IC_{50} 0.15 μM) than that of the mixture of (*E*)- and (*Z*)-rosmarinic acid (**1**) (IC_{50} 0.60 μM). We further investigated the anti-hemorrhage activity of saturated rosmarinic acid (**4**), but no activity was observed. From the results, the existence of the *E* configuration of a double bond seemed to be required to exhibit anti-hemorrhagic activity.

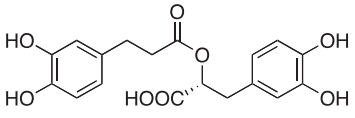
2.2. Anti-hemorrhagic activity of caffeic acid (2), and 3-(3,4-dihydroxyphenyl)lactic acid (3) as components of rosmarinic acid (1) and the contribution of the carboxylic group

As mentioned above, rosmarinic acid (**1**) consists of caffeic acid (**2**) and 3-(3,4-dihydroxyphenyl)lactic acid (**3**) moieties. We interested in the anti-hemorrhagic activities of these compounds which might contribute to the activity. The activity of caffeic acid (**2**) (0.19 μM) was as potent as that of (*E*)-rosmarinic acid (**1a**) (Table 1). On the other hand, we tried to prepare compound **3** to examine its activity, but were unsuccessful. As an alternative, we tested 3-(4-hydroxy-3-methoxyphenyl)lactic acid (**3b**) since, as we will go on to mention in the next section, the methoxyl group on the benzene ring is not expected to affect the activity. As expected, compound **3** exhibited no activity. Moreover, in our previous report, rosmarinic acid methyl ester (**5**), which was also a mixture of *Z*- and *E*-isomers (ca. 1:9), did not show anti-hemorrhagic activity, so the presence of a free carboxylic group might be critical for anti-hemorrhagic activity. To test this hypothesis, caffeic acid methyl ester (**6**) was subjected to anti-hemorrhage tests, for which it showed no activity. The caffeic acid moiety in rosmarinic acid (**1**) is esterified by the hydroxyl group of 3-(3,4-dihydroxyphenyl)lactic acid (**3**), but the activity might be performed by the double bond of caffeic acid moiety and the carboxylic group.

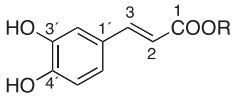
Table 1
Anti-hemorrhagic activity of rosmarinic acid and its analogues



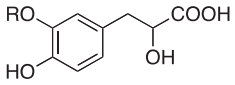
R=H, a mixture of 7*E* and 7*Z* Rosmarinic acid (**1**)
R=H, 7*E* (*E*)-Rosmarinic acid (**1a**)
R=CH_3, a mixture of 7*E* and 7*Z* Rosmarinic acid methyl ester (**5**)



Saturated rosmarinic acid (**4**)



R=H Caffeic acid (**2**)
R=CH_3 Methyl caffeate (**6**)



R=H 3-(3,4-Dihydroxyphenyl)lactic acid (**3**)
R=CH_3 3-(4-Hydroxy-3-methoxyphenyl)lactic acid (**3b**)

Compounds	IC_{50} (μM)
Rosmarinic acid (**1**)	0.60
(*E*)-Rosmarinic acid (**1a**)	0.15
Caffeic acid (**2**)	0.19
3-(4-Hydroxy-3-methoxyphenyl)lactic acid (**3b**)	NA[a]
Saturated rosmarinic acid (**4**)	NA
Rosmarinic acid methyl ester (**5**)	NA
Methyl caffeate (**6**)	NA

[a] NA denotes 'not active'.

2.3. Contribution of phenolic hydroxyl groups to anti-hemorrhagic activity

Caffeic acid (**2**) is suspected to play an important role to exhibit anti-hemorrhagic activity. Because of this, the participation of phenolic hydroxyl groups in relation to this activity should be discussed. Anti-hemorrhagic activities of various kinds of cinnamic acid analogues were investigated as demonstrated in Table 2. As a result, it was revealed that this activity does not depend on the numbers or positions of the phenolic hydroxyl groups when the configuration of a double bond is *E*. This is the result of cinnamic acid (**7**), which possesses no phenolic hydroxyl group, does not show a significant change of activity from that of caffeic acid. Moreover, compounds with a methoxyl group on phenyl group (**16** and **17**) exhibited just slightly lower activities than that of caffeic acid (**2**).

2.4. Contribution of the carboxyl group and side chain to anti-hemorrhagic activity

Compounds **13**–**15** with three phenolic hydroxyl groups, whose configurations of double bonds are all *Z*, did not show the activity (Table 2). When the carboxyl group is reduced to aldehyde and alcohol, the resultant compounds (**18** and **19**) were also inactive. Moreover, methyl cinnamate **20** showed no activity. On the other hand, compound **21**, which possessed a conjugated *E* diene structure, showed similar activity (0.22 μM) to that of caffeic acid while saturated caffeic acid (**22**, 3-(3,4-dihydroxyphenyl)propanoic acid) did not. As a result, this evidence suggests that the configuration of the double bond and the existence of the carboxyl group were more effective to the activity than the positions of oxygen substituents on the phenyl group.

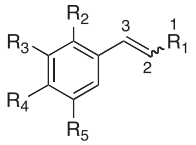
2.5. Anti-hemorrhagic activity of chlorogenic acid

As shown in Table 3, chlorogenic acid (**23**), an ester of caffeic acid and quinic acid, did not change the IC_{50} value (0.20 μM) significantly in comparison to that of *E*-caffeic acid (**2**). The carboxyl group of chlorogenic acid (**23**) is esterified by quinic acid, but the compound exhibited the activity by the combination of the double bond of caffeic acid moiety and the carboxyl group of quinic acid, as in the case with rosmarinic acid (**1a**).

2.6. Contribution of the phenyl group to anti-hemorrhagic activity

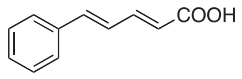
As mentioned above, the hydroxyl group(s) on the phenyl group is not critical to exhibit anti-hemorrhagic activity, but the *E*-enoic acid moiety is. Then we tested the anti-hemorrhagic activity of the simplest compounds, crotonic acid (**24**) and sorbic acid (2,4-hexadienoic acid) (**25**). As a result, as shown in Table 4, these two compounds also exhibited comparable activity as that of

Table 2
Anti-hemorrhagic activity of cinnamic acid and its analogues

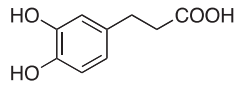


Compounds	2*EZ*	R_1	R_2	R_3	R_4	R_5	IC_{50} (μM)
Cinnamic acid (**7**)	*E*	COOH	H	H	H	H	0.18
2-Hydroxycinnamic acid (**8**)	*E*	COOH	OH	H	H	H	0.19
3-Hydroxycinnamic acid (**9**)	*E*	COOH	H	OH	H	H	0.18
4-Hydroxycinnamic acid (**10**)	*E*	COOH	H	H	OH	H	0.19
2,4-Dihydroxycinnamic acid (**11**)	*E*	COOH	OH	H	OH	H	0.21
3,4,5-Trihydroxycinnamic acid (**12**)	*E*	COOH	H	OH	OH	OH	0.18
2,3,4-Trihydroxycinnamic acid (**13**)	*Z*	COOH	OH	OH	OH	H	NA
2,4,5-Trihydroxycinnamic acid (**14**)	*Z*	COOH	OH	H	OH	OH	NA
3,4,5-Trihydroxycinnamic acid (**15**)	*Z*	COOH	H	OH	OH	OH	NA
Ferulic acid (**16**)	*E*	COOH	H	OCH_3	OH	H	0.20
3,4-Dimethoxycinnamic acid (**17**)	*E*	COOH	H	OCH_3	OCH_3	H	0.24
Cinnamaldehyde (**18**)	*E*	CHO	H	H	H	H	NA
Cinnam alcohol (**19**)	*E*	CH_2OH	H	H	H	H	NA
Methyl cinnamate (**20**)	*E*	$COOCH_3$	H	H	H	H	NA

Table 3
Anti-hemorrhagic activity of cinnamic acid analogues



(2*E*,4*E*)-5-Phenylpenta-2,4-dienoic acid (**21**)



3-(3,4-Dihydroxyphenyl)propanoic acid (**22**)

Compounds	IC_{50} (μM)
(2*E*,4*E*)-5-Phenylpenta-2,4-dienoic acid (**21**)	0.22
3-(3,4-Dihydroxyphenyl)propanoic acid (**22**)	NA
Chlorogenic acid (**23**)	0.20

Table 4
Anti-hemorrhagic activity of aliphatic enoic acids

Compounds	IC_{50} (μM)
Crotonic acid (**24**)	0.22
Sorbic acid (**25**)	0.21

cinnamic acid (**7**). From this result, it revealed that phenolic group is not also so effective to the activity.

3. Conclusion

In this study, the structural requirement for cinnamic acid analogues to exhibit anti-hemorrhagic activity was investigated. As a result, compounds possessing a carboxylic group and an *E*-double bond showed high potency with respect to their activities. Moreover, even the compounds without the phenyl group exhibit the activity. It does not seem that the carboxylic group and the double bond need to be conjugated in order for this to occur. Rosmarinic acid (**1**) along with caffeic acid (**2**) and chlorogenic acid (**23**) are known to have antivenom activity.[6] However, the contribution of functional groups was not discussed. Lans et al. reported that caffeic acid (**2**) might strongly bind to proteins through hydrophobic interactions and hydrogen bonds with subsequent conformational changes in the proteins.[7,8] However, according to our results, phenolic hydroxyl groups on the phenyl group would not be critical. We speculate that the anti-hemorrhagic compound should bind the venom allosterically through the carboxylic group and the double bond to cause conformational change so as to exhibit activity. However, further studies are needed to investigate the mode of action of these compounds. We have already reported on the mechanism of action of rosmarinic acid (**1**) with respect to its anti-hemorrhagic activity.[9] The investigation of the methods for its administration is currently ongoing with the goal of developing a potent alternative antidote compound for snake envenomation.

4. Experimental

4.1. General

IR spectra were recorded on FT-IR-410 spectrophotometer. ^1H and ^{13}C NMR spectra were recorded on a JEOL ECA-500 (^1H: 500 MHz and ^{13}C: 125 MHz). Chemical shifts for ^1H and ^{13}C NMR are given in parts per million (δ) relative to solvent signal (methanol-d_4: δ_H 3.30 and δ_C 49.0) as internal standard. EI- and FAB-MS were obtained with a JEOL JMS MS-700 and HX-110 and *m*-nitrobenzyl alcohol was used for FAB-MS as a matrix. Analytical TLC was performed on Silica gel 60 F_{254} (Merck). Column chromatography was carried out on silica gel BW-820MH (Fuji Silysia Chemicals, Co. Ltd, Seto, Japan). Develosil ODS UG-5 (ϕ20 × 250 mm, Nomura Chemical, Seto, Japan), and COSMOSIL Cholester (ϕ 20 × 250 mm, Nacalai Tesque, Kyoto, Japan) columns were used for preparative HPLC, and Develosil ODS UG-5 (ϕ4.6 × 250 mm, Nomura Chemical, Seto, Japan), and Cosmosil Cholester (ϕ4.6 × 250 mm, Nacalai Tesque, Kyoto, Japan) columns were used for the analytical HPLC. All experiments involving the use of animals were carried out in compliance with the guidelines for animal experiments of Faculty of Pharmacy, Meijo University.

4.2. Venoms and chemicals

P. flavoviridis venom (Okinawa) was purchased from Japan Snake Institute, Gunma. Chlorogenic acid was isolated from *Chrysanthemum coronarium* L. (garland chrysanthemum, Compositae).

Cinnamic acid, 2-hydroxy cinnamic acid, 4-hydroxy cinnamic acid, caffeic acid, 3-(4-hydroxy-3-methoxyphenyl)lactic acid, 4-hydroxy-3-methoxycinnamic acid, 3,4-dimethoxycinnamic acid, 3,4-dihydroxyphenyllactic acid, 3,4,5-trimethoxycinnamic acid, 2,4,5-trimethoxycinnamic acid, 2,3,4-trimethoxycinnamic acid, crotonic acid and sorbic acid were obtained from Tokyo Chemical Industry Co., Ltd (Tokyo, Japan). 3-Hydroxy cinnamic acid was purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). 2,4-Dihydroxycinnamic acid was purchased from Sigma–Aldrich Co. (St. Louis, MO, U.S.A.). Other chemicals used were of analytical grade. Rosmarinic acid (**1**, *Z* and *E*-mixture) and rosmarinic methyl ester (**5**) were isolated from methanolic extract of *A. argentea* as described in Ref. 5.

4.3. Separation of *Z*- and *E*-isomers of rosmarinic acid

A mixture of *Z*- and *E*-isomers of rosmarinic acid were separated using HPLC equipped with COSMOSIL Cholester ($\phi 20 \times 250$ mm) eluted by a mixed solvent of acetonitrile–water–trifluoroacetic acid (2:8:0.05) at a flow rate of 5 mL/min. The column was maintained at a constant temperature of 30 °C. UV monitoring was done at 220 nm. (*Z*)- and (*E*)-Rosmarinic acid (**1a** and **1b**) was eluted at 68 min and 76 min, respectively.

4.4. Anti-hemorrhagic activity assay

Anti-hemorrhagic activity was examined under the modified method reported previously.[5,10] Male ddY mice of 20 g average weight were used for this experiment. Test solutions were prepared as follows: Crude snake venom solution of *P. flavoviridis* (0.14 mg/mL in saline, 50 μL) and sample solution in 10% dimethylsulfoxide (DMSO)–saline, 50 μL) were mixed and incubated at 37 °C for 10 min. The test solution (100 μL) was injected subcutaneously into the abdomen of the mice. Mice injected the vehicle solution only served as a control group. After 24 h, mice were euthanized by inhalation of chloroform, the skin covering the abdomen was removed, and hemorrhagic lesions were determined as follows. We evaluated the lesions by calculating a value of major axes x minor axes, just as that of an ellipse, since the shapes of the lesions are always amorphous.

4.5. Preparation of saturated rosmarinic acid (4)

Rosmarinic acid (**1**, 105 mg) was dissolved in acetone (1 mL), and 5% palladium on activated carbon (20 mg) was added to the solution. The heterogeneous mixture was stirred under a hydrogen atmosphere at room temperature for 3 h. The mixture was filtered through celite, affording a saturated rosmarinic acid (**4**) as a yellow crystal (98 mg, 93%).

Saturated rosmarinic acid (**4**): IR ν_{max} (film) 3364 (br) cm^{-1}; ^1H NMR (CD_3OD, 500 MHz): δ 6.71 (1H, d, J = 1.5 Hz; H-2), 6.61 (1H, d, J = 7.6 Hz; H-5), 6.55 (1H, dd, J = 7.6, 1.5 Hz; H-6), 4.97 (1H, dd, J = 9.8, 3.0 Hz; H-8), 2.84 (1H, dd, J = 14.4, 9.8 Hz; H-7), 3.04 (1H, dd, J = 14.4, 3.0 Hz; H-7), 6.58 (1H, d, J = 1.5 Hz; H-2′), 6.66 (1H, d, J = 8.3 Hz; H-5′), 6.36 (1H, dd, J = 8.3, 1.5 Hz; H-6′), 2.65 (2H, t, J = 7.6 Hz; H-7′), 2.52 (2H, t, J = 7.6 Hz; H-8′); FAB-MS m/z 385 $[M+Na]^+$.

4.6. Preparation of saturated caffeic acid (22)

Five percentage palladium on activated carbon (20 mg) was added to caffeic acid (**2**, 100 mg) in acetone (l mL). The heterogeneous mixture was stirred under a hydrogen atmosphere at room temperature for 3 h. The mixture was filtered through celite which afforded a saturated caffeic acid (**22**, 95 mg, 95%) as a brown crystal.

Saturated caffeic acid (= 3-(3,4-Dihydroxyphenyl)propanoic acid) (**22**): IR ν_{max} (film) 1715 cm^{-1}; ^1H NMR (CD_3OD, 500 MHz): δ 6.62 (1H, d, J = 7.6 Hz; H-5′), 6.59 (1H, d, J = 1.5 Hz; H-2′), 6.48 (1H, dd, J = 7.6, 1.5 Hz; H-6′), 2.71 (2H, t, J = 7.6 Hz; H-3), 2.47 (2H, t, J = 7.6 Hz; H-2); EIMS m/z 182 $[M]^+$, 123 (base).

4.7. Methylation of caffeic acid (2)

Trimethylsilyldiazomethane (174 μL) was added to caffeic acid (**2**, 215 mg, 1.19 mmol) in 10 mL of acetone, and the solution was stirred at 0 °C for 20 min. The solution was evaporated, and the residue was purified by SiO_2 column chromatography to afford methyl caffeate (**6**, 145 mg, 67.4%).

*Methyl caffeate (**6**):* IR ν_{max} (film) 3363 (br), 1684 cm^{-1}; ^1H NMR (CD_3OD, 500 MHz): δ 7.52 (1H, d, J = 15.9 Hz; H-3), 7.01 (1H, d, J = 1.5 Hz; H-2′), 6.92 (1H, dd, J = 8.3, 2.2 Hz; H-6′), 6.75 (1H, d, J = 8.3 Hz; H-5′), 6.24 (1H, d, J = 15.9 Hz; H-2), 3.73 (3H, s; $COOCH_3$); EIMS m/z 194 $[M]^+$.

4.8. Preparation of (*E*)-3,4,5-trihydroxycinnamic acid (12)

Aniline (50 μL) was added to a solution of 3,4,5-trihydroxybenzaldehyde (250 mg) and malonic acid (250 mg) in anhydrous pyridine (1.25 mL) and the reaction mixture was stirred at 50 °C for 12 h. After cooling, diethylether (20 mL) was added to the solution. The organic phase was washed with 2 M hydrochloric acid and water, and dried over anhydrous magnesium sulfate. The product was purified by HPLC, which afforded the desired product (**12**, 160 mg, 64%) as a brown crystal.

*(E)-3,4,5-Trihydroxycinnamic acid (**12**):* ^1H NMR (500 MHz, CD_3OD): δ 7.42 (1H, d, J = 15.9 Hz; H-2), 6.58 (2H, s; H-2′ and H-6′), 6.15 (1H, d, J = 15.9 Hz; H-3); EIMS m/z 196 $[M]^+$, 122 (base).

4.9. Preparation of *Z*-trihydroxycinnamic acid

E-Trimethoxycinnamic acid was dissolved to dry CH_2Cl_2 under an argon atmosphere and cooled to −78 °C. 1 M BBr_3 in CH_2Cl_2 solution (2.8 equiv) was added dropwise to the solution and was stirred at −78 °C for 1 h. Then the reaction temperature was gradually raised to 0 °C. When the starting material disappeared on TLC, methanol was added to the solution, and the reaction mixture was evaporated. Methanol was added again to the residue and the solution was evaporated to remove methylborate. The product was purified by SiO_2 chromatography, and the desired product was obtained.

*(Z)-2,3,4-Trihydroxycinnamic acid (**13**) from E-2,3,4-trimethoxycinnamic acid (8.9% yield):* ^1H NMR (CD_3OD, 500 MHz): δ 7.4 (1H, d, J = 9.8 Hz; H-3), 6.87 (1H, d, J = 8.3 Hz; H-6′), 6.47 (1H, d, J = 8.3; H-5′), 6.15 (1H, d, J = 9.8 Hz; H-2); ^{13}C NMR (CD_3OD, 125 MHz): δ 162.1, 149.8, 145.3, 143.6, 132.1, 118.8, 112.6, 112.4, 110.8; EIMS m/z 178 $[M-H_2O]^+$, 150 (base).

*(Z)-2,4,5-Trihydroxycinnamic acid (**14**) from E-2,4,5-trimethoxycinnamic acid (5.7% yield):* ^1H NMR (CD_3OD, 500 MHz): δ 7.75 (1H, d, J = 8.3 Hz; H-3), 6.91 (1H, s, H-6′), 6.73 (1H, d, J = 8.3, H-5′), 6.15 (1H, d, J = 8.3 Hz; H-2); ^{13}C NMR (CD_3OD, 125 MHz): δ 162.9, 150.7, 149.1, 144.7, 143.2, 111.7, 111.5, 111.1, 102.3; EIMS m/z 178 $[M-H_2O]^+$, 150 (base).

*(Z)-3,4,5-Trihydroxycinnamic acid (**15**) from E-3,4,5-trimethoxycinnamic acid (0.5% yield):* ^1H NMR (CD_3OD, 500 MHz): δ 7.6 (1H, d, J = 9.1 Hz; H-3), 6.91 (1H, s; H-6′), 6.72 (1H, s; H-2′), 6.14 (1H, d, J = 9.1 Hz; H-2); EIMS m/z 178 $[M-H_2O]^+$, 150 (base).

4.10. Preparation of methyl cinnamate (20)

To a solution of (*E*)-cinnamic acid (**7**, 5.1 g) in 50 mL of acetone, 23.7 g of K_2CO_3 and 3.25 mL of dimethyl sulfate were added. The

mixture was stirred at 80 °C for 6 h. The mixture was then filtered to remove K_2CO_3. The filtrate was acidified with 1 M of hydrochloric acid and was extracted with ethyl acetate. The organic layer was washed with water and brine, dried with anhydrous magnesium sulfate, filtered and evaporated to afford methyl (*E*)-cinnamate (**20**, 4.6 g, 90%).

*Methyl cinnamate (**20**):* IR ν_{max} (film) 3062, 2950, 1716 cm^{-1}; ^1H NMR (CD_3OD, 500 MHz): δ 7.65 (1H, d, J = 15.9 Hz; H-3), 7.56–7.35 (5H, m; aromatic), 6.48 (1H, d, J = 15.9 Hz; H-2), 3.75 (3H, s; $COOCH_3$); EIMS m/z 162 $[M]^+$, 131 (base).

4.11. Preparation of (2*E*,4*E*)-5-phenylpenta-2,4-dienoic acid (21)

A mixture of methyl chloroacetate (4.8 g, 44 mmol) and triethylphosphite (7.3 g, 44 mmol) was heated at 135 °C for 10 h. The mixture was allowed to cool to room temperature to afford the desired product, methyl 2-(diethylphosphoryl)acetate, in 98% yield (6.2 g) as a mixture of rotamers.

To a suspension of sodium hydride (7.8 mmol) in dry THF (10 mL), methyl 2-(diethyl phosphoryl)acetate (420 mg) in 5 mL of THF was added dropwise at −60 °C. The reaction mixture was then stirred at 0 °C for 15 min and cooled to −60 °C again. Next cinnamaldehyde (**18**, 300 mg) in 2 mL of THF was added to the reaction solution and stirred for 90 min. The reaction mixture was stirred at 0 °C for 16 h, then at room temperature for 2 h. Next the reaction was acidified with 2 M hydrochloric acid and extracted with ethyl acetate. The organic fractions were collected, washed with brine, dried with anhydrous magnesium sulfate and filtered. The crude product was purified using a SiO_2 column to afford 240 mg of (2*E*,4*E*)-5-phenylpenta-2,4-dienoic acid (**21**, 61% yield).

*(2E,4E)-5-Phenylpenta-2,4-dienoic acid (**21**):* IR ν_{max} (film) 3022, 1679 cm^{-1}; ^1H NMR (CD_3OD, 500 MHz): δ 7.55–7.29 (5H, m; aromatic), 7.44 (1H, dd, J = 15.9, 10.6 Hz; H-3), 7.11 (1H, dd, J = 15.9, 10.6 Hz; H-4), 7.03 (1H, d, J = 15.9 Hz; H-5), 6.03 (1H, d, J = 15.9 Hz; H-2); EIMS m/z 174 $[M]^+$, 129 (base).

Acknowledgments

This work was supported by the bounty for academic research from the Research Institute of Meijo University. One of the authors (H.T.A.) is supported by the Japanese Government (MEXT) Scholarship 2007.

Supplementary data

Supplementary data (profiles of HPLC anlyses of the mixture of *Z*- and *E*-rosmarinic acid (**1**) and purified *E*-rosmarinic acid (**1a**). ^1H NMR spectra of these compounds are also obtained) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.02.013.

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