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Synthesis, anti-fungal and 1,3-β-D-glucan synthase inhibitory activities of caffeic and quinic acid derivatives

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ABSTRACT

New derivatives of caffeic acid and quinic acid were synthesized and their anti-fungal and inhibitory activities on fungal 1,3-β-glucan synthase were determined in comparison with those of the corresponding chlorogenic acid derivatives. All the chlorogenic, quinic and caffeic acid derivatives that were coupled with an H_2N-orn-4-(octyloxy) aniline group (**1**, **1b** and **1c**) displayed potent activities in both anti-fungal and inhibition of 1,3-glucan synthase assays. Compounds **1** and **1c** inhibited the fungal membrane enzyme with the potency comparable to that of a known 1,3-β-D-glucan synthase inhibitor, aculeacin A. The results revealed that the anti-fungal activity of the chlorogenic acid derivative with a free amino group was at least partly due to inhibition of the fungal 1,3-β-glucan synthase. These results suggest that further investigation on caffeic acid derivatives may lead to the discovery of novel anti-fungal agents with drug-like properties.

1. Introduction

The rapid increase of life-threatening fungal infections along with the emergence of drug-resistant fungal strains demands the development of novel anti-fungal agents with new structural features or new mechanisms of action. 1,3-β-D-Glucan synthase is an essential enzyme for the synthesis of 1,3-β-glucan component of fungal cell wall. This enzyme is unique to fungi and is not found in mammalian cells, thus has been viewed as a promising target for the development of low toxic anti-fungal agents.[1,2] Some chlorogenic acid derivatives containing lipophilic chains have been reported to have potent anti-fungal activity.[3] These compounds were initially designed to mimic the pharmacophores of a well known inhibitor of fungal 1,3-β-D-glucan synthase, echinocadin. Our recent research showed that though chlorogenic acid derivatives could be absorbed as an intact molecule in vivo, the ester bond easily broke in vivo to release quinic acid derivatives into the blood. This absorption and metabolism pattern agreed with that reported for chlorogenic acid.[4] Thus, it is important to investigate if the quinic acid derivatives are active as well. It will be equally important to search for anti-fungal agents that are more stable in vivo. Caffeic and quinic acid derivatives with no ester bond in their structures are expected to be more stable in vivo than the parent chlorogenic acid derivatives. The present research was carried out to synthesize new caffeic and quinic acid derivatives and to evaluate their 1,3-β-D-glucan synthase inhibitory and anti-fungal activities.

2. Results and discussion

2.1. Synthesis of quinic acid derivatives

Compounds (**1**–**4** and **1p**) (Fig. 1) were synthesized using the previously described procedure with chlorogenic acid as starting material.[3] Aculeacin, a known 1,3-β-D-glucan synthase inhibitor, is used as a positive control in this research.

Alkaline hydrolysis of **2**–**4** and **1p** yielded the corresponding quinic acid derivatives **2b**–**4b** and the protected quinic acid derivative **1bp** which was de-protected with 90% TFA to yield **1b** (Scheme 1).

The caffeic acid derivatives (**1c**–**4c**) were synthesized by allowing 3,4-diacetoxycinnamoyl chloride to react with 4-(octyloxy) aniline or H_2N-aa-4-(octyloxy) aniline followed by de-protection (Scheme 2). The aa represents an amino acid with the functional

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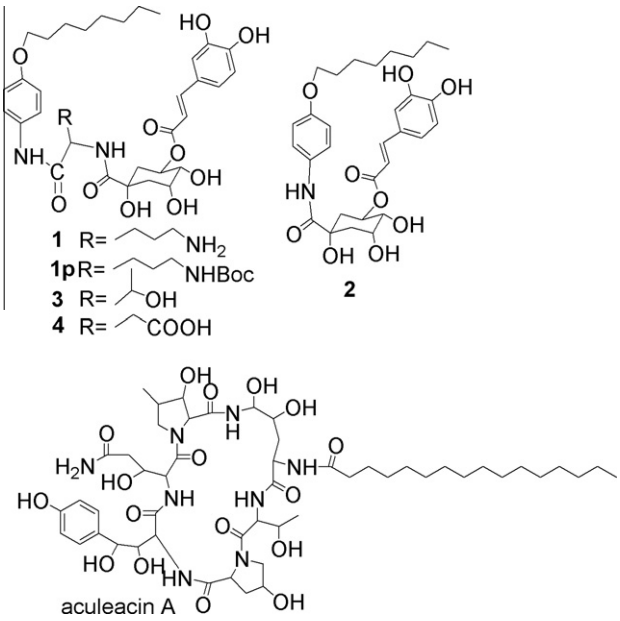


Figure 1. Structures of chlorogenic acid derivatives (**1**–**4**) and aculeacin A.

group on its side chain being protected. The H_2N-aa-4-(octyloxy) anilines were synthesized using the procedure described previously.[3]

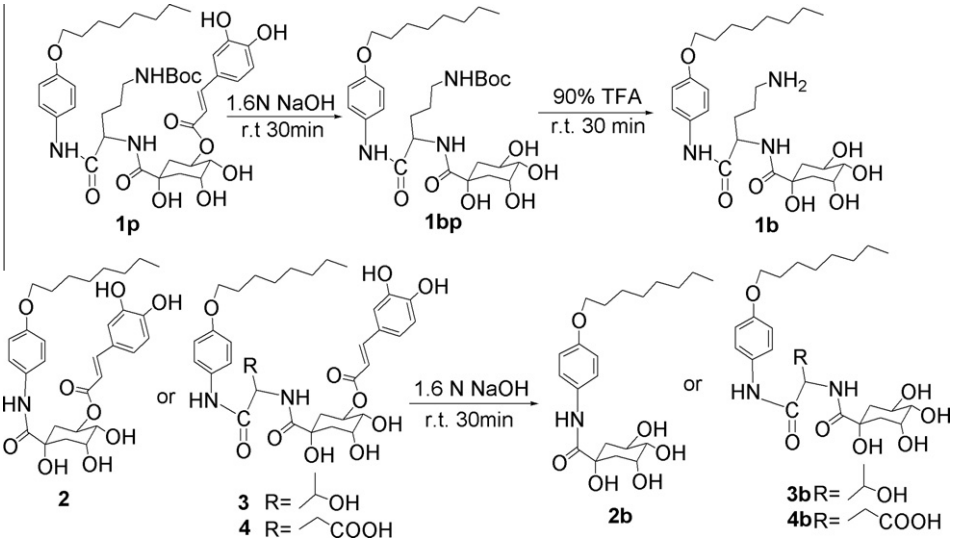
2.2. Assessment of anti-fungal and 1,3-β-D-glucan synthase inhibitory activities

The anti-fungal activity on *Candida albicans* NBRC 1594 was evaluated according to the NCCLS method.[5] The 1,3-β-D-glucan synthase assay was carried out mainly in two steps. The first step was to synthesize the glucan in the presence and absence of the testing compounds by incubating a mixture of 50 mM Tris–HCl (pH 7.5), 0.5 mM EDTA, 25 mM KF, 20 μM GTP γ-S, 0.75% BSA, 5.0 mM UDP-Glc with the membrane-bound 1,3-β-D-glucan synthase. The second step was to measure the amount of the produced glucan using a 1,3-β-D-glucan-dependent enzyme cascade reaction and a β-glucan determination kit (BGSTAR).

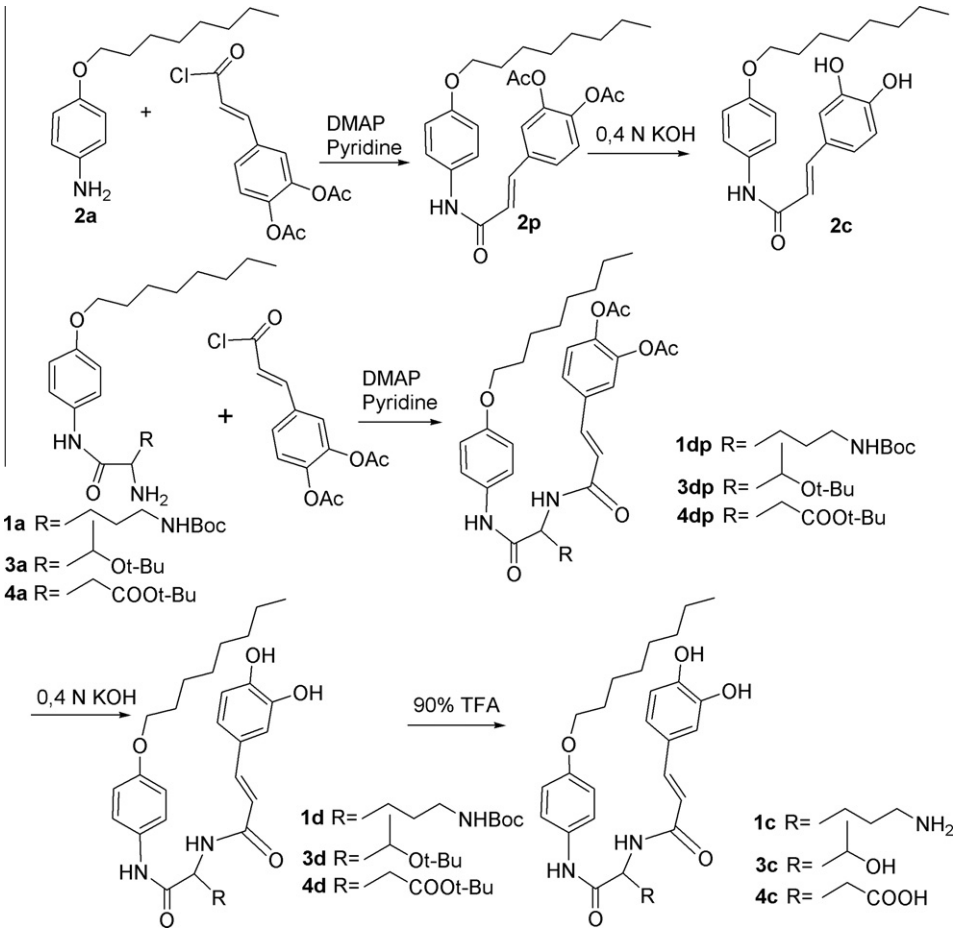
The bioassay results of the synthesized caffeic, quinic and chlorogenic acid derivatives are listed in Table 1. The inhibitory activities on 1,3-β-D-glucan synthase were shown as IC_{50} representing the concentrations that inhibited 50% of the enzyme activity. The anti-fungal activities were expressed as MIC. All the chlorogenic and caffeic acid derivatives showed anti-fungal activity. Of the three types of compounds, those with a free amino group in their structures showed stronger activities than those without a free amino group (**1** vs **2**–**4**, **1b** vs **2b**–**4b** and **1c** vs **2c**–**4c**). The strong inhibition on 1,3-β-glucan synthase of **1** revealed that the anti-fungal activity of the chlorogenic acid derivative with a free amino group was at least partly due to its inhibition on the fungal 1,3-β-glucan synthase. The 1,3-β-glucan synthase inhibitory activity of chlorogenic acid derivative (**1**) was similar to that of caffeic acid derivative (**1c**) but stronger than that of the quinic acid derivative (**1b**). The inhibitory potencies of compounds **1** and **1c** on the fungal membrane 1,3-glucan synthase were similar to that of a known 1,3-β-D-glucan synthase inhibitor, aculeacin A tested in the same experiment. The activities demonstrated by the quinic acid derivative **1b** suggested that the chlorogenic acid derivatives may also display in vivo anti-fungal activity since they release the corresponding quinic acid derivatives into blood as the major metabolites according to our recent research results and that reported for chlorogenic acid.[4] In addition, the similarity of the 1,3-β-D-glucan synthase inhibitory and cell-based anti-fungal activities of the caffeic acid derivative (**1c**) with that of chlorogenic acid derivative (**1**) suggest that further structural modification of caffeic acid derivatives may lead to the discovery of novel small molecule anti-fungal agents.

3. Conclusion

The present study provided synthetic methods for new caffeic and quinic acid derivatives linking with an amino acid residue and a lipophilic chain. Quinic acid amides can also be synthesized using quinic acid as starting material.[8,9] Due to the lower cost of quinic acid than that of chlorogenic acid, the methods described in the literatures are more economic. When chlorogenic acid is available as a starting material, the quinic acid derivatives can be produced with a shorter synthetic route. The starting material, chlorogenic acid, can be synthesized efficiently from quinic acid.[10] An alternative synthetic method for cafffeic acid amides is to use free caffeic acid to react with amines using benzotriazol-1-yloxy-



Scheme 1. Synthesis of quinic acid derivatives.



Scheme 2. Synthesis of caffeic acid derivatives.

Table 1
Anti-fungal and 1,3-β-D-glucan synthase inhibitory activities

Compound	IC_{50} (μg/ml)	MIC[a] (μg/ml)	IC_{50} (μM)	MIC[a] (μM)
1	10	4	15	6
2	23	8	41	14
3	>32	8	>49	12
4	>32	32	>48	48
1b	27	8	53	16
2b	>32	32	>81	81
3b	>32	>64	>65	>129
4b	>32	>64	>63	>125
1c	11	2	22	4
2c	>32	32	>84	84
3c	>32	4	>66	8.3
4c	>32	32	>64	64
1d	31	16	52	27
3d	20	8	37	15
4d	32	16	58	29
Chl	>32	>64	>90	>181
A	9	0.25	8	0.23

Chl: chlorogenic acid.
A: aculeacin A.
[a] The MIC values were determined by NCCLS method using *Candida albicans* NBRC 1594.

tris(dimethylamino)phosphonium hexafluorophosphate (BOP) as the coupling reagent.[11,12]

Among the compounds described in this paper, all those with an ornithine residue in their structures exhibited stronger cell-based anti-fungal and enzyme inhibitory activities. The quinic acid derivatives are considered as the in vivo metabolites of the corresponding chlorogenic acid derivatives which were previously reported to have strong anti-fungal activity in vitro.[3] The caffeic and quinic acid derivatives are expected to be more stable in vivo than the corresponding chlorogenic acid derivatives since as a metabolic rule, amides are more stable to esterase hydrolysis than are esters.[13] The promising in vitro anti-fungal and enzyme inhibitory activities of the caffeic acid derivatives, along with their possible in vivo metabolic stability suggest the potential of these compounds as new leads for the discovery of novel small molecule anti-fungal agents. Further structural optimization on caffeic acid derivatives and the investigation of their metabolic and pharmacokinetic profiles are planned to be carried out in our group, shortly.

4. Experimental

4.1. Materials

The β-glucan determination kit (BGSTAR) was purchased from Wako, Osaka, Japan. The chemical reagents were purchased from Sigma-Aldrich, Inc. Column chromatography was carried out on Wakogel 50C18 (38–63 μm, Wako Pure Chemical Industries, Ltd). Preparative HPLC was performed on a Tosoh CCPM-II system (Tosoh Co., Tokyo) equipped with a UV 8020 detector and a 5C18-AR-II waters HPLC column (20 × 200 mm). NMR spectra were measured on a Varian Unity 500 (^{1}H, 500 MHz; ^{13}C, 125 MHz) or a Varian Gemini 300 (^{13}C, 75 MHz) NMR spectrometer. TMS was used as an internal standard and *J* values were reported in Hertz. Electrospray ionization mass (ESI-MS) spectra were obtained on an Esquire 3000^{plus} spectrometer (Bruker

Daltonik GmbH, Bremen, Germany). High-resolution-FAB-MS spectra were measured on a Jeol JMS-700 with a resolution of 5000 using *m*-nitrobenzyl alcohol as the matrix. The purities of synthesized compounds were verified with ^{1}H NMR, ^{13}C NMR and HPLC.

4.2. General method for the synthesis of quinic acid derivatives (1b–4b)

Compounds **1p** and **2–4** were synthesized using the method described previously.[3] To a methanol solution (6 ml) of compound **1p** or **2–4** (0.05 mmol) was added 4 ml of 4 M NaOH. The mixture was stirred at rt for 30 min, neutralized with 1 M HCl and condensed under reduced pressure. The remaining mixture was purified by chromatography on ODS or on SiO_2 to obtain **1bp**, and **2b–4b**.

4.2.1. Compound 1bp

After being synthesized from **1p** and purified on an ODS column, **1bp** was obtained from the MeOH–H_2O (8:2) eluted part (yield: 91%).^{1}H NMR ($CDCl_3$) δ 0.87 (t, J = 6.5 Hz, 3H, –$(CH_2)_7CH_3$), 1.28 (19H, m) and 1.49 (2H, m) and 1.70 (3H, m) and 1.85–2.08 (5H, m) (–$CH_2(CH_2)_6CH_3$ and Boc and H2 and H6 and H-γ and H-β), 2.85 (2H, m, H-δ), 3.51 (1H, br s, H-4), 3.84 (2H, t, J = 6.5 Hz, –$CH_2(CH_2)_6CH_3$), 4.10 (1H, m, H-5), 4.19 (1H, m, H-3), 4.88 (1H, br s, H-α), 6.73 (2H, d, J = 8.5 Hz, H-3′,5′), 7.37 (2H, d, J = 8.5 Hz, H-2′,6′) and some signals for the OH and NH groups. ^{13}C NMR ($CDCl_3$, 75 MHz) δ 14.2 (–$(CH_2)_7CH_3$), 22.7 and 26.1 and 28.5 and 29.3 and 29.4 and 29.5 and 29.8 and 30.2 and 31.9 (–$CH_2(CH_2)_6CH_3$ and Boc-CH_3 and C-γ), 37.3 (C-2), 39.3 (C-β), 41.4 (C-6), 52.6 (C-α), 67.1 (C-5), 68.2 (–$OCH_2(CH_2)_6CH_3$), 71.0 (C-3), 75.8 (C-4), 79.3 (C-1), 114.5 (C-3′,5′), 121.9 (C-2′,6′), 130.5 (C-1′), 155.9 (Boc-C), 156.7 (C-4′), 170.2 (–PhNHCO–), 175.1 (C-7). ESI-MS (Negative): 608.4 ([M−H]$^-$, 10%).

Compound **1bp** was treated with 90% TFA at rt for 30 min. The mixture was concentrated to dryness followed by the addition of 2 ml of H_2O. The pH of the mixture was then adjusted to 10 by NH_4OH, followed by extraction with EtOAc. The EtOAc layer was concentrated to dryness to obtain **1b**.

4.2.2. Compound 1b

Yield: 45%, ^{1}H NMR (CD_3OD) δ 0.93 (t, J = 6.5 Hz, 3H, –$(CH_2)_7CH_3$), 1.36 (9H, m) and 1.47 (2H, m) and 1.55 (2H, m) and 1.76 (3H, m) and 1.99 (4H, m) (–$CH_2(CH_2)_6CH_3$ and H2 and H6 and H-γ and H-β), 2.82 (2H, m, H-δ), 3.41 (1H, dd, J = 3.5, 9.0 Hz, H-4), 3.96 (2H, t, J = 6.5 Hz, –$CH_2(CH_2)_6CH_3$), 4.04 (1H, m, H-5), 4.17 (1H, m, H-3), 4.51 (1H, dd, J = 5.5, 8.0 Hz, H-α), 6.88 (2H, d, J = 8.5 Hz, H-3′,5′), 7.45 (2H, d, J = 8.5 Hz, H-2′,6′). ^{13}C NMR (CD_3OD, 75 MHz) δ 14.5 (–$(CH_2)_7CH_3$), 23.7 and 27.2 and 27.8 and 30.4 and 30.5 and 31.2 and 33.0 (–$CH_2(CH_2)_6CH_3$ and C-γ), 38.6 (C-2), 41.8 (C-β, C-δ), 42.6 (C-6), 54.3 (C-α), 68.1 (C-5), 69.2 (–$OCH_2(CH_2)_6CH_3$), 72.1 (C-3), 76.9 (C-4), 78.0 (C-1), 115.5 (C-3′,5′), 123.0 (C-2′,6′), 131.9 (C-1′), 157.4 (C-4′), 171.4 (–PhNHCO–), 176.9 (C-7). ESI-MS (Positive): 510.3 ([M+H]$^-$, 100%); ESI-MS (Negative): 544.6 ([M+Cl]$^-$, 100%). HR-FAB-MS [M+H]$^+$ *m/z* 510.31879 (calcd for $C_{26}H_{44}O_7N_3$, requires 510.31792).

4.2.3. Compound 2b

After synthesis from **2** and purification on a SiO_2 column, **2b** was obtained from the EtOAc eluted part (yield: 95%). ^{1}H NMR (CD_3OD) δ 0.91 (3H, t, J = 6.5 Hz, –$(CH_2)_7CH_3$), 1.35 (8H, m) and 1.46 (2H, m) and 1.75 (2H, m) (–$CH_2(CH_2)_6CH_3$), 2.05 (4H, m, H-2 and H-6), 3.42 (1H, dd, J = 3.5, 9.0 Hz, H-4), 3.95 (2H, t, J = 6.5 Hz, –$CH_2(CH_2)_6CH_3$), 4.04 (1H, m, H-5), 4.17 (1H, m, H-3), 6.87 (2H, d, J = 8.5 Hz, H-3′,5′), 7.45 (2H, d, J = 8.5 Hz, H-2′,6′). ^{13}C NMR (CD_3OD, 75 MHz) δ 14.5 (–$(CH_2)_7CH_3$), 23.8 and 27.2 and 30.4 and 30.5 and 33.0 (–$CH_2(CH_2)_6CH_3$), 38.8 (C-2), 42.6 (C-6), 68.1 (C-5), 69.2 (–$OCH_2(CH_2)_6CH_3$), 72.3 (C-3), 77.0 (C-4), 78.1 (C-1), 115.4 (C-3′,5′), 123.3 (C-2′,6′), 131.6 (C-1′), 157.5 (C-4′), 175.0 (C-7). ESI-MS (Positive): 396.1 ([M+H]$^-$, 100%); ESI-MS (Negative): 430.5 ([M+Cl]$^-$, 100%). HR-FAB-MS [M+H]$^+$ *m/z* 396.23741 (calcd for $C_{21}H_{34}O_6N$, requires 396.23861).

4.2.4. Compound 3b

After synthesis from **3** and purification on a SiO_2 column, **3b** was obtained from the EtOAc–MeOH (8:2) eluted part (yield: 94%).^{1}H NMR (CD_3OD) δ 0.90 (3H, t, J = 6.5 Hz, –$(CH_2)_7CH_3$), 1.21 (3H, d, J = 6.0 Hz, Thr-CH_3), 1.35 (10H, m) and 1.73 (3H, m) and 2.02 (3H, m) (–$CH_2(CH_2)_6CH_3$), H-2 and H-6), 3.42 (1H, br d, J = 9.0 Hz, H-4), 3.95 (2H, t, J = 6.5 Hz, –$CH_2(CH_2)_6CH_3$), 4.04 (1H, m, H-5), 4.17 (1H, br s, H-α), 4.20 (1H, m, H-3), 4.39 (1H, m, H-β), 6.86 (2H, d, J = 8.5 Hz, H-3′,5′), 7.41 (2H, d, J = 8.5 Hz, H-2′,6′). ^{13}C NMR (CD_3OD + $CDCl_3$, 125 MHz) δ 14.5 (–$(CH_2)_7CH_3$), 20.3 and 23.7 and 27.2 and 30.4 and 30.5 and 33.0 (–$CH_2(CH_2)_6CH_3$ and Thr-CH_3), 38.8 (C-2), 42.6 (C-6), 59.1 (C-α), 67.3 (C-β, 68.7 (C-5), 69.2 (–$OCH_2(CH_2)_6CH_3$), 72.3 (C-3), 77.0 (C-4), 78.1 (C-1), 115.4 (C-3′,5′), 123.3 (C-2′,6′), 131.6 (C-1′), 157.5 (C-4′), 171.6 (–PhNHCO–), 176.0 (C-7). ESI-MS (Positive): 497.2 ([M+H]$^-$, 100%); ESI-MS (Negative): 531.5 ([M+Cl]$^-$, 100%). HR-FAB-MS [M+H]$^+$ *m/z* 497.28642 (calcd for $C_{25}H_{41}O_8N_2$, requires 497.28628).

4.2.5. Compound 4b

After synthesis from **4** and purification on an ODS column, **4b** was obtained from the MeOH–H_2O (8:2) eluted part (yield: 93%). ^{1}H NMR (CD_3OD) δ 0.91 (t, J = 6.5 Hz, 3H, –$(CH_2)_7CH_3$), 1.35 (8H, m) and 1.46 (2H, m) and 1.75 (2H, m) (–$CH_2(CH_2)_6CH_3$), 1.90 (1H, dd, J = 11.0, 13.5 Hz, H-6a), 2.00 (3H, m, H-6b and H-2), 2.83 (2H, m, H-β (–CH_2COOH)), 3.38 (1H, dd, J = 3.5, 9.0 Hz, H-4), 3.95 (2H, t, J = 6.5 Hz, –$CH_2(CH_2)_6CH_3$), 4.01 (1H, m, H-5), 4.14 (m, 1H, H-3), 4.78 (1H, t, J = 6.5 Hz, H-α), 6.86 (2H, d, J = 8.5 Hz, H-3′,5′), 7.41 (2H, d, J = 8.5 Hz, H-2′,6′). ^{13}C NMR (CD_3OD, 75 MHz) δ 14.5 (–$(CH_2)_7CH_3$), 23.8 and 27.2 and 30.5 and 33.0 (–$CH_2(CH_2)_6CH_3$), 38.0 (C-β), 38.6 (C-2), 42.4 (C-6), 51.8 (C-α), 68.1 (C-5), 69.2 (–$OCH_2(CH_2)_6CH_3$), 72.1 (C-3), 77.0 (C-4), 78.0 (C-1), 115.5 (C-3′,5′), 123.2 (C-2′,6′), 132.0 (C-1′), 157.4 (C-4′), 170.7 (–PhNHCO–), 174.4 (C-7), 177.0 (COOH). ESI-MS (Positive): 511.2 ([M+H]$^-$, 100%); ESI-MS (Negative): 545.5 ([M+Cl]$^-$, 100%). HR-FAB-MS [M+H]$^+$ *m/z* 511.26470 (calcd for $C_{25}H_{39}O_9N_2$, requires 511.26553).

4.3. General method for the synthesis of protected caffeic acid derivatives (1dp, 2p, 3dp and 4dp)

To a solution of **1a–4a** (1.5 mmol) and DMAP (68 mg) in CH_2Cl_2 (10 ml), pyridine (4 ml) and 3,4-diacetoxycinnamoyl chloride (3.5 mmol) were added at room temperature. The reaction mixture was stirred overnight and then acidified with 1 M HCl to pH 3. The aqueous phase was re-extracted with CH_2Cl_2. The combined organic extracts were concentrated and purified by SiO_2 column chromatography eluted with hexane–EtOAc to yield the products.

4.3.1. Compound 1dp

After synthesis from **1a** and purification on SiO_2 column, **1dp** was eluted out with hexane–EtOAc (2:8) (yield: 88%). ^{1}H NMR ($CDCl_3$) δ 0.88 (3H, t, J = 6.5 Hz, –$(CH_2)_7CH_3$), 1.25 (8H, m) and 1.64–1.84 (6H, m) and 2.32 (2H, m) (–$CH_2(CH_2)_6CH_3$ and H-β and H-γ), 1.37 (9H, m, H-Boc), 2.27 (3H, s) and 2.28 (3H, s) (2 × –$COCH_3$), 3.13 (1H, m) and (3.35 (1H, m) (H-δ), 3.87 (2H, t, J = 6.5 Hz, –$CH_2(CH_2)_6CH_3$), 4.99 (1H, br s, H-α), 6.48 (1H, d, J = 15.5 Hz, H-8), 6.77 (2H, J = 8.5 Hz, H-3′,5′), 7.10 (d, J = 8.5, H-5), 7.26 (1H, d, J = 2.5 Hz, H-2), 7.40 (3H, m, H-2′,6′,6), 7.50 (1H, d, J = 15.5 Hz, H-7), and NH signal. ^{13}C NMR ($CDCl_3$, 125 MHz) δ 14.2 (–$(CH_2)_7CH_3$), 20.6 and 20.7 and 22.7 and 26.1 and 26.7 and 28.5 and 29.3 and 29.4 and 30.0 and 31.8

(–$CH_2(CH_2)_6CH_3$ and Boc-CH_3 and C-β and C-γ and 2 × –$COCH_3$), 39.4 (C-δ), 53.0 (C-α), 68.2 (–$OCH_2(CH_2)_6CH_3$), 79.3 (Boc-C), 114.5 (C-3′,5′), 121.4 (C-6), 121.8 (C-2′,6′), 122.4 (C-8), 123.7 (C-2), 126.0 (C-5), 130.5 (C-1), 133.4 (C-1′), 139.5 (C-7), 142.1 (C-4), 142.8 (C-3), 155.8 (C-4′), 156.8 (Boc-CO), 165.8 (C-9), 167.8 and 167.9 (2 × –COCH), 170.1 (Orn-CO).

4.3.2. Compound 2p

After synthesis from **2a** and purification on a SiO_2 column, **2p** was obtained from hexane–EtOAc (6:4) eluted part (yield: 91%).^{1}H NMR ($CDCl_3$) δ 0.89 (t, *J* = 6.5 Hz, 3H, –$(CH_2)_7CH_3$), 1.30 (8H, m) and 1.44 (2H, m) and 1.77 (2H, m) (–$CH_2(CH_2)_6CH_3$), 2.31 (6H, s, 2 × –$COCH_3$), 3.94 (2H, t, *J* = 6.5 Hz, –$CH_2(CH_2)_6CH_3$), 6.43 (1H, d, *J* = 15.5 Hz, H-8), 6.87 (2H, d, *J* = 8.5 Hz, H-3′,5′), 7.20 (1H, d, *J* = 8.0 Hz, H-5), 7.38 (2H, m, H-2,6), 7.50 (2H, d, *J* = 8.5 Hz, H-2′,6′), 7.66 (1H, d, *J* = 15.5 Hz, H-7). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 14.1 (–$(CH_2)_7CH_3$), 20.6 and 22.6 and 26.0 and 29.2 and 29.3 and 31.8 (–$CH_2(CH_2)_6CH_3$ and 2 × –$COCH_3$), 68.3 (–$OCH_2(CH_2)_6CH_3$), 114.8 (C-3′,5′), 121.6 (C-2′,6′), 122.3 (C-8), 122.4 (C-1), 123.8 (C-2), 126.3 (C-5), 130.9 (C-1′), 122.8 (C-6), 139.7 (C-7), 142.3 (C-4), 142.9 (C-3), 156.1 (C-4′), 163.3 (C-9), 168.2 (2 × –$COCH_3$).

4.3.3. Compound 3dp

After synthesis from **3a** and purification on a SiO_2 column, **3dp** was eluted out with hexane–EtOAc (1:1) (yield: 89%). ^{1}H NMR ($CDCl_3$) δ 0.89 (3H, t, *J* = 6.5 Hz, –$(CH_2)_7CH_3$), 1.08 (3H, d, *J* = 6.5 Hz, H-γ), 1.31 (8H, m) and 1.43 (2H, m) and 1.76 (2H, m) (–$CH_2(CH_2)_6CH_3$), 1.38 (9H, s, *t*Bu-CH_3), 2.28 (3H, s) and 2.29 (3H, s) (2 × –$COCH_3$), 3.90 (2H, t, *J* = 6.5 Hz, –$CH_2(CH_2)_6CH_3$), 4.35 (1H, m, H-β), 4.63 (1H, br s, H-α), 6.54 (1H, d, *J* = 15.5 Hz, H-8), 6.85 (1H, d, *J* = 8.5 Hz, H-3′,5′), 7.19 (2H, m, H-2,5), 7.41 (1H, dd, *J* = 2.0, 8.5 Hz, H-6), 7.39 (2H, d, *J* = 8.5 Hz, H-2′,6′), 7.57 (1H, d, *J* = 15.5 Hz, H-7). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 14.0 (–$(CH_2)_7CH_3$), 16.8 and 20.4 and 20.5 and 22.5 and 25.9 and 28.0 and 29.1 and 29.2 and 31.7 (–$CH_2(CH_2)_6CH_3$, *t*Bu-CH_3 and 2 × –$COCH_3$), 57.8 (C-α), 66.4 (C-β), 68.1 (–$OCH_2(CH_2)_6CH_3$), 114.7 (C-3′,5′), 121.1 (C-2′,6′), 121.4 (C-6), 122.3 (C-8), 123.7 (C-2), 126.1 (C-5), 130.4 (C-1), 133.5 (C-1′), 139.3 (C-7), 142.2 (C-4), 142.9 (C-3), 155.8 (C-4′), 165.2 (C-9), 167.2 (Thr-CO), 167.9 (2 × –$COCH_3$).

4.3.4. Compound 4dp

After synthesis from **4a** and purification on a SiO_2 column, **4dp** was eluted out with hexane–ethyl acetate (1:1) (yield: 90%). $[\alpha]_D$ +20.9 (*c* 0.2, MeOH); ^{1}H NMR ($CDCl_3$) δ 0.88 (3H, t, *J* = 6.5 Hz, –$(CH_2)_7CH_3$), 1.29 (8H, m) and 1.45 (11H, m) and 1.76 (2H, m) (–$CH_2(CH_2)_6CH_3$ and *t*-Bu), 2.30 (6H, s) (2 × –$COCH_3$), 2.69 (1H, dd, *J* = 7.0, 16.5 Hz) and 2.97 (1H, dd, *J* = 4.5, 16.5 Hz) (H-β), 3.90 (2H, t, *J* = 6.5 Hz, –$CH_2(CH_2)_6CH_3$), 5.00 (1H, m, H-α), 6.40 (1H, d, *J* = 15.5 Hz, H-8), 6.83 (2H, d, *J* = 8.5 Hz, H-3′,5′), 7.20 (1H, d, *J* = 8.0 Hz, H-5), 7.26 (1H, d, *J* = 2.0 Hz, H-2), 7.33 (1H, dd, *J* = 2.0, 8.0 Hz, H-6), 7.39 (2H, d, *J* = 8.5 Hz, H-2′,6′), 7.59 (1H, d, *J* = 15.5 Hz, H-7). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 14.1 (–$(CH_2)_7CH_3$), 20.6 and 22.6 and 26.0 and 28.0 and 29.2 and 29.3 and 31.8 (–$CH_2(CH_2)_6CH_3$, *t*-Bu-CH_3 and 2 × –$COCH_3$), 36.5 (C-β), 50.2 (C-α), 68.1 (–$OCH_2(CH_2)_6CH_3$), 82.1 (*t*Bu-C), 114.7 (C-3′,5′), 120.9 (C-6), 121.6 (C-2′,6′), 122.5 (C-8), 123.9 (C-2), 126.3 (C-5), 130.5 (C-1), 133.4 (C-1′), 140.3 (C-7), 142.4 (C-4), 143.2 (C-3), 156.0 (C-4′), 165.7 (C-9), 168.1 (Asp-CONH, 2 × –$COCH_3$), 171.8 (Asp-COO*t*Bu).

4.4. General method for the synthesis of 1d, 2c, 3d and 4d

Compound **1dp**, **2p**, **3dp** or **4dp** (0.5 mmol) was dissolved in THF (1.5 ml) and MeOH 1.5 ml. To the mixture was added 0.2 M KOH (H_2O–MeOH 9:1 solution, 0.8 ml). The mixture was stirred at rt for 1 h followed by the addition of 1 M HCl (0.5 ml). The mixture was partitioned with $CHCl_3$ and H_2O. The $CHCl_3$ layer was concentrated to dryness to obtain the products.

4.4.1. Compound 1d

Yield: 93%, ^{1}H NMR ($CDCl_3$) δ 0.88 (3H, t, *J* = 6.5 Hz, –$(CH_2)_7CH_3$), 1.25–1.93 (25 H, m) (–$CH_2(CH_2)_6CH_3$ and H-β and H-γ and H-Boc), 3.09 (1H, m) and 3.35 (1H, m) (H-δ), 3.87 (2H, t, *J* = 6.5 Hz, –$CH_2(CH_2)_6CH_3$), 4.81 (1H, br s, H-α), 6.20 (1H, d, *J* = 15.5 Hz, H-8), 7.72 (2H, br s, H-5,6), 6.77 (2H, d, *J* = 8.5 Hz, H-3′,5′), 6.93 (1H, br s, H-2), 7.38 (1H, d, *J* = 15.5 Hz, H-7), 7.39 (2H, d, *J* = 8.5 Hz,, H-2′,6′), and NH signals. ^{13}C NMR ($CDCl_3$, 125 MHz) δ 14.2 (–$(CH_2)_7CH_3$), 22.7 and 26.1 and 26.6 and 28.4 and 29.3 and 29.5 and 29.8 and 31.9 (–$CH_2(CH_2)_6CH_3$ and Boc-CH_3 and C-β and C-γ), 39.5 (C-δ), 53.5 (C-α), 68.2 (–$OCH_2(CH_2)_6CH_3$), 79.6 (Boc-C), 114.3 (C-3′,5′), 115.5 (C-2, 8), 116.7 (C-6), 122.3 (C-2′,6′,5), 126.9 (C-1), 130.1 (C-1′), 142.2 (C-7), 144.1 (C-4), 146.6 (C-3), 156.0 (C-4′), 156.8 (Boc-CO), 167.6 (C-9), 171.1 (Orn-CO). ESI-MS (Positive): 598.2 ([M+H]$^-$, 100%); ESI-MS (Negative): 632.6 ([M+Cl]$^-$, 100%). HR-FAB-MS [M+H]$^+$ *m/z* 598.35019 (calcd for $C_{33}H_{48}O_7N_3$, requires 598.34922).

4.4.2. Compound 2c

Yield: 97%, ^{1}H NMR ($CDCl_3$ + CD_3OD) δ 0.89 (3H, t, *J* = 6.5 Hz, –$(CH_2)_7CH_3$), 1.30 (8H, m) and 1.44 (2H, m) and 1.77 (2H, m) (–$CH_2(CH_2)_6CH_3$), 3.93 (2H, t, *J* = 6.5 Hz, –$CH_2(CH_2)_6CH_3$), 6.43 (1H, d, *J* = 15.5 Hz, H-8), 6.80 (1H, d, *J* = 7.5 Hz, H-6), 6.85 (2H, d, *J* = 8.5 Hz, H-3′,5′), 6.92 (1H, d, *J* = 7.5 Hz, H-5), 7.05 (1H, br s, H-2), 7.51 (3H, m, H-2′,6′,7). ^{13}C NMR ($CDCl_3$ + CD_3OD, 125 MHz) δ 13.7 (–$(CH_2)_7CH_3$), 22.4 and 25.8 and 29.0 and 29.1 and 31.6 (–$CH_2(CH_2)_6CH_3$), 68.1 (–$OCH_2(CH_2)_6CH_3$), 113.6 (C-2), 114.3 (C-3′,5′), 114.9 (C-6), 117.5 (C-8), 121.1 (C-5), 121.3 (C-2′,6′), 126.9 (C-1), 131.2 (C-1′), 141.3 (C-7), 144.4 (C-4), 146.6 (C-3), 155.3 (C-4′), 165.2 (C-9). ESI-MS (Positive): 384.2 ([M+H]$^-$, 100%); ESI-MS (Negative): 382.3 ([M−H]$^-$, 100%). HR-FAB-MS [M+H]$^+$ *m/z* 384.21764 (calcd for $C_{23}H_{30}O_4N$, requires 384.21748).

4.4.3. Compound 3d

Synthesis from **3dp** (yield: 89%), ^{1}H NMR ($CDCl_3$) δ 0.88 (t, *J* = 6.5 Hz, 3H, –$(CH_2)_7CH_3$), 1.07 (3H, d, *J* = 6.0 Hz, H-Thr-CH_3), 1.34 (m, 17H) and 1.61 (m, 2H) and 1.77 (m, 2H) (–$CH_2(CH_2)_6CH_3$ and H-*t*-Bu), 3.78 (2H, br s, –$CH_2(CH_2)_6CH_3$), 4.35 (1H, m, H-β), 4.62 (1H, t, *J* = 5.0 Hz, H-α), 6.31 (1H, d, *J* = 15.5 Hz, H-8), 6.88 (3H, m, H-6, 3′,5′), 6.94 (1H, d, *J* = 8.0 Hz, H-5), 7.02 (1H, d, *J* = 3.0 Hz, H-2), 7.38 (2H, d, *J* = 8.5 Hz, H-2′,6′), 7.54 (1H, d, *J* = 15.5 Hz, H-7) and some NH signals. ^{13}C NMR ($CDCl_3$, 125 MHz) δ 14.2 (–$(CH_2)_7CH_3$), 17.7 and 18.2 and 22.9 and 26.3 and 28.4 and 29.5 and 29.6 and 32.7 (–$CH_2(CH_2)_6CH_3$ and *t*Bu-CH_3 and Thr-CH_3), 58.4 (C-α), 67.2 (C-β), 68.6 (–$OCH_2(CH_2)_6CH_3$), 76.4 (C-*t*-Bu), 114.5 (C-3′,5′), 115.1 (C-2), 115.7 (C-8), 117.0 (C-6), 122.0 (C-2′,6′,5), 127.3 (C-1), 130.4 (C-1′), 142.8 (C-7), 144.6 (C-4), 147.2 (C-3), 156.5 (C-4′), 167.6 (C-9), 168.3 (Thr-CO). ESI-MS (Positive): 541.2 ([M+H]$^-$, 100%); ESI-MS (Negative): 575.5 ([M+Cl]$^-$, 100%). HR-FAB-MS [M+H]$^+$ *m/z* 541.29310 (calcd for $C_{31}H_{45}O_6N_2$, requires 541.32775).

4.4.4. Compound 4d

Synthesis from **4dp** (yield: 95%). ^{1}H NMR ($CDCl_3$) δ 0.87 (t, *J* = 6.5 Hz, 3H, –$(CH_2)_7CH_3$), 1.27 (m, 8H) and 1.32 (m, 11H) and 1.67 (m, 2H) (–$CH_2(CH_2)_6CH_3$ and *t*-Bu), 2.87 (2H, m, H-β), 3.81 (2H, t, *J* = 6.5 Hz, –$CH_2(CH_2)_6CH_3$), 5.11(1H, m, H-α), 6.16 (1H, d, *J* = 15.5 Hz, H-8), 6.68 (4H, m, H-5, 6, 3′,5′), 6.88 (1H, br s, H-2), 7.29 (2H, d, *J* = 8.5 Hz, H-2′,6′), 7.34 (1H, d, *J* = 15.5 Hz, H-7), and some NH signals. ^{13}C NMR ($CDCl_3$, 125 MHz) δ 14.1 (–$(CH_2)_7CH_3$), 22.6 and 26.0 and 28.0 and 29.2 and 29.4 and 31.8 (–$CH_2(CH_2)_6CH_3$ and *t*Bu-CH_3), 37.4 (C-β), 50.5 (C-α), 68.2 (–$OCH_2(CH_2)_6CH_3$), 82.3 (*t*Bu-C), 114.0 (C-2), 114.6 (C-3′,5′), 115.4 (C-8), 116.4 (C-6),

122.4 (C-2′,6′,5), 126.9 (C-1), 129.9 (C-1′), 142.7 (C-7), 144.1 (C-4), 146.8 (C-3), 156.4 (C-4′), 167.5 (C-9), 169.5 (ASP-*C*OO*t*Bu), 171.1 (Asp-*C*ONH). ESI-MS (Positive): 555.2 ([M+H]$^-$, 100%). HR-FAB-MS [M+H]$^+$ *m/z* 555.30644 (calcd for $C_{31}H_{43}O_7N_2$, requires 555.30703).

4.5. General method for the synthesis of 1c, 3c and 4c

Compound **1d**, **3d** or **4d** (0.5 mmol) was treated with 90% TFA at rt for 30 min. The mixture was concentrated to dryness to obtain **1c**, **3c** or **4c**.

4.5.1. Compound 1c

Yield: 92%, ^{1}H NMR ($CDCl_3$ + CD_3OD) δ 0.87 (3H, t, *J* = 6.5 Hz, –$(CH_2)_7CH_3$), 1.26 (8H, m) and 1.38 (2H, m) and 1.69 (5H, m) and 1.89 (1H, m) (–$CH_2(CH_2)_6CH_3$ and H-β and H-γ), 2.90 (2H, br s, H-δ), 3.84 (2H,t, *J* = 6.5 Hz, –$CH_2(CH_2)_6CH_3$), 4.59 (1H, br s, H-α), 6.28 (1H, d, *J* = 15.5 Hz, H-8), 6.69 (2H, br s, H-5,6), 6.75 (2H, d, *J* = 8.5 Hz, H-3′,5′), 6.97 (1H, br s, H-2), 7.28 (1H, d, *J* = 15.5 Hz, H-7), 7.35 (2H, d, *J* = 8.5 Hz, H-2′,6′), and some OH and NH signals. ^{13}C NMR ($CDCl_3$ + CD_3OD, 75 MHz) δ 14.0 (–$(CH_2)_7CH_3$), 22.6 and 23.6 and 26.0 and 29.2 and 29.3 and 31.8 (–$CH_2(CH_2)_6CH_3$ and C-β and C-γ), 39.1 (C-δ), 53.4 (C-α), 68.2 (–$OCH_2(CH_2)_6CH_3$), 113.8 (C-2), 114.4 (C-3′,5′), 115.3 (C-6), 116.2 (C-8), 122.0 (C-2′,6′), 122.1 (C-5), 126.7 (C-1), 129.9 (C-1′), 142.1 (C-7), 144.3 (C-4), 146.8 (C-3), 156.0 (C-4′), 167.8 (C-9), 170.2 (Orn-*C*O). ESI-MS (Positive): 498.3 ([M+H]$^-$, 100%); ESI-MS (Negative): 532.6 ([M+Cl]$^-$, 100%). HR-FAB-MS [M+H]$^+$ *m/z* 498.29615 (calcd for $C_{28}H_{40}O_5N_3$, requires 498.29679).

4.5.2. Compound 3c

Yield: 98%, ^{1}H NMR ($CDCl_3$ + CD_3OD) δ 0.88 (3H, t, *J* = 6.5 Hz, –$(CH_2)_7CH_3$), 1.23 (3H, d, *J* = 6.5 Hz, H-γ), 1.28 (8H, m) and 1.40 (2H, m) and 1.71 (2H, m) (–$CH_2(CH_2)_6CH_3$), 3.86 (2H, t, *J* = 6.5 Hz, –$CH_2(CH_2)_6CH_3$), 4.36 (1H, m, H-β), 4.65 (1H, br s, H-α), 6.32 (1H, d, *J* = 15.5 Hz, H-8), 6.74 (1H, d, *J* = 8.5 Hz, H-5), 6.78 (3H, m, H-3′,5′,6), 6.99 (1H, br s, H-2), 7.38 (2H, d, *J* = 8.5 Hz, H-2′,6′), 7.42 (1H, d, *J* = 15.5 Hz, H-7). ^{13}C NMR ($CDCl_3$ + CD_3OD, 125 MHz) δ 13.9 (–$(CH_2)_7CH_3$), 18.5 and 22.5 and 25.9 and 29.1 and 29.2 and 31.7 (–$CH_2(CH_2)_6CH_3$ and C-γ), 58.3 (C-α), 67.3 (C-β), 68.2 (–$OCH_2(CH_2)_6CH_3$), 113.8 (C-2), 114.6 (C-3′,5′), 115.2 (C-6), 116.4 (C-8), 122.0 (C-2′,6′), 122.2 (C-5), 126.8 (C-1), 129.9 (C-1′), 142.5 (C-7), 144.5 (C-4), 146.9 (C-3), 156.2 (C-4′), 168.0 (C-9), 169.2 (Thr-*C*O). ESI-MS (Positive): 485.1 ([M+H]$^-$, 100%). HR-FAB-MS [M+H]$^+$ *m/z* 485.26525 (calcd for $C_{27}H_{37}O_6N_2$, requires 485.26517).

4.5.3. Compound 4c

Yield: 98%, ^{1}H NMR ($CDCl_3$ + CD_3OD) δ 0.88 (3H, t, *J* = 6.5 Hz, –$(CH_2)_7CH_3$), 1.28 (8H, m) and 1.41 (2H, m) and 1.75 (2H, m) (–$CH_2(CH_2)_6CH_3$), 2.89 (m, partially overlapped with H_2O signal, H-β), 3.91 (2H, t, *J* = 6.5 Hz, –$CH_2(CH_2)_6CH_3$), 5.03 (1H, t, d, *J* = 6.5 Hz, H-α), 6.30 (1H, d, *J* = 15.5 Hz, H-8), 6.82 (3H, m, H-3′,5′,5), 6.91 (1H, dd, *J* = 1.5, 8.0 Hz, H-6), 7.04 (1H, 1H, d, *J* = 1.5 Hz, H-2), 7.40 (2H, d, *J* = 8.5 Hz, H-2′,6′), 7.49 (1H, d, *J* = 15.5 Hz, H-7). ^{13}C NMR ($CDCl_3$ + CD_3OD, 125 MHz) δ 14.0 (–$(CH_2)_7CH_3$), 16.9 and 22.5 and 25.9 and 29.1 and 29.2 and 31.7 (–$CH_2(CH_2)_6CH_3$), 35.7 (C-β), 50.0 (C-α), 68.2 (–$OCH_2(CH_2)_6CH_3$), 113.5 (C-2), 114.6 (C-3′,5′), 115.1 (C-6), 116.4 (C-8), 121.8 (C-2′,6′,5), 126.8 (C-1), 130.3 (C-1′), 142.6 (C-7), 144.6 (C-4), 147.0 (C-3), 156.1 (C-4′), 167.3 (C-9), 168.8 (ASP-*C*ONH), 173.6 (ASP-*C*OOH). ESI-MS (Positive): 499.1 ([M+H]$^-$, 100%); ESI-MS (Negative): 497.1 ([M−H]$^-$, 100%). HR-FAB-MS [M+H]$^+$ *m/z* 499.24404 (calcd for $C_{27}H_{35}O_7N_2$, requires 499.24443).

4.6. 1,3-β-D-Glucan synthase assay

The fungal membrane enzyme was prepared from *Candida albicans* using a previously reported procedure.[6,7] The 1,3-β-D-glucan synthase reaction mixture contained 50 mM Tris–HCl (pH 7.5), 0.5 mM EDTA, 25 mM KF, 20 μM GTP γ-S, 0.75% BSA, 5.0 mM UDP-Glc and the membrane-bound 1, 3-β-D-glucan synthase. The reaction was initiated by adding of the enzyme, reacted for 20 min by incubation at 30 °C and was terminated by heating at 96 °C for 10 min. The 1,3-β-D-glucan-dependent enzyme cascade reaction was carried out using a β-glucan determination kit (BGSTAR) following the procedure attached to the kit.

4.7. Anti-fungal assay

The anti-fungal activities were determined by broth dilution method according to NCCLS using *Candida albicans* NBRC 1594 (from NITE, Tokyo, Japan) as the organism.[5] The concentration of the anti-fungal agents was in the range between 0.12 and 64 μg/ml.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.08.022.

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