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Hanasanagin: a new antioxidative pseudo-di-peptide, 3,4-diguanidinobutanoyl-DOPA, from the mushroom, *Isaria japonica*

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Abstract—The 'Hanasanagitake' mushroom, *Isaria japonica*, is a folk medicine and a traditional health food. Fractionation of the 60% ethanol extract of the mushroom, guided by the antioxidant activity test, led to the isolation of a new pseudo-di-peptide, and it was called 'hanasanagin'. Spectral analysis and chemical transformation determined the structure of hanasanagin as 3,4-diguanidinobutanoyl-DOPA.

The entomogenous 'Hanasanagitake' mushroom, *Isaria japonica*,[1] is a folk medicine and a traditional health food called 'To-Chu-Ka-So' in Japanese, and is used for perennial youth. In our search for naturally occurring, biologically active compounds from the mushroom, guided by the free radical scavenging test[2] and the superoxide dismutase (SOD) like activity test,[3,4] one novel pseudo-di-peptide was isolated. The pseudopeptide was called 'hanasanagin'. Its structure was clarified by spectral analysis and chemical transformation. This paper describes the isolation, structure elucidation and the antioxidant activity of hanasanagin.

The fruiting bodies of *I. japonica*, cultivated on silkworm pupae were collected and extracted with 60% EtOH. The concentrated aqueous residue of the extract was adsorbed on a reversed phase column packed with Diaion HP-20 (Mitsubishi Chemical Co.). The column was eluted with 5% MeOH after washing with water. The 5% MeOH fraction was purified by reversed phase HPLC (Mightysil RP-18 GP, 20 mm × 250 mm, 5 μm, Kanto Chemicals Co., gradient elution between 0.1% TFA and 50% acetonitrile containing 0.1% TFA, 120 min) to give the pure antioxidant, hanasanagin. The yield was 35.2 mg from 193 g (wet weight) of the mushroom.

Hanasanagin, a pale yellow oil, showed the protonated molecular ion at $m/z = 382$ in the MALDI-TOF-MS analysis. The molecular formula of hanasanagin was analyzed as $C_{15}H_{23}N_7O_5$ on the basis of its HR-FAB-MS spectra (positive, in glycerin, $m/z = 382.1819$, $\Delta -1.9$ mmu). Its UV spectrum (λ_{max} 282 nm) suggested that it had a phenol moiety.

The ^{1}H NMR, ^{13}C NMR, ^{1}H–^{1}H COSY and HMBC spectra of hanasanagin were measured in D_2O, and these results are summarized in Table 1. The ^{1}H–^{13}C correlations were assigned by the HSQC analysis. The partial structures elucidated from the ^{1}H–^{1}H COSY spectrum were indicated by bold lines and the important long range ^{1}H–^{13}C correlations were shown by arrows in Figure 1.

The methylene proton signals at 2.66 and 3.01 ppm (β-H of DOPA) that had a ^{1}H–^{1}H COSY correlation with the methine signal at 4.47 ppm (α-H) showed HMBC correlations with the carbon at 55.8 ppm (α-C), carboxyl carbon at 176.8 ppm and three aromatic carbons at 117.6,

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Table 1. NMR spectral data of hanasanagin in D_2O

Residue	Position	δ_C	δ_H	^1H–^1H COSY	HMBC ($^1H \rightarrow {}^{13}C$)
DOPA	C=O	176.8			
	α	55.8	4.47 (1H, dd, $J = 10.0$, 5.0 Hz)	β-Ha, Hb	CO, β-C, C-1 (DOPA), C-1 (But)
	β	37.2	a 2.66 (1H, dd, $J = 14.0$, 10.0 Hz)	α-H, β- Hb	CO, α-C, C-1, C-2, C-6 (DOPA)
			b 3.01[a] (1H, m)	α-H, β- Ha	CO, α-C, C-1, C-2, C-6 (DOPA)
	1	130.6			
	2	117.6	6.67 (1H, d, $J = 2.0$ Hz)	H-6	β-C, C-3, C-4, C-6 (DOPA)
	3	144.6			
	4	143.6			
	5	116.9	6.72 (1H, d, $J = 8.0$ Hz)	H-6	C-1, C-3 (DOPA)
	6	122.3	6.58 (1H, dd, $J = 8.0$, 2.0 Hz)	H-2, H-5	β-C, C-2, C-4 (DOPA)
But	1	172.0			
	2	38.1	a 2.35 (1H, dd, $J = 15.5$, 6.6 Hz)	Hb-2, H-3	C-1, C-3, C-4 (But)
			b 2.41 (1H, dd, $J = 15.5$, 8.0 Hz)	Ha-2, H-3	C-1, C-3, C-4 (But)
	3	49.8	3.84 (1H, br s)	Ha-2, Hb-2, H-4	C-1, C-2, C-4, C-5 (But)
	4	44.8	3.01[a] (2H, m)	H-3	C-2, C-3, C-6 (But)
	5	157.5			
	6	157.8			

But = diguanidinobutanoyl moiety; DOPA = dihydroxyphenylalanine moiety.
[a] Overlapped CH_2 and CH signals were separately observed and assigned in the spectrum measured in D_2O–pyridine-d_5.

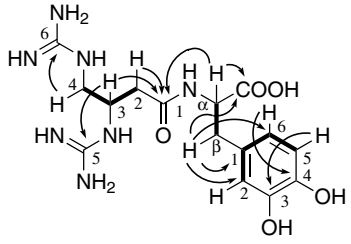

Figure 1. Structure of hanasanagin. Bold lines are partial structures identified by ^1H–^1H COSY and arrows indicate the important HMBC correlations.

122.3 and 130.6 ppm (C-2, C-6 and C-1 of DOPA). The methine proton at 4.47 ppm (α-H), whose chemical shift suggested that it could be the α-proton of the amino acid with the acylated N-terminal, possessed HMBC correlations with the carboxyl carbon at 176.8 ppm, aliphatic carbon at 37.2 ppm (β-C) and aromatic carbon at 130.6 ppm (C-1). These analyses clarified the partial structure, HOOC–CH(N)–CH_2–phenyl, in hanasanagin. The aromatic proton at 6.58 ppm (H-6), which coupled with the protons at 6.67 (H-2, $J = 2.0$ Hz) and 6.72 ppm (H-5, $J = 8.0$ Hz), showed HMBC correlation peaks with the aromatic carbons at 117.6 (C-2) and 143.6 ppm (C-4). The aromatic proton at 6.72 ppm (H-5) showed HMBC cross peaks with the carbons at 130.6 (C-1) and 144.6 ppm (C-3). The chemical shifts of aromatic carbons, C-3 (144.6 ppm) and C-4 (143.6 ppm), indicated that they were connected to the oxygen atoms. Thus, the aromatic signals were assigned, and the DOPA moiety in hanasanagin was proved.

The 3,4-disubstituted butanoyl moiety in hanasanagin was elucidated by ^1H–^1H COSY analysis and HMBC analysis (Fig. 1 and Table 1). The H-3 methine proton at 3.84 ppm showed ^1H–^1H COSY signals with the methylene protons at 2.35 and 2.41 ppm (Ha-2 and Hb-2) and the methylene protons at 3.01 ppm (H-4). HMBC cross peaks were observed between the H-2 methylene protons and the C-1 carbonyl carbon at 172.0 ppm and between the H-3 methine proton and the same carbonyl carbon. Thus, the 3,4-disubstituted butanoyl partial structure was established.

As the C-1 carbonyl carbon of the butanoyl moiety showed an HMBC correlation with the α-proton of DOPA at 4.47 ppm, the 3,4-disubstituted butanoyl moiety was proved to be connected to the DOPA moiety via this carbonyl group. The chemical shifts of the α-proton (4.47 ppm), α-carbon of the DOPA moiety (55.8 ppm) and the C-1 carbonyl carbon of the butanoyl moiety (172.0 ppm) indicated that the butanoyl moiety was connected to the DOPA moiety via the amide bond.

The substituting groups on the butanoyl moiety were speculated to be guanidine groups because the chemical shifts of C-5 (157.5 ppm) and C-6 (157.8 ppm), which showed HMBC correlations with H-3 and H-4 of the butanoyl moiety, respectively, were similar to that of the guanidine carbon of the other guanidino compounds such as arginine (159.4 ppm).

This speculation was proved by the chemical transformation as shown in Figure 2. Hanasanagin was treated with 2,4-pentanedione in 1% K_2CO_3 at 70 °C for two days. The reaction is known to convert the guanidine group into the 4,6-dimethylpyrimidinylamino group.[5] The structure of the reaction product was confirmed by the MALDI-TOF-MS and ^1H NMR spectra (Table 2). The protonated molecular ion of the derivative was observed at $m/z = 510$ in the mass spectrum and the value was consistent with the value calculated for the desired product. The ^1H NMR spectrum showed two 6H singlet signals at 2.17 and 2.21 ppm, assignable to four methyl groups of two 4,6-dimethylpyrimidine groups, and two 1H singlet signals at 6.12 and 6.14 ppm, assignable to the aromatic protons on two 4,6-dimethylpyrimidine rings. The H-4 methylene signal (3.01 ppm) and H-3 methine signal (3.84 ppm) of the butanoyl moiety

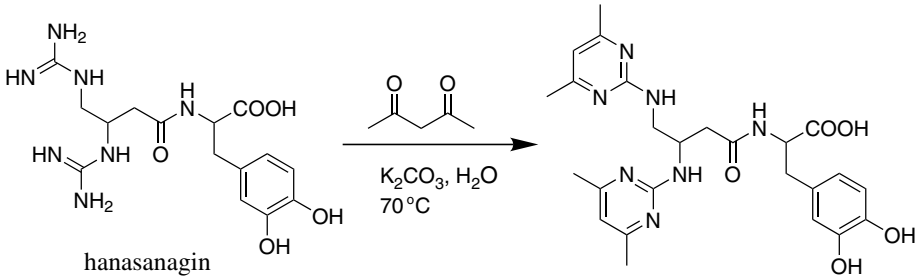

Figure 2. Transformation of hanasanagin into its pyrimidine derivative.

Table 2. ^{1}H NMR data of 3,4-di-(4,6-dimethylpyrimidinylamino)-butanoyl-DOPA in C_5D_5N

δ_H	Assignment
2.17 (6H, s)	CH_3 (Pyr)
2.21 (6H, br s)	CH_3 (Pyr)
3.02 (1H, dd, $J = 14.4$, 7.0 Hz)	CH_2CO (But)
3.17 (1H, dd, $J = 14.4$, 5.0 Hz)	CH_2CO (But)
3.33 (1H, dd, $J = 14.0$, 7.5 Hz)	β-H (DOPA)
3.50 (1H, dd, $J = 14.0$, 5.5 Hz)	β-H (DOPA)
4.15 (2H, bm)	$NHCH_2$ (But)
5.17[a]	NHCH (But)
5.42[a]	α-H (DOPA)
6.12 (1H, s)	H (Pyr)
6.14 (1H, s)	H (Pyr)
7.06 (1H, dd, $J = 7.9$, 2.1 Hz)	arom-H (DOPA)
7.18 (1H, d, $J = 7.9$ Hz)	arom-H (DOPA)
7.35 (1H, d, $J = 2.1$ Hz)	arom-H (DOPA)

Pyr = dimethylpyrimidine moiety; But = butanoyl moiety; DOPA = dihydroxyphenylalanine moiety; arom = aromatic.
[a] Hidden by DHO signal and found in ^{1}H–^{1}H COSY spectrum.

in the starting material, hanasanagin, shifted to 4.15 and 5.17 ppm, respectively (Tables 1 and 2), after conversion. All of the other ^{1}H signals were assigned to the other moiety of the derivative (Table 2) based on the ^{1}H–^{1}H COSY analysis. These results clearly suggested that the two guanidine groups were converted into the 4,6-dimethylpyrimidinylamino groups and that the structure of the product was 3,4-di-(4,6-dimethylpyrimidinylamino)-butanoyl-DOPA. Thus, the structure of hanasanagin was established to be 3,4-diguanidinobutanoyl-DOPA, except for its stereochemistry. Synthesis of hanasanagin aiming at determining its stereochemistry is in progress.

The free radical scavenging activity of hanasanagin was evaluated against the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical.[2] The relative concentration of the radical was measured by ESR (JES-RE1X, JEOL Co.), and the scavenging activity, EC_{50}, was defined as the amount of scavenger necessary to decrease the concentration of the initial DPPH radical by 50%. The EC_{50} value of hanasanagin was 8.1 μM, and this value was 2.6 times greater than that of ascorbic acid.

The SOD like activity of hanasanagin was measured using the ESR spin trapping method according to Yeşilada et al. and Sekine et al.[3,4] Superoxide anion radicals generated by the hypoxanthine–xanthine oxidase system were trapped by DMPO (5,5-dimethyl-1-pyrroline-*N*-oxide) in the presence or absence of the test sample, and the effect of the test sample was compared to that of the standard SOD. The SOD like activity of hanasanagin was 152 SOD unit/μmol and that of ascorbic acid was 80 SOD unit/μmol. This result indicated that the SOD like activity of hanasanagin was 1.9 times stronger than that of ascorbic acid.

In the present study, one novel antioxidant was isolated from the Hanasanagitake mushroom, and was called hanasanagin. It was a new pseudo-di-peptide composed of a unique 3,4-diguanidinobutanoic acid and DOPA. The DOPA moiety containing a catechol group is expected to be the origin of the antioxidant activity, because many natural products having this moiety show such an activity.[3,4,6,7] Reactive oxygen species are suspected to cause the peroxidative disintegration of cells, which is implicated in various pathological processes and are involved in the pathogenesis of diseases such as cancer initiation and the aging process. The antioxidant activity of hanasanagin from the Hanasanagitake mushroom could contribute to reducing the reactive oxygen species, and make the mushroom popular as a folk medicine and a traditional health food.

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