Syntheses of Carnosic Acid and Carnosol, Anti-oxidants in Rosemary, from Pisiferic Acid, the Major Constituent of Sawara

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Received July 1, 2009; accepted October 8, 2009; published online October 9, 2009

Carnosic acid (2), a major anti-oxidant in rosemary (*Rosmarinus officinalis*), was synthesized from pisiferic acid (1), the major constituent of Sawara (*Chamaecyparis pisifera*), *via ortho*-oxidation of the phenol using *meta*-chlorobenzoyl peroxide (*m*CBPO), chloroacetyl *meta*-chlorobenzoyl peroxide (CAMCBPO) or 2-iodoxybenzoic acid (IBX). Carnosol (3), another anti-oxidant in rosemary, was synthesized from carnosic acid by oxidation with silver oxide. Potent antibacterial activities against *Propionibacterium acnes* (ATCC 6919) (minimum inhibitory concentration (MIC) μg/ml) and *Staphylococcus aureus* ME/GM/TC Resistant (ATCC 33592) (MIC μg/ml) of carnosic acid and carnosol were reported.

Key words carnosic acid; carnosol; synthesis; rosemary; anti-*Propionibacterium acnes*; anti-methicillin-resistant *Staphylococcus aureus* (MRSA)

Many species of the *Salvia* family have been used for herbs and spices, folk medicines and anti-oxidants for foods.[1] Rosemary and sage, well known herbs in this family, contain many anti-oxidant diterpenes, *e.g.* carnosic acid (**2**), carnosol (**3**), and rosmanol (**4**).[2,3] The German Commission E recognized that the extract from rosemary is effective for indigestion when used internally or to quicken the circulation of the blood when used externally. Recently, a major anti-oxidant in rosemary, carnosic acid, has attracted much attention because of its neuron protective effects.[4] Many other biological activities of carnosic acid have been reported, *e.g.* nerve growth factor forming promoters,[5] therapeutic agents for amnesia, dementia, Alzheimer's disease,[6] and lipid absorption inhibitors.[7] Despite these various biological activities, there is no efficient method for synthesizing carnosic acid. Consequently, carnosic acid is produced by extraction from natural rosemary and is very expensive (>$630/500 mg).[8] Sawara (*Chamaecyparis pisifera*) is a popular and useful tree in Japan and its leaves contain the phenolic diterpene, pisiferic acid (**1**), as the major constituent.[9,10] Pisiferic acid **1** has the same abietane carbon skeleton as carnosic acid **2** with a carboxyl group at C-10 and a phenolic hydroxyl group at C-12. Carnosic acid **2** has an additional phenolic hydroxyl group at C11. Previously we reported an efficient *ortho*-oxidation of phenols with *meta*-chlorobenzoyl peroxide (*m*CBPO)[11] and the synthesis of an anti-microbial diterpene, abietaquinone methide, active against drug resistant bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus* (VRE).[12,13] In this report, we describe the facile synthesis of the major anti-oxidant in rosemary, carnosic acid **2**, from pisiferic acid **1**, which is the major constituent of Sawara (*Chamaecyparis pisifera*). The other rosemary anti-oxidants, *e.g.*, carnosol **3**, rosmanol **4**, and other antioxidant diterpenes were synthesized from carnosic acid **2**.[14,15] This method will thus be applicable for the synthesis of these anti-oxidant diterpenes of rosemary. The potent antimicrobial activities (MIC, μg/ml) of carnosic acid and carnosol against *Propionibacterium acnes* (ATCC 6919) and *Staphylococcus aureus* ME/GM/TC resistant (ATCC 33592) were measured to show the potency of the antimicrobial compounds.

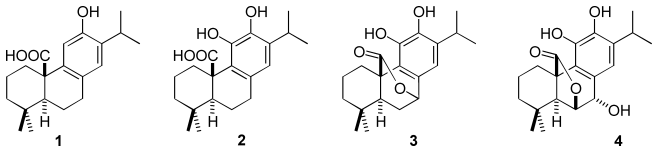

Fig. 1. Major Diterpenes of Sawara and Rosemary

Results and Discussion

Isolation of Pisiferic Acid (1) from Sawara (*Chamaecyparis pisifera*) The leaves of *Chamaecyparis pisifera* were extracted with methanol under reflux for 24 h. The extract was evaporated and the residue was chromatographed on a silica gel column with hexane–ethyl acetate. The crude fraction of pisiferic acid was rechromatographed on a silica gel column with hexane–ethyl acetate. Light green crystals of pisiferic acid were obtained after crystallization from hexane–ethyl acetate (0.6% of the leaves). The spectroscopic data of the isolated pisiferic acid were identical with those of the authentic pisiferic acid.[9,10]

The oxidation of pisiferic acid was examined with three reagents, *meta*-chlorobenzoyl peroxide (*m*CBPO), chloroacetyl *meta*-chlorobenzoyl peroxide (CAMCBPO),[11] and 2-iodoxybenzoic acid (IBX).[16,17]

Synthesis of Carnosic Acid (2) *via* Oxidation with *m*CBPO Pisiferic acid **1** was treated with 3 molar equivalents of *m*CBPO in methylene chloride for 16 h at ambient temperature. The crude *meta*-chlorobenzoic acid (*m*CBA) monoester (**5**) of carnosic acid was obtained and the crude monoester was hydrolyzed with sodium hydroxide and

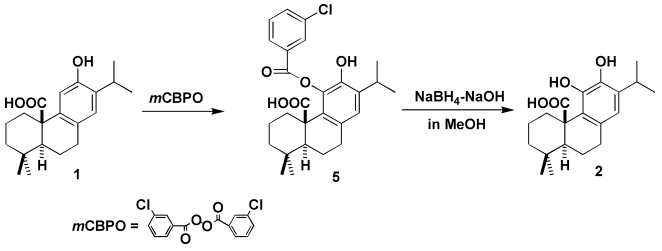

Chart 1. Synthesis of Carnosic Acid from Pisiferic Acid *via* Oxidation with *m*CBPO

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sodium borohydride ($NaBH_4$) in methanol. The products were separated by chromatography to give **2** in 11% yield from **1**. The spectral properties (^{1}H- and ^{13}C-NMR) of the synthetic carnosic acid were identical to those of the natural carnosic acid reported in the literature.[4] The yield of carnosic acid *via* oxidation with *m*CBPO was calculated to be more than 50% by ^{1}H-NMR spectroscopy, however, the isolated yield was very low (11%), due to the difficulty of separating carnosic acid from *m*CBA.

Synthesis of Carnosic Acid (2) *via* Oxidation with CAMCBPO A solution of CAMCBPO in CH_2Cl_2 was prepared as previously reported and applied to the oxidation of pisiferic acid **1** at 0 °C for 66 h.[11] The products were treated with NaOH–$NaBH_4$ in methanol as in the oxidation with *m*CBPO to give **2** (23.3 mg, 0.07 mmol, 22.4% from **1**). The yield of carnosic acid was not a considerable improvement over the previous synthesis with *m*CBPO.

Synthesis of Carnosic Acid (2) *via* Oxidation with IBX Pisiferic acid **1** was oxidized with 1.2 equivalents of IBX in $CHCl_3$–CH_3OH (4 : 1) at ambient temperature for 1 h under argon. As the product was presumed to be the unstable *ortho*-quinone (**A**), it was reduced without isolation by 10 equivalents of $NaBH_4$ under argon for 4 h. 2-Iodobenzoic acid was easily crystallized in hexane, the mother liquid was evaporated, and the residue was chromatographed to afford **2** (72%). Oxidation of pisiferic acid with IBX in DMF, followed by reduction with $NaBH_4$, produced less carnosic acid than the oxidation in $CHCl_3$–CH_3OH (4 : 1).

Synthesis of Carnosol (3) from Carnosic Acid (2) Carnosic acid **2** was oxidized with silver oxide in CH_2Cl_2 for 1.5 h at ambient temperature under Ar. The reaction mixture was filtered through Celite and the filtrate was washed with aqueous saturated $NaHCO_3$. The oxidation product, quinone methide **C**, the tautomeric isomer of *ortho*-quinone **B**, could be transformed to the lactone *via* nucleophilic addition of the carboxylic acid group to carbon C-7. After purification by column chromatography, carnosol **3** was obtained in 67% yield.

Biological Activities Minimun inhibitory concentration (MIC μg/ml) of the synthetic carnosic acid **2** and carnosol **3** were measured against *Propionibacterium acnes* (ATCC 6919)[18] and *Staphylococcus aureus* ME/GM/TC Resistant (ATCC 33592)[19] to show the potential of these compounds as antibacterial drugs. Both of the synthetic compounds showed potent antibacterial activities against *P. acnes* and *S. aureus* (Table 1). The anti-*P. acnes* activity of synthetic carnosic acid was more potent than that of the reported activity of natural carnosic acid by Weckesser *et al*.[20] As the different species of *P. acnes* might be used, it would be difficult to compare the strength of the two carnosic acid. The isolation and purification of carnosic acid and carnosol from natural plant is very difficult in general.[3] Our synthesis could afford pure carnosic acid reasonably. The MIC of well known antibiotics, Ampicillin and Vancomycin were measured as the reference compounds against *P. acnes* (ATCC 6919) and *S. aureus* respectively.

In conclusion, we synthesized the major rosemary anti-oxidant, carnosic acid, from the major constituent of Sawara, pisiferic acid, in a two-step one-pot synthesis. Carnosol, another anti-oxidant in rosemary, was synthesized from carnosic acid. The other anti-oxidant diterpenes of rosemary, rosmanol and isorosmanol, have previously been synthesized from carnosic acid.[14,15] Synthetic carnosic acid showed potent antibacterial activities against *Propionibacterium acnes* and *Staphylococcus aureus* ME/GM/TC Resistant. Thus, these syntheses provide efficient methods to synthesize the anti-oxidant diterpenes of rosemary and these syntheses also demonstrate the utility of Sawara as a natural resource.[9,10]

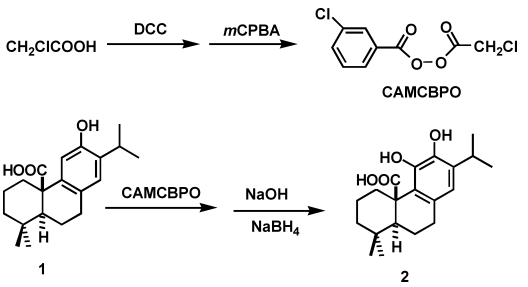

Chart 2. Synthesis of Carnosic Acid from Pisiferic Acid *via* Oxidation with CACBPO

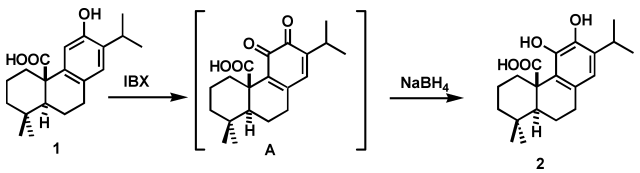

Chart 3. Synthesis of Carnosic Acid from Pisiferic Acid *via* Oxidation with IBX

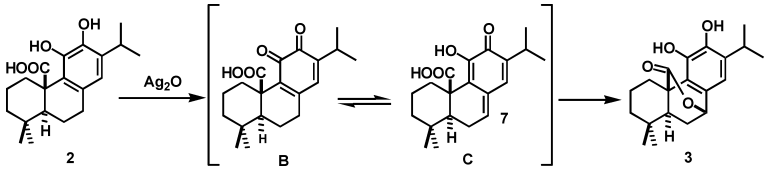

Chart 4. Synthesis of Carnosol from Carnosic Acid

Experimental

General Procedures NMR spectra were measured on a JEOL alpha-600 (^{1}H: 600 MHz, ^{13}C: 150.8 MHz) spectrometer in $CDCl_3$ using tetramethylsilane as an internal standard (*J*-values in Hz). IR spectra were meas-

Table 1. Antimicrobial Activities of Carnosic Acid and Carnosol

Compounds	*Propionibacterium acnes* (ATCC 6919) (MIC μg/ml)	*Staphylococcus aureus* ME/GM/TC Resistant (ATCC 33592) (MIC μg/ml)
Carnosic acid	1	10
Carnosol	10	30
Vancomycin	Not measured	1
Ampicillin	0.1	Not measured

ured on a JEOL JIR-WINSPEC 50 infrared spectrometer. Mass spectra were recorded on a JEOL JMS-SX102A spectrometer. Optical rotations were measured on JASCO DIP-360 polarimeter. Melting points were measured on a MEL-TEMP (Laboratory Device) and were uncorrected. TLC was carried out on Silica gel 60 (0.25 mm thickness) with fluorescent indicator (Macherey-Nagel). Silica gel (6 nm, BW-127ZH, Fuji Silysia Chemical Ltd.) was used for column chromatography.

Pisiferic Acid (1) from Sawara (*Chamaecyparis pisifera*) Leaves of *Chamaecyparis pisifera* were collected in the Fuchu campus of Tokyo University of Agriculture and Technology in January. The leaves (1.2 kg) were extracted with methanol (2.5 l) under reflux for 24 h. The extract was evaporated and the residue was extracted with ethyl acetate and water. Organic layer was evaporated and the residue was chromatographed on a short column of silica gel with hexane–ethyl acetate (3 : 1). The crude fraction of pisiferic acid was chromatographed on a silica gel column with hexane–ethyl acetate (3 : 1) again. Crystallization from ethyl acetate–hexane gave light green crystals (7.3 g, 0.6% of the leaves) of **1**. The isolated pisiferic acid; green solid; mp 174—180 °C was found to be identical with authentic pisiferic acid by the ^{1}H-NMR (600 MHz, $CDCl_3$) and ^{13}C-NMR (150 MHz, $CDCl_3$).[10]

Synthesis of Carnosic Acid (2) by Oxidation with *m*CBPO To the solution of **1** (200 mg: 0.63 mmol) in methylene chloride (10 ml), 3 equivalent molar of *m*CBPO (590 mg: 1.9 mmol) was added. The mixture was allowed at ambient temperature for 16 h. The reaction mixture was evaporated and the residue was dissolved in ethyl acetate. To the solution, hexane was added to give white crystals of *m*CBA. After filtration of the precipitate, the solution was dissolved in MeOH and treated with $NaBH_4$ at ambient temperature for 1 h. The solution was evaporated and the residue was chromatographed on a silica gel column with ethyl acetate–hexane to give a mixture of 11-carnosyl *m*-chlorobenzoate **5**, light yellow powder; ^{1}H-NMR (600 MHz, $CDCl_3$) δ: 8.20 (1H, s), 8.10 (1H, dd, J=7.5, 1.8 Hz), 7.60 (1H, dd, J=8.4, 1.8 Hz), 7.45 (1H, t, J=8.4 Hz), 6.69 (1H, s), 3.35—3.32 (1H, m), 2.97—2.86 (3H, m), 2.40—2.33 (1H, m), 1.91—1.87 (2H, m), 1.61—1.59 (2H, m), 1.52—1.48 (1H, m), 1.34—1.25 (2H, m), 1.20 (3H, d, J=6.6 Hz), 1.17 (3H, d, J=6.6 Hz), 1.01 (3H, s), 0.89 (3H, s); ^{13}C-NMR (150 MHz, $CDCl_3$) δ: 180.17, 163.70, 140.06, 136.69, 135.71, 134.81, 133.62, 130.97, 130.32, 129.94, 128.43, 124.77, 119.2, 53.82, 48.36, 41.59, 34.32, 34.29, 32.59, 31.99, 27.64, 22.94, 22.75, 21.16, 20.05, 18.54.

The monoesters mixture was dissolved in methanol (9 ml)–1% NaOH (1 ml) and then 3 equivalent molar of $NaBH_4$ (72 mg, 1.9 mmol) was added. The mixture was heated under reflux for 2 h. The reaction mixture was acidified with 1 M-HCl and extracted with brine–hexane. The organic layer was washed with brine, dried over $MgSO_4$ and evaporated. The residue was chromatographed on a silica gel column with hexane–ethyl acetate (10 : 1) to give **2** (23 mg: 0.07 mmol, 11% from pisiferic acid).

Synthesis of Carnosic Acid (2) by Oxidation with CAMCBPO A solution of chloroacetic acid (59.7 mg, 0.63 mmol) and dicyclohexyl carbodiimide (143.5 mg, 0.70 mmol) in CH_2Cl_2 (15 ml) was stirred for 15 min and then *m*CPBA (725 mg, less than 0.88 mmol, content >68%, Tokyo Kasei) was added. The solution was stirred for an additional 30 min and then pisiferic acid **1** (100 mg, 0.32 mmol) was added. After stirring at 0 °C to ambient temperature for 66 h, the reaction mixture was filtered to remove solids of dicyclohexylurea. The filtrate was evaporated and the residue was dissolved in EtOAc. The EtOAc solution was successively washed with saturated aqueous $NaHCO_3$ and brine, dried over $MgSO_4$ and evaporated. The solution was evaporated and the residue was chromatographed on a silica gel column with ethyl acetate–hexane. The product was dissolved in methanol (9 ml)–1% NaOH (1 ml) and then 3 equivalent molar of $NaBH_4$ (35.9 mg, 0.95 mmol) was added. The mixture was heated under reflux for 2 h. The reaction mixture was acidified with 1 M-HCl and extracted with brine–hexane. The organic layer was washed with brine, dried over $MgSO_4$ and evaporated. The residue was chromatographed on a silica gel column with hexane–ethyl acetate (10 : 1) to give **2** (23.3 mg, 0.07 mmol, 22.4% from pisiferic acid).

Synthesis of Carnosic Acid (2) by Oxidation with IBX To a solution of **1** (2.0 g, 6.32 mmol) in $CHCl_3$–CH_3OH (4 : 1, 30 ml), IBX (2.1 g, 7.58 mmol) was added and the solution was stirred for 1 h at ambient temperature under Ar. To the reaction mixture, $NaBH_4$ (2.4 g, 63.2 mmol) was added and stirred for 4 h at ambient temperature under Ar. The reaction was quenched by addition of 1 M HCl, extracted with hexane. The produced 2-iodobenzoic acid was easily crystallized from hexane. The hexane solution was washed with brine, dried over $MgSO_4$ and evaporated. The residue was chromatographed on a silica gel column with hexane–ethyl acetate (10 : 1) to give carnosic acid **2** (1.5 g, 4.60 mmol, 72% from pisiferic acid), yellow powder, mp 185—188 °C (crystallized from hexane–ethyl acetate); $[\alpha]_D$(MeOH)=+133 (lit. mp 185—190 °C; $[\alpha]_D$ +191).[21]

Synthesis of Carnosol (3) from Carnosic Acid (2) To a solution of **2** (60 mg, 0.18 mmol) in CH_2Cl_2 (5 ml), silver oxide (85 mg, 0.36 mmol) was added and the mixture was stirred for 1.5 h at ambient temperature under Ar. The mixture was filtered through Celite with hexane. The filtrate was washed with $NaHCO_3$, dried and evaporated. The residue was chromatographed on a silica gel column with hexane–ethyl acetate (5 : 1), to give carnosol **3** (40.2 mg, 0.12 mmol, 67%), yellow powder, mp 212—213 °C (crystallized from hexane–ethyl acetate) (lit. synthetic carnosol, 212—213 °C[15]; natural carnosol, 219.5 °C[22]).

Methods of Anti-microbial Activities *in vitro* Assays Minimun inhibitory concentration (MIC μg/ml) of the synthetic carnosic acid **2** and carnosol **3** were performed under conditions described in the literatures for each assay against *Propionibacterium acnes* (ATCC 6919)[18] and *Staphylococcus aureus* ME/GM/TC Resistant (ATCC 33592).[19] Two compounds **2** and **3** were tested at half-log concentrations ranging from 0.03 to 100 mg/ml in the *P. acnes* and *S. aureus in vitro* growth inhibition assays.

Propionibacterium acnes (ATCC 6919): Culture Medium: Reinforced Clostridial Medium, Vehicle: 1% DMSO, Incubation Time/Temp: 2 d at 37 °C, Incubation Volume: 3 ml, Time of Assessment: 2 d, Quantitation Method: Turbidity measurement and plating count of subculture.

Staphylococcus aureus ME/GM/TC Resistant (ATCC 33592): Culture Medium: Mueller-Hinton Broth, Vehicle: 1% DMSO, Incubation Time/Temp: 20 h at 37 °C, Incubation Volume: 1 ml, Time of Assessment: 1 d, Quantitation Method: Turbidity. Measurement.

Acknowledgement We are grateful to Professor M. Kuroyanagi of Hiroshima Prefectural University for providing the authentic pisiferic acid.[10]

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