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Mulberry anthracnose antagonists (iturins) produced by *Bacillus amyloliquefaciens* RC-2

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Abstract

Bacillus amyloliquefaciens strain RC-2 produced seven antifungal compounds (**1**–**7**) secreted into the culture filtrate. These compounds inhibited the development of mulberry anthracnose caused by the fungus, *Colletotrichum dematium*. Chemical structural analyses by NMR and FAB-MS revealed that all these compounds were iturins (cyclic peptides with the following sequence: L-Asn → D-Tyr → D-Asn → L-Gln → L-Pro → D-Asn → L-Ser → D-β-amino acid →) and compounds **1**–**6** are identical to iturins A-2–A-7, respectively. Compound **7** (iturin A-8) is a new iturin, which has a $-(CH_2)_{10}CH(CH_3)CH_2CH_3$ group as a side chain in the β-amino acid in the molecule.

Keywords: *Bacillus amyloliquefaciens*; Iturin; Cyclic peptide; β-amino acid; *Colletotrichum dematium*; Mulberry anthracnose

1. Introduction

For silk production, mulberry is widely cultivated as a feed for silkworms. Recently, mulberry anthracnose, a common disease caused by *Colletotrichum dematium*, has been found to be widespread in Japan, which leads to large losses in mulberry leaf production. Dense planting and mechanical harvesting could be the cause of this epidemic. Yoshida et al. (2001) searched antagonistic micro-organisms against mulberry anthracnose from healthy mulberry leaves and found *Bacillus amyloliquefaciens* strain RC-2 as one of the most powerful antagonists. This strain produces antifungal compounds which inhibit the development of mulberry anthracnose by preventing conidial germination of the fungus. Furthermore, the aseptic culture filtrate of the strain shows inhibitory effects on the growth of several other phytopathogenic fungi and bacteria, such as *Rosellinia necatrix*, *Pyricularia oryzae*, *Agrobacterium tumefaciens*, and *Xanthomonas campestris* pv. *campestris*, in vitro (Yoshida et al., 2001). Therefore, this strain and the antibiotic compounds produced could be useful for biological control of some diseases. One of these antimicrobial compounds was isolated from the culture filtrate of *B. amyloliquefaciens* strain RC-2 and identified as iturin A-2, a cyclic peptide with following sequence: Asn → Tyr → Asn → Gln → Pro → Asn → Ser → 3-amino-tetradecanoic acid (β-amino acid) →, although chemical structures of the other compounds were not clarified (Yoshida et al., 2001). In this study, antifungal compounds **1**–**7** were isolated from the culture filtrate of strain RC-2 by monitoring antifungal activity against *C. dematium*, and their chemical structures were elucidated.

2. Results and discussion

The bacterial strain RC-2 was cultured in a 0.5% peptone-potato dextrose medium, which promotes the production and accumulation of the antifungal compounds (Yoshida et al., 2001). By determining the antifungal activity against *C. dematium* using a placing assay (see below), bioactivity-directed purification of

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antifungal compounds in the culture filtrate was conducted. Fractionations by solvent extraction, reversed-phase solid extraction, and preparative reversed-phase HPLC gave seven pure antifungal compounds (**1**–**7**). All these compounds showed the same UV-visible absorption spectra with absorption maximum at 276 nm.

2.1. Compound *1*

The HRFAB-MS spectrum of compound 1 showed a protonated molecular ion $[M+H]^+$ at m/z 1043.5498, indicating a molecular formula $C_{48}H_{74}N_{12}O_{14}$. The ^1H NMR (600 MHz, DMSO-d_6) of compound **1** indicated the presence of N-binding protons (δ 8.68–6.82) and α-protons (δ 4.51–3.97) in a peptide, one *para*-substituted benzene ring (δ 7.01, 6.65, each *d*, $J=8.4$ Hz, 2H), long methylene chain (δ 1.40–1.08) and one terminal methyl group (δ 0.84, *t*, $J=7.0$ Hz, 3H). The ^{13}C NMR and DEPT spectra indicated the presence of 12 carbonyl groups (δ 174.0–170.3), one *para*-substituted benzene (δ 155.8, 1C; 129.7, 2C; 127.9, 1C; 115.0, 2C), methylene carbons (δ 61.3–22.0), and one methyl carbon (δ 13.9). After determining the connectivity between each carbon and proton by analysis of the $^{13}C-{}^1H$ COSY spectrum, these C – H units were constructed according to the results of ^1H–^1H COSY, HOHAHA and HMBC spectral analyses. These indicated that compound **1** is a cyclic peptide composed of eight amino acids with the following sequence: Asn → Tyr → Asn → Gln → Pro → Asn → Ser → β-amino acid with a–$(CH_2)_{10}CH_3$ group as a side chain (3-amino-tetradecanoic acid) → (Fig. 1). All ^1H and ^{13}C NMR signals were assigned as indicated in Fig. 1. The atomic arrangement of compound **1** is same as that of iturin A-2, which has been reported as an antibiotic agent from *Bacillus species* (Isogai et al., 1982). It was confirmed that the retention time on HPLC analysis, UV-visible spectrum, and ^1H and ^{13}C NMR spectrum of compound **1** were identical to those of an authentic sample of iturin A-2. Furthermore, the optical rotation of compound **1** ($[\alpha]_D^{26.7}$ −2.2, MeOH; *c* 0.37) was also identical to that of iturin A ($[\alpha]_D$ −1.7, MeOH; *c* 5.0, Peypoux et al., 1978). Iturin A is a mixture of two iturins, whose side chain structure in the β-amino acid are –$(CH_2)_8CH(CH_3)CH_2CH_3$ (same as iturin A-3, compound **2**) and–$(CH_2)_8CH(CH_3)_2$. Therefore, it is concluded that the absolute configuration of compound **1** is identical to iturin A (L-Asn → D-Tyr → D-Asn → L-Gln → L-Pro → D-Asn → L-Ser → D-β-amino acid →, Peypoux et al., 1978; Nagai, 1979) and compound **1** is iturin A-2.

2.2. Compounds *2*–*7*

A series of NMR and UV spectral analyses revealed that compounds **2**–**7** are composed of the same amino acid sequence as compound **1** (iturin A-2), except for the side chain structure of the β-amino acid, indicating they are all classified as iturins. FAB-MS spectra of compounds **2**–**7** showed $[M+H]^+$ ion peaks at m/z 1057, 1057, 1057, 1071, 1071, and 1085, respectively. ^1H NMR, ^{13}C NMR and HMBC spectra of compound **2** showed the presence of one terminal *sec*-butyl group, indicating that the side-chain of β-amino acid was –$(CH_2)_8CH(CH_3)CH_2CH_3$ (iturin A-3). Likewise, compound **3** was shown to contain one terminal isopropyl group and to be iturin A-4 (side-chain of β-amino acid was −$(CH_2)_9CH(CH_3)_2$). Compound **4** contained one terminal methyl group so that the side-chain of β-amino acid was –$(CH_2)_{11}CH_3$ (iturin A-5), whereas compound **5** contained one terminal isopropyl group so that the side-chain of β-amino acid was

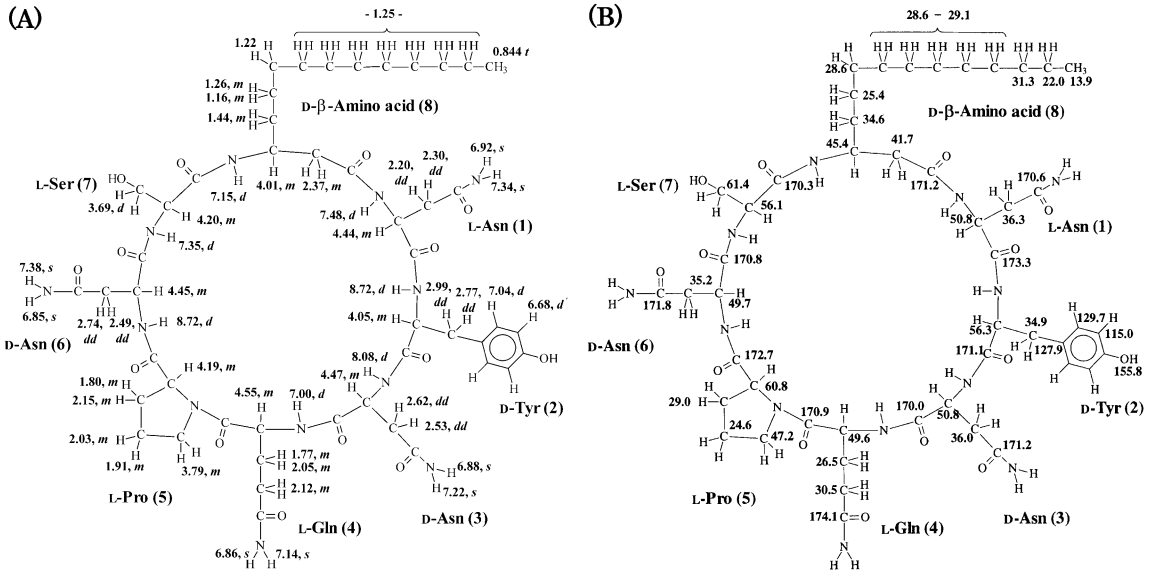


Fig. 1. Chemical structure and assignment of ^1H (A) and ^{13}C (B) NMR signals (DMSO-d_6) of compound **1** (iturin A-2).

$-(CH_2)_{10}CH(CH_3)_2$ (iturin A-6). Compound **6** contained one terminal methyl group so that the side-chain of β-amino acid was $-(CH_2)_{12}CH_3$ (iturin A-7), and compound **7** contained one terminal *sec*-butyl group so that the side-chain of β-amino acid was $-(CH_2)_{10}CH(CH_3)CH_2CH_3$ (Table 1). Results of FAB-MS spectra and major assignment of ^1H and ^{13}C NMR signals of the side-chain of β-amino acid were summarized in Table 1. Compound **7** is a novel compound and may be identical with iturin A-8, previously isolated from *Bacillus* species by Isogai et al. (1982) but whose chemical structure was not elucidated. The lipid moiety of compound **7** (3-amino-15-methylpentadecanoic acid, anteiso C_{17}) was previously identified in mycosubtilin (Peypoux et al., 1986) and bacillomycin F (Peypoux et al., 1985), which belong to the iturin family.

Peypoux et al. (1973, 1978) also reported that *B. subtilis* produced iturins in which the side-chain structures of the β-amino acid are mainly $-(CH_2)_8CH(CH_3)CH_2CH_3$ (iturin A-3, anteiso C_{15}) and $-(CH_2)_8CH(CH_3)_2$ (iso C_{14}). In the present study, it was found that the latter iturin (iso C_{14}) was not obtained and the main iturin product of *B. amyloliquefaciens* was iturin A-2. This result is very similar to the research on *Bacillus* species done by Isogai et al. (1982). The composition of iturins produced would depend on the species of *Bacillus* used. Conformational studies (Garbay-Jaureguiberry et al., 1978; Marion et al., 1986) and mode of action (Quentin et al., 1982; Maget-Dana et al., 1985; Maget-Dana and Peypoux, 1994) of iturins were also reported. *Bacillus subtilis* produces other antimicrobial substances, such as subtilin (Gross et al., 1973), bacilysin (Walker and Abraham, 1970), mycobacillin (Sengupta et al., 1971), bacillomycin (Besson et al., 1977; Peypoux et al., 1985), and mycosubtilin (Peypoux et al., 1976, 1986), and some of their antifungal activities are compared (Besson et al., 1979). *Bacillus subtilis* is similar to *B. amyloliquefaciens* but distinguished by slightly lower G+C content in DNA in *B. subtilis* (Yoshida et al., 2001). Despite their similarity, the antifungal activity of *B. amyloliquefaciens* is not frequently reported. In this study, *B. amyloliquefaciens* strain RC-2 was found to produce iturins A-2–A-8 as antifungal agents, especially against mulberry anthracnose fungus, *C. dematium*, and the chemical structure of the new iturin (A-8) was elucidated from spectroscopic analyses. It is likely that the iturins are produced by *B. amyloliquefaciens* on mulberry leaves and the development of *C. dematium* is suppressed by these chemicals, resulting in protection of mulberry leaves by *B. amyloliquefaciens*. *Bacillus amyloliquefaciens* strain RC-2 could possibly be used as a biocontrol agent or iturin producer for regulating the mulberry anthracnose disease.

3. Experimental

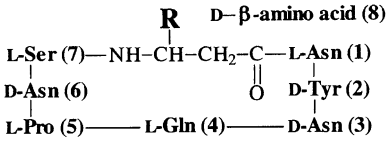
3.1. General

1D (^1H, ^{13}C and DEPT) and 2D (^1H–^1H COSY, HOHAHA, ^1H–^{13}C COSY, and HMBC) NMR spectra were recorded in DMSO-d_6 at 303 K on a JEOL JNM α-600 spectrometer (^1H: 600.05 MHz, ^{13}C: 150.80 MHz) using standard JEOL software (Alpha Data System). Chemical shifts are reported in ppm relative to TMS but were recorded relative to the residual signal of the solvent (^1H: 2.490 ppm, ^{13}C: 39.50 ppm) and coupling constant in Hz. FAB-MS spectra were recorded on a JEOL SX102A spectrometer. Samples were mixed with glycerol as a matrix and analyzed using a direct inlet system. For solid phase extraction, chemically modified silica gel (reversed phase, Analytichem

Table 1
Chemical structures and selected FAB-MS and NMR (DMSO-d_6) spectral data of compounds **1**–**7** (iturins A-2–A-8)

Compound No. (name)	R[c]	MS $(M+H)^+$	^{13}C NMR of β-amino acid moiety, δ (ppm)						^1H NMR δ (ppm of CH_3)
			C-12	C-13	C-14	C-15	C-16	CH_3[a]	
Compound **1** (iturin A-2)	$-(CH_2)_{10}CH_3$	1043	31.3	22.0	13.9				0.84 (*t*, 3H)
Compound **2** (iturin A-3)	$-(CH_2)_8CH(CH_3)CH_2CH_3$	1057	33.7	29.1	11.2			19.1	0.81 (*d*, 3H) 0.82 (*t*, 3H)
Compound **3** (iturin A-4)	$-(CH_2)_9CH(CH_3)_2$	1057	38.5	27.3	22.5			22.5	0.83 (*d*, 6H)
Compound **4** (iturin A-5)	$-(CH_2)_{11}CH_3$	1057	–[b]	31.2	22.0	13.9			0.84 (*t*, 3H)
Compound **5** (iturin A-6)	$-(CH_2)_{10}CH(CH_3)_2$	1071	–	38.4	27.3	22.5		22.5	0.83 (*d*, 6H)
Compound **6** (iturin A-7)	$-(CH_2)_{12}CH_3$	1071	–	–	31.2	22.0	13.9		0.84 (*t*, 3H)
Compound **7** (iturin A-8)	$-(CH_2)_{10}CH(CH_3)CH_2CH_3$	1085	26.4	36.0	33.7	29.1	11.1	19.0	0.81 (*d*, 3H) 0.82 (*t*, 3H)

[a] Branched methyl.
[b] Not specified.
[c] The side chain structure of β-amino acid as below.



BONDESIL C18, 40 μm, preparative grade, Varian) was filled in a glass column (200 × 22 mm i.d.). Preparative HPLC was performed with a Waters 626 pump and 996 photodiode array detector, equipped with a reversed phase column (Shim-pack PREP-ODS(H), 20 mm i.d. × 250 cm, Shimadzu), and eluted with MeOH–H_2O (7:3, v/v) at a flow rate of 8 ml min^{-1} at 40 °C. Optical rotations were measured at room temp (~27 °C) by using HORIBA SEPA-300 polarimeter.

3.2. Bacterial growth

Bacillus amyloliquefaciens strain RC-2 was originally isolated from healthy mulberry leaves attached to the trees in the field at National Institute of Sericultural and Entomological Science, Tsukuba, Japan (Yoshida et al., 2001). Strain RC-2 was identified as *B. amyloliquefaciens* by using API 50 CHB test strip (Bio Merieux S. A., France), which tests the bacterial catabolism of 49 kinds of substrates to identify *Bacillus* strains. The bacterial strain was incubated with 50 ml of 0.5% peptone-PD broth (per liter: polypeptone, 5 g; potato, 200 g; dextrose, 20 g) in a 300 ml Erlenmeyer flask with shaking (130 rpm) for 2 days at 25 °C in the dark. By repeating the incubation, ca. 11 l of incubation mixture of the bacterial strain were obtained.

3.3. Bioassay (placing assay)

The sample solution was evaporated, and MeOH–H_2O (1:1, v/v) was used to prepare appropriate concentration (1–8-fold dilution of original culture filtrate). *Colletotrichum dematium* was grown on an agar plate (PSA; per liter: potato, 200 g; sucrose, 20 g; agar, 18 g), and 20 μl of prepared test solution were placed onto a small mycelial block (0.5–1 mm^3) of *C. dematium*. After incubation for 3 days at 25 °C in the dark, antifungal activity was evaluated by measuring the diameter of the mycelial colony developed from the mycelial block.

3.4. Isolation of antifungal compounds

Incubation mixture of *B. amyloliquefaciens* strain RC-2 (ca. 11 l) was centrifuged at 8000 × *g* for 10 min. Freeze-dried culture filtrate (60 g) was washed with EtOAc (4 l), and the residue was extracted with MeOH (4 l) overnight at room temp. The MeOH extract was separated into five fractions by solid phase extraction eluted successively with H_2O, H_2O–MeOH (60:40; 45:55; 25:75) and pure MeOH, respectively. The active fraction (H_2O–MeOH, 25:75) was further purified with solid phase extraction eluted stepwise with H_2O–MeOH (40:60; 30:70), and pure MeOH. The active fraction (eluted with H_2O–MeOH, 30:70) was evaporated to dryness in vacuo and successively extracted with pure MeOH, MeOH–H_2O (80:20; 60:40; 40:60; 20:80) and distilled water. The most active fraction (eluted with MeOH–H_2O, 80:20) was subjected to preparative HPLC, resulting in seven active fractions (compound **1**: 30.0 mg, 10.0 min retention time; compound **2**: 15.7 mg, 12.5 min; compound **3**: 20.0 mg, 13.5 min; compound **4**: 9.0 mg, 14.5 min; compound **5**: 3.5 mg. 20.0 min; compound 6: 3.7 mg, 22.5 min; compound 7: 2.5 mg, 30.0 min).

3.5. Compound *1* (iturin A-2)

$[\alpha]_D^{26.7}$ −2.2° (*c* 0.37, MeOH). ^{1}H NMR (600 MHz, DMSO-d_6, δ): 8.68 (*d*, 2H, Tyr and Asn-6 NH), 8.04 (*d*, 1H, Asn-3 NH), 7.70 (*d*, 1H, Asn-1 NH), 7.35 (*s*, 1H, Asn-6 NH_2), 7.31 (*d*, 1H, Ser NH), 7.31 (*s*, 1H, Asn-1 NH_2), 7.19 (*s*, 1H, Asn-3 NH_2), 7.12 (*d*, 1H, β-amino acid NH), 7.10 (*s*, 1H, Gln NH_2), 7.01 (*d*, J = 8.4 Hz, 2H, Tyr δ), 6.91 (*d*, 1H, Gln NH), 6.89 (*s*, 1H, Asn-1 NH_2), 6.85 (*s*, 1H, Asn-3 NH_2), 6.82 (*s*, 2H, Asn-6 and Gln NH_2), 6.65 (*d*, J = 8.4 Hz, 2H, Tyr ε), 4.83 (*br*, 1H, Ser O*H*), 4.51 (*m*, 1H, Gln α), 4.43 (*m*, 1H, Asn-3 α), 4.42 (*m*, 2H, Asn-1 and Asn-6 α), 4.16 (*m*, 2H, Pro and Ser α), 4.02 (*m*, 1H, Tyr α), 3.97 (*m*, 1H, β-amino acid C_3H), 3.75 (*m*, 2H, Pro δ), 3.66 (*d*, 2H, Ser β), 2.96 (*dd*, J = 3.7, 14.3 Hz, 1H, Tyr β), 2.73 (*dd*, 1H, Tyr β), 2.71 (*dd*, 1H, Asn-6 β), 2.58 (*dd*, J = 9.5, 15.4 Hz, 1H, Asn-3 β), 2.47 (*dd*, Asn-3 β), 2.45 (*dd*, Asn-6 β), 2.33 (*m*, 2H, β-amino acid C_2H_2), 2.29 (*dd*, J = 8.4, 15.8 Hz, 1H, Asn-1 β), 2.16 (*dd*, 1H, Asn-1 β), 2.12 (*m*, 1H, Pro β), 2.09 (*m*, 2H, Gln γ), 2.03 (*m*, 1H, Gln β), 1.98 (*m*, 1H, Pro γ), 1.88 (*m*, 1H, Pro γ), 1.75 (*m*, 1H, Gln β), 1.74 (*m*, 1H, Pro β), 1.40 (*m*, 2H, β-amino acid C_4H_2), 1.28–1.08 (*m*, β-amino acid aliphatic CH_2), 0.84 (*t*, J = 7.0 Hz, 3H, β-amino acid $C_{14}H_3$); ^{13}C NMR (151 MHz, DMSO-d_6, δ): 174.0 (C=O), 173.3 (C=O), 172.7 (C=O), 171.8 (C=O), 171.2 (C=O), 171.2 (C=O), 171.1 (C=O), 171.0 (C=O), 170.9 (C=O), 170.8 (C=O), 170.6 (C=O), 170.3 (C=O), 155.8 (Tyr, ζ), 129.7 (Tyr, δ, 2C), 127.9 (Tyr, γ), 115.0 (Tyr, ε, 2C), 61.3 (Ser, β), 60.8 (Pro, α), 56.3 (Tyr, α), 56.1 (Ser, α), 50.8 (Asn-6, α), 50.8 (Asn-1, α), 49.7 (Asn-3, α), 49.6 (Gln, α), 47.2 (Pro, δ), 45.3 (β-amino acid, C_3), 41.7 (β-amino acid, C_2), 36.3 (Asn-1, β), 36.0 (Asn-3, β), 35.2 (Asn-6, β), 34.9 (Tyr, β), 34.6 (β-amino acid, C_4), 31.3 (β-amino acid, C_{12}), 30.5 (Gln, γ), 29.1 (β-amino acid), 29.1 (β-amino acid), 29.0 (β-amino acid), 29.0 (β-amino acid), 29.0 (Pro, β), 28.7 (β-amino acid), 28.6 (β-amino acid), 26.5 (Gln, β), 25.3 (β-amino acid, C_5), 24.6 (Pro, γ), 22.0, (β-amino acid, C_{13}), 13.9 (β-amino acid, C_{14}). HRFAB-MS m/z 1043.5498 $[M+H]^+$, m/z 1043.5526 calculated for $C_{48}H_{75}N_{12}O_{14}$.

3.6. Compound *2* (iturin A-3)

^{1}H NMR signals of the β-amino acid moiety (600 MHz, DMSO-d_6, δ): 7.12 (*d*, 1H, β-amino acid NH), 3.98 (*m*, 1H, β-amino acid C_3H), 2.33 (*m*, 2H, β-amino

acid C_2H_2), 1.40 (*m*, 2H, β-amino acid C_4H_2), 1.29 (*m*, β-amino acid $C_{12}H$), 1.32–1.02 (*m*, β-amino acid aliphatic CH_2), 0.82 (*t*, $J=7.3$ Hz, 3H, β-amino acid $C_{14}H_3$), 0.81 (*d*, $J=7.3$ Hz, 3H, β-amino acid $C_{12}Me$); ^{13}C NMR (DMSO-d_6, δ): 45.4 (β-amino acid, C_3), 41.7 (β-amino acid, C_2), 36.0 (β-amino acid, C_{11}), 34.6 (β-amino acid, C_4), 33.7 (β-amino acid, C_{12}), 29.4 (β-amino acid), 29.1 (β-amino acid, C_{13}), 28.9 (β-amino acid), 28.9 (β-amino acid), 28.6 (β-amino acid), 26.5 (β-amino acid, C_{10}), 25.3 (β-amino acid, C_5), 19.1 (β-amino acid, $C_{12}Me$), 11.2 (β-amino acid, C_{14}). FAB-MS $[M+H]^+$, *m*/*z* 1057 calculated for $C_{49}H_{77}N_{12}O_{14}$.

3.7. Compound **3** (iturin A-4)

^1H NMR signals of the β-amino acid moiety (600 MHz, DMSO-d_6, δ): 7.12 (*d*, 1H, β-amino acid NH), 3.98 (*m*, 1H, β-amino acid C_3H), 2.33 (*m*, 2H, β-amino acid C_2H_2), 1.49 (*m*, 1H, β-amino acid $C_{13}H$), 1.40 (*m*, 2H, β-amino acid C_4H_2), 1.25–1.10 (*m*, β-amino acid aliphatic CH_2), 0.83 (*d*, $J=6.6$ Hz, 6H, β-amino acid $C_{14}H_3$); ^{13}C NMR (DMSO-d_6, δ): 45.4 (β-amino acid, C3), 41.7 (β-amino acid, C_2), 38.5 (β-amino acid, C_{12}), 34.6 (β-amino acid, C_4), 29.3 (β-amino acid), 29.1 (β-amino acid), 29.0 (β-amino acid), 28.9 (β-amino acid), 28.9 (β-amino acid), 28.6 (β-amino acid), 27.3 (β-amino acid, C_{13}), 26.8 (β-amino acid), 25.4 (β-amino acid, C_5), 22.5 (β-amino acid, C_{14}, 2C). FAB-MS $[M+H]^+$, *m*/*z* 1057 calculated for $C_{49}H_{77}N_{12}O_{14}$.

3.8. Compound **4** (iturin A-5)

^1H NMR signals of the β-amino acid moiety (600 MHz, DMSO-d_6, δ): 7.12 (*d*, 1H, β-amino acid NH), 3.97 (*m*, 1H, β-amino acid C_3H), 2.33 (*m*, 2H, β-amino acid C_2H_2), 1.40 (*m*, 2H, β-amino acid C_4H_2), 1.28–1.08 (*m*, β-amino acid aliphatic CH_2), 0.84 (*t*, $J=6.6$ Hz, 3H, β-amino acid $C_{15}H_3$); ^{13}C NMR (DMSO-d_6, δ): 45.4 (β-amino acid, C_3), 41.7 (β-amino acid, C_2), 34.6 (β-amino acid, C_4), 31.2 (β-amino acid, C_{13}), 29.1 (β-amino acid), 28.9 (β-amino acid), 28.7 (β-amino acid), 28.6 (β-amino acid), 25.3 (β-amino acid, C_5), 22.0 (β-amino acid, C_{14}), 13.9 (β-amino acid, C_{15}). FAB-MS $[M+H]^+$, *m*/*z* 1057 calculated for $C_{49}H_{77}N_{12}O_{14}$.

3.9. Compound **5** (iturin A-6)

^1H NMR signals of the β-amino acid moiety (600 MHz, DMSO-d_6, δ): 7.12 (*d*, 1H, β-amino acid NH), 3.98 (*m*, 1H, β-amino acid C_3H), 2.34 (*m*, 2H, β-amino acid C_2H_2), 1.49 (*m*, 1H, β-amino acid $C_{14}H$), 1.40 (*m*, 2H, β-amino acid C_4H_2), 1.25–1.10 (*m*, β-amino acid aliphatic CH_2), 0.83 (*d*, $J=6.6$ Hz, 6H, β-amino acid $C_{15}H_3$); ^{13}C NMR (DMSO-d_6, δ): 45.4 (β-amino acid, C_3), 41.7 (β-amino acid, C_2), 38.4 (β-amino acid, C_{13}), 34.6 (β-amino acid, C_4), 29.3 (β-amino acid), 29.1 (β-amino acid), 28.9 (β-amino acid), 28.9 (β-amino acid), 28.6 (β-amino acid), 27.3 (β-amino acid, C_{14}), 26.7 (β-amino acid), 25.3 (β-amino acid, C_5), 22.5 (β-amino acid, C_{15}, 2C). FAB-MS $[M+H]^+$, *m*/*z* 1071 calculated for $C_{50}H_{79}N_{12}O_{14}$.

3.10. Compound **6** (iturin A-7)

^1H NMR signals of the β-amino acid moiety (600 MHz, DMSO-d_6, δ): 7.12 (*d*, 1H, β-amino acid NH), 3.97 (*m*, 1H, β-amino acid C_3H), 2.33 (*m*, 2H, β-amino acid C_2H_2), 1.40 (*m*, 2H, β-amino acid C_4H_2), 1.28–1.08 (*m*, β-amino acid aliphatic CH_2), 0.84 (*t*, $J=6.6$ Hz, 3H, β-amino acid $C_{16}H_3$); ^{13}C NMR (DMSO-d_6, δ): 45.4 (β-amino acid, C_3), 41.7 (β-amino acid, C_2), 34.6 (β-amino acid, C_4), 31.2 (β-amino acid, C_{14}), 29.1 (β-amino acid), 29.0 (β-amino acid), 28.9 (β-amino acid), 28.7 (β-amino acid), 28.6 (β-amino acid), 28.6 (β-amino acid), 28.6 (β-amino acid), 25.3 (β-amino acid, C_5), 22.0 (β-amino acid, C_{15}), 13.9 (β-amino acid, C_{16}). FAB-MS $[M+H]^+$, *m*/*z* 1071 calculated for $C_{50}H_{79}N_{12}O_{14}$.

3.11. Compound **7** (iturin A-8)

^1H NMR (600 MHz, DMSO-d_6, δ): 8.68 (*d*, 2H, Tyr and Asn-6 NH), 8.04 (*d*, 1H, Asn-3 NH), 7.70 (*d*, 1H, Asn-1 NH), 7.35 (*s*, 1H, Asn-6 NH_2), 7.31 (*d*, 1H, Ser NH), 7.30 (*s*, 1H, Asn-1 NH_2), 7.19 (*s*, 1H, Asn-3 NH_2), 7.12 (*d*, 1H, β-amino acid NH), 7.10 (*s*, 1H, Gln NH_2), 7.01 (*d*, $J=8.4$ Hz, 2H, Tyr δ), 6.96 (*d*, 1H, Gln NH), 6.88 (*s*, 1H, Asn-1 NH_2), 6.84 (*s*, 1H, Asn-3 NH_2), 6.82 (*s*, 2H, Asn-6 and Gln NH_2), 6.65 (*d*, $J=8.4$ Hz, 2H, Tyr ε), 4.84 (*br*, 1H, Ser O*H*), 4.51 (*m*, 1H, Gln α), 4.44 (*m*, 1H, Asn-3 α), 4.42 (*m*, 2H, Asn-1 and Asn-6 α), 4.16 (*m*, 2H, Pro and Ser α), 4.02 (*m*, 1H, Tyr α), 3.98 (*m*, 1H, β-amino acid C_3H), 3.76 (*m*, 2H, Pro δ), 3.66 (*d*, 2H, Ser β), 2.96 (*dd*, $J=3.8$, 14.1 Hz, 1H, Tyr β), 2.74 (*dd*, 1H, Tyr β), 2.72 (*dd*, 1H, Asn-6 β), 2.58 (*dd*, $J=9.5$, 14.8 Hz, 1H, Asn-3 β), 2.48 (*dd*, Asn-3 β), 2.46 (*dd*, Asn-6 β), 2.33 (*m*, 2H, β-amino acid C_2H_2), 2.29 (*dd*, $J=8.2$, 15.9 Hz, 1H, Asn-1 β), 2.16 (*dd*, 1H, Asn-1 β), 2.11 (*m*, 1H, Pro β), 2.09 (*m*, 2H, Gln γ), 2.02 (*m*, 1H, Gln β), 1.99 (*m*, 1H, Pro γ), 1.88 (*m*, 1H, Pro γ), 1.77 (*m*, 1H, Gln β), 1.73 (*m*, 1H, Pro β), 1.40 (*m*, 2H, β-amino acid C_4H_2), 1.29 (*m*, β-amino acid $C_{14}H$), 1.32–1.02 (*m*, β-amino acid aliphatic CH_2), 0.82 (*t*, $J=7.3$ Hz, 3H, β-amino acid $C_{16}H_3$), 0.81 (*d*, $J=7.7$ Hz, 3H, β-amino acid $C_{14}Me$); ^{13}C NMR (DMSO-d_6, δ): 174.0 (C=O), 173.2 (C=O), 172.7 (C=O), 171.8 (C=O), 171.2 (C=O), 171.2 (C=O), 171.1 (C=O), 171.0 (C=O), 170.9 (C=O), 170.8 (C=O), 170.6 (C=O), 170.2 (C=O), 155.8 (Tyr, ζ), 129.7 (Tyr, δ, 2C), 127.9 (Tyr, γ), 115.0 (Tyr, ε, 2C), 61.3 (Ser, β), 60.7 (Pro, α), 56.3 (Tyr, α), 56.2 (Ser, α), 50.8 (Asn-6, α), 50.7 (Asn-1, α), 49.7 (Asn-3, α)49.6 (Gln, α) 47.2 (Pro, δ), 45.4 (β-amino acid, C_3), 41.7 (β-amino acid, C_2), 36.3 (Asn-1, β), 36.0 (Asn-3, β), 36.0 (β-amino acid, C_{13}), 35.3 (Asn-6, β), 34.9 (Tyr, β), 34.6

(β-amino acid, C_4), 33.7 (β-amino acid, C_{14}), 30.6 (Gln, γ), 29.4 (β-amino acid), 29.1 (β-amino acid, C_{15}), 29.0 (Pro, β), 28.9 (β-amino acid), 28.9 (β-amino acid), 28.7 (β-amino acid), 28.6 (β-amino acid), 28.6 (β-amino acid), 26.4 (β-amino acid, C_{12}), 26.4 (Gln, β), 25.3 (β-amino acid, C_5), 24.6 (Pro, γ), 19.0 (β-amino acid, $C_{14}Me$), 11.1 (β-amino acid, C_{16}). HRFAB-MS m/z 1085.5917 $[M+H]^+$, m/z 1085.5995 calculated for $C_{51}H_{81}N_{12}O_{14}$.

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