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NOTE

PJS, a novel isocoumarin with hexahydropyrimidine ring from *Bacillus subtilis* PJS

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Amicoumacin group of antibiotics, such as baciphelacin,[1] amicoumacins,[2] AI-77s,[3] xenocoumacins,[4] Y-05460M-A,[5] PM-94128,[6] Sg17-1-4,[7] bacilosarcins A, B[8] and lipoamicoumacins A–D,[9] is a small family of isocoumarin, which possesses the common chromophore, 3, 4-dihydro-8-hydroxyisocoumarin. Most members of amicoumacin group of antibiotics are produced by the genus *Bacillus* and exhibit various important bioactivities. The chromophore shows specific UV absorbance at 247 and 314 nm in methanol. Thus, a project based on HPLC-diode array screening and HPLC-MS dereplication was carried out to find new amicoumacin analogues from the secondary metabolites of *Bacillus* spp. As a result, PJS (**1**), a new isocoumarin antibiotic was discovered from the fermentation broth of *Bacillus subtilis* PJS. In this study, we wish to report the fermentation, isolation, physico−chemical properties, structural elucidation and biological activities of (**1**) (Figure 1).

The producing strain *Bacillus subtilis* PJS was isolated from the leaf of an unidentified plant collected at Luopu county, Hetian area, Xinjiang Province, People's Republic of China. On the basis of analysis of 16S rRNA, it was identified as *Bacillus subtilis* subsp. *inaquosorum*. A stock culture of the strain *Bacillus subtilis* PJS was maintained on modified Gause's no. 1 agar slant consisting of soluble starch (Beijing Qi Te Xin Chemical Co. Ltd. China) 20.0 g, NaCl 50.0 g, K_2HPO_4 0.5 g, KNO_3 1.0 g, $MgSO_4$ 1.0 g, $FeSO_4$ 0.02 g, glucose 1.0 g, peptone 0.5 g, tryptone 0.3 g and agar 20.0 g in 1.0 l distilled water (pH 8.0) at 4 °C. The stock culture was inoculated into 250 ml Erlenmeyer flasks containing 50 ml of seed medium, which was the same modified Gause's no.1 liquid medium as above, but no agar. The culture was incubated on a rotary shaker (180 r.p.m.) at 28 °C for 24 h. Fifty millilitres of the seed culture was transferred to a 5000 ml Erlenmeyer flask containing 1000 ml of the producing medium, which was the same as the seed medium. The fermentation was carried out at 28 °C for 48 h on a rotary shaker (180 r.p.m.).

Eighty liters of the fermentation broth was centrifuged at 4500 r.p.m. for 20 min, then, the supernatant was obtained and was extracted twice with 40 l of ethyl acetate each time. The organic layer was pooled and concentrated under reduced pressure at 37 °C to give yellow syrup (2.3 g). It was then separated by preparative thin layer chromatography on 10 × 10 cm plates (silica gel $60F_{254}$, Merck KGaA, Darmstadt, Germany) using $CHCl_3$-MeOH, 65:35 (v/v) as the developing solvent. Under UV 365 nm, bands with light blue fluorescence at $R_f = 0.35$ were scraped and then eluted with methanol to yield a semipurified sample (260 mg). After dissolved in 1 ml methanol, the sample was filtered through a 0.22 μm membrane and was further purified by HPLC on a shim-Pack PRC-ODS column (250 × 20 mm, Shimadzu Corp., Tokyo, Japan) with MeOH-H_2O, 55:45 (v/v) at 2 ml min^{-1}. Peak at $R_t = 26$ min, showed UV absorbance at 247 and 314 nm detected by prominence diode array detector (SPD-M20A, Shimadzu), was collected and pooled to yield 10.4 mg of (**1**) as a white powder.

Compound (**1**) was soluble in dimethyl sulfoxide (DMSO), MeOH, $CHCl_3$ and pyridine. Its m.p. was 79–80 °C. MW of (**1**) was found to be 435 by high-resolution ESI-MS, which showed $[M+H]^+$ at *m/z* 436.2089 (calcd 436.2079). The molecular formula was then established as $C_{21}H_{29}O_7N_3$, which has nine degrees of unsaturation. ^{13}C-NMR and DEPT spectra of (**1**) indicated 21 carbon signals could be attributed to three carbonyl carbons, six aromatic carbons and 12 aliphatic carbons including six carbons bonded to nitrogen or oxygen. The UV absorption of (**1**) at λ_{max}^{MeOH} nm (ε): 202(29 930), 246(6491) and 314(3491) was almost identical with that of isocoumarin compounds, such as Sg17-1-4, PM-94128, Y-05460M-A, bacilosarcins and AI-77-B. The IR spectrum (film) of (**1**) exhibited absorption bands at 3343, 2956, 1668, 1620, 1539, 1231, 807, 697 cm^{-1}, which indicated the presence of a benzoic acid moiety with a phenolic hydroxyl group and an amide group. All data above revealed (**1**) had a

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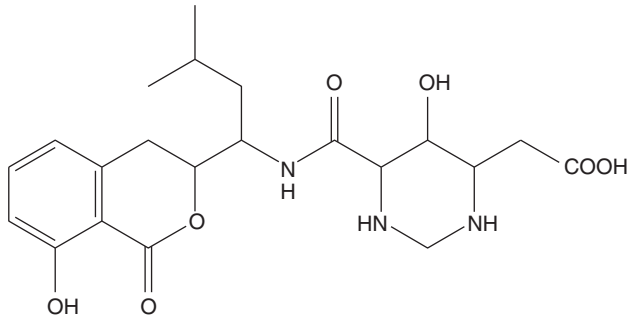
chromophore similar to the 3, 4-dihydro-8-hydroxyisocoumarin skeleton in its structure. NMR data of (**1**) in DMSO-d_6 (I) and CD_3OD (II) were listed in Table 1. By careful analysis of ^1H and ^{13}C-NMR, ^1H–^1H COSY, DEPT, ^{13}C–^1H COSY, HMBC spectra in DMSO-d_6 and in CD_3OD together with NOESY and D_2O exchange experiment in DMSO-d_6, chemical structure of (**1**) was elucidated. It consisted of structural fragment L and R, as shown in Figure 2.



Structure of PJS (**1**)

Figure 1 Structure of PJS (**1**).

Identification of structural fragment L was started from H-5, H-6 and H-7. The three aromatic protons were readily observed and displayed the coupling patterns for 1, 2, 3-trisubstituted benzenoid ring in ^1H-NMR. Their corresponding carbons were assigned by ^{13}C–^1H COSY. Other three aromatic carbons were observed and assigned by tracing cross peaks from H-5 and H-7 to C-8a, from H-6 to C-4a and C-8 in HMBC. Chemical shift of C-8 at 161.5 suggested a hydroxyl group should attach to C-8, which was confirmed by NOESY correlation from 8-OH proton at δ 7.24 to H-7 at δ 6.82 in DMSO-d_6. Identification of isopentyl group in structural fragment L was started from two methyl protons, H-1′ at δ 0.84 and H-2′ at δ 0.89 in ^1H-NMR in DMSO-d_6, which showed HMBC correlation to one another, meanwhile, to C-4′ at δ 39.1. H-1′, H-2′ and H-4′ showed cross peaks with a methine proton H-3′ at δ 1.61 in ^1H–^1H COSY. These observations suggested 3′-CH linked with 1′-CH_3, 2′-CH_3 and 4′-CH_2. A cross peak observed between H-4′ and H-5′, a methine proton at δ 4.20 in ^1H–^1H COSY confirmed the presence of the isopentyl group. H-5′ had a cross peak with H-3, a methine proton at δ 4.66 in ^1H–^1H COSY in DMSO-d_6, in turn, H-3 showed COSY correlation with H-4. H-4 showed HMBC correlation with C-8a in DMSO-d_6, meanwhile, H-4 also showed HMBC correlation with C-5 in CD_3OD. Thus, connectivity between C-4 and C-4a was established. Chemical shift of H-3 and its corresponding carbon, C-3 at δ 81.0 in low field indicated C-3 should attach to the oxygen of lactone

Table 1 NMR data of 1 in DMSO-d_6 (I) and CD_3OD (II)

	I		*II*	
Position	δ_C[a]	δ_H[b] *(mult, J Hz)*	δ_C[a]	δ_H[b] *(mult, J Hz)*
1	169.0	—	170.9	—
3	81.0	4.66 (brd, 12.5)	82.6	4.60 (dt, 12.5, 3.0, 2.5)
4	29.2	2.83 (m)	31.0	2.85 (dd, 16.2, 2.5)
		3.00 (dd, 16.0,13.0)		3.03 (dd,16.2, 12.5)
4a	140.7	—	141.7	—
5	118.1	6.77 (d, 7.0)	119.1	6.72 (d, 7.5)
6	136.2	7.45 (dd, 8.0, 7.0)	137.4	7.38 (dd, 7.5, 8.5)
7	115.5	6.82 (d, 8.0)	117.0	6.77 (d, 8.5)
8	161.5	—	160.7	—
8-OH	—	7.24 (s)	—	—
8a	108.3		109.5	—
1′	21.5	0.84 (d, 6.0)	22.0	0.88 (d, 7.0)
2′	23.3	0.89 (d, 6.5)	23.7	0.92 (d, 6.5)
3′	24.1	1.61 (m)	25.9	1.63 (m)
4′	39.1	1.33 (m)	40.8	1.38 (ddd, 14.0, 9.5, 4.0)
		1.67 (m)		1.78 (ddd, 14.0, 11.0, 4.5)
5′	48.1	4.20 (m)	50.3	4.30 (dt,10.5, 3.5, 3.0)
6′-NH	—	7.95 (d, 9.5)	—	—
7′	169.7	—	173.0	—
8′	80.4	3.69 (d, 9.0)	81.3	3.75 (d, 9.5)
9′	68.1	3.17 (m)	70.5	3.23 (t, 9.5, 9.5)
9′-OH	—	5.15 (d, 5.0)	—	—
10′	57.3	2.78 (m)	58.8	2.90 (ddd, 9.5, 9.0, 3.5)
11′	37.3	2.10 (dd, 15.0, 8.0)	38.2	2.23 (dd,15.0, 9.0)
		2.39 (dd, 15.0, 3.5)		2.62 (dd,15.0, 3.5)
12′-COOH	173.1	10.80 (brd)	176.8	—
13′-NH	—	6.82 (overlapped)		—
14′	78.0	3.99 (dd, 12.5, 10.5)	79.4	4.15 (d, 10.5)
		4.41 (dd, 10.5, 3.5)		4.50 (d, 10.5)
15′-NH	—	2.85 (dt, 12.5, 12.5, 5.0)		—

[a]Recorded at 125 MHz.
[b]Recorded at 500 MHz.

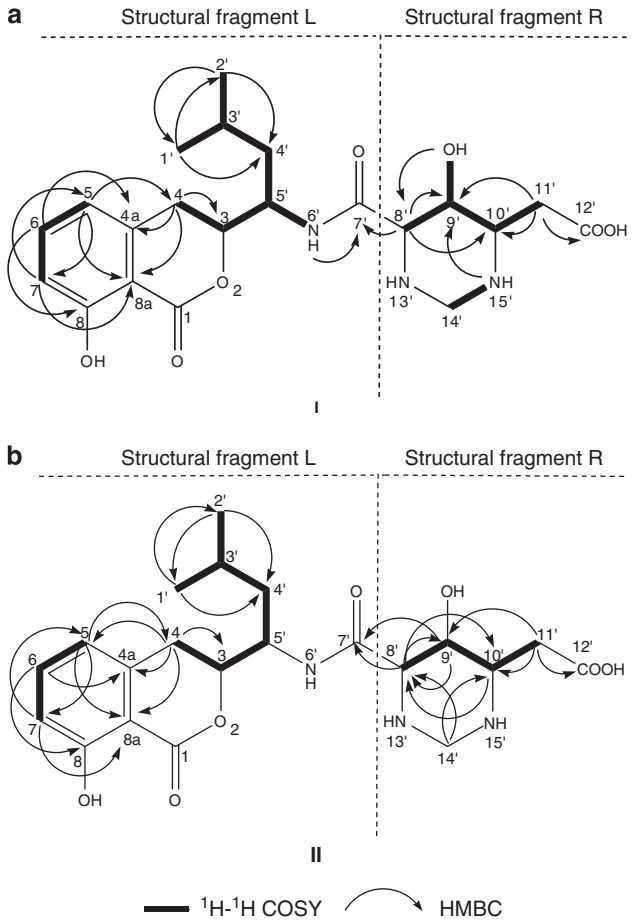


Figure 2 Summary of ^{1}H–^{1}H COSY and HMBC experiments of (**1**) in DMSO-d_6 (I) and CD_3OD (II).

ring to form 3, 4-dihydro-8-hydroxyisocoumarin skeleton. Furthermore, a cross peak observed between H-5′ and 6′-XH proton at δ 7.95 in ^{1}H–^{1}H COSY in DMSO-d_6 revealed 6′-XH bound with C-5′ at δ 48.1. Chemical shift of H-5′and C-5′ in downfield, as well as a cross peak observed between 6′-XH proton and 7′-C = O at δ 169.7 in HMBC in DMSO-d_6 revealed XH was NH, which bound to 7′-C = O to form an amide group. All data above revealed (**1**) had methylbutylamino-3, 4-dihydro-8-hydroxyisocoumarin, which was the common structural moiety of amicoumacin group of antibiotics. It was further confirmed by comparison of ^{1}H and ^{13}C-NMR data of (**1**) with that of known amicoumacin group of compounds such as PM-94128, Y-05460M-A, xenocoumacins and AI-77-B.

In structural fragment L of (**1**), there were seven degrees of unsaturation. Thus, two degrees of unsaturation were left in structural fragment R. Elucidation of structural fragment R was started from H-8′, a methine proton, which was readily observed as a doublet signal at δ 3.69 in ^{1}H-NMR in DMSO-d_6. By tracing the cross peaks from H-8′ to H-9′, from H-9′ to H-10′, from H-10′ to H-11′ in ^{1}H–^{1}H COSY, structural moiety as -CH(8′)-CH(9′)-CH(10′)-CH_2(11′)- was identified, and was further confirmed by cross peaks in HMBC between H-8′ and C-10′, between H-11′ and C-9′ in DMSO-d_6, in addition, between H-10′ and C-8′ in CD_3OD. A broad single signal in downfield at δ 10.8 in ^{1}H-NMR and a quaternary carbon signal in downfield at δ 173.1 in ^{13}C-NMR in DMSO-d_6 suggested the presence of 12′-COOH. A cross peak observed between H-11′ and C-12′ in HMBC suggested 12′-COOH was attached to C-11′.

Table 2 Antimicrobial activity of PJS

Test organisms	*MIC(μg ml^{-1})*
Staphylococcus aureus ATCC 25923	1
Oxacillin-resistant *Staphylococcus aureus* (ORSA)	2
Oxacillin-resistant *Staphylococcus epidermidis* (ORSE)	1
Enterococcus faecalis ATCC 29212	64
Enterococcus faecalis ATCC 33186	128
Bacillus subtilis ATCC 6633	64
Micrococcus luteus FDA 1001	0.25
Streptococcus pneumoniae CMCC 31001	0.5
Escherichia coli ATCC 25922	64
Pseudomonas aeruginosa ATCC 27853	>128
Salmonella enteritidis CMCC 50041	128
Klebsiella pneumoniae ATCC 700603	>128
Shigella flexneri CMCC 51571	2
Acinetobacter baumannii 2799	>128
Candida parapsilosis ATCC 22019	128
Candida albicans CCTCC AY 93025	128
Candida tropicalis CCTCC AY 93006	>128
Penicillium marneffei FRR 2161	>128

On the basis of chemical shift of H-9′ at 3.17, C-9′ at 68.1 and H-10′ at 2.78, C-10′ at 57.3 in DMSO-d_6, it was speculated that a hydroxyl group attached to C-9′ and a NH attached to C-10′. It was confirmed by D_2O exchange experiment. When drops of D_2O added into DMSO-d_6, a proton at δ 5.15 (d, J = 5.0), showed COSY correlation with H-9′ and HMBC correlation with C-8′ in DMSO-d_6, was absent. Thus, the proton was attributed to 9′-OH. Meanwhile, another proton at δ 2.85 (dt, J = 12.5, 12.5, 5.0), showed COSY correlation to H-14′ and HMBC correlation to C-9′ in DMSO-d_6, was also absent. Thus, the proton was attributed to 15′-NH. The absence of 15′-NH proton in D_2O exchange experiment led the multiplicity of H-10′ at δ 2.78(m), H-14′ at δ 3.99 (dd, J = 10.5, 12.5) and δ 4.41 (dd, J = 10.5, 3.5) simplified as the corresponding data in ^{1}H-NMR in CD_3OD. The phenomena suggested that 15′-NH not only attached to C-14′, but also attached to C-10′. By the calculation of element composition and degrees of unsaturation of (**1**), it was speculated that a hexahydropyrimidine ring must exist in structural fragment R and was composed of 8′-CH, 9′-CH, 10′-CH, 15′-NH, 14′-CH_2, and left 13′-NH at δ 6.82 overlapped by the aromatic proton of H-7. Cross peaks between H-14′ and C-8′, between H-14′and C-10′ in HMBC spectrum in CD_3OD further supported the speculation. Thus, the structural fragment R was elucidated.

HMBC correlations from H-9′ to C-7′ in CD_3OD revealed the linkage between the structural fragment L and structural fragment R was through C-8′ to C-7′ and completed the elucidation of the planar chemical structure of (**1**), which was further confirmed by downfield shift of 13′-NH proton, which could form H-bonding with 7′-C = O.

By analysis of ^{1}H–^{1}H coupling constants and cross peaks observation in NOESY, the relative configuration of H-3, H-8′, H-9′, H-10′ and H-15′ were elucidated. In the ^{1}H-NMR in CD_3OD, large diaxial coupling constants between H-3 and H-4, between H-8′ and H-9′, between H-9′ and H-10′ were observed as 12.5, 9.5 and 9.5 Hz, separatively, which indicated all protons including H-3, H-8′, H-9′, H-10′ had axial orientation. In the ^{1}H-NMR in DMSO-d_6, two large diaxial coupling constants between H-10′ and H-15′, between H-14′ and H-15′ were observed as 12.5 and 12.5 Hz, respectively. It indicated H-15′ also had axial orientation. As for H-13′overlapped by H-7, careful analysis of its vicinal protons in DMSO-d_6 in ^{1}H-NMR

showed H-13′ had no contribution to multiplicity and coupling constants of H-8′ and H-14′. In addition, no cross peak can be observed between H-13′ and H-8′ or H-14′ in NOESY in DMSO-d_6. Therefore, H-13′ either had an equatorial orientation or couldn't be defined, due to rapid interchange in DMSO-d_6.

PJS was active against Gram-positive and less active against Gram-negative bacteria and fungi. MICs were determined by broth microdilution method according to National Committee for Clinical Laboratory Standards guidelines.[10,11] The MIC values of PJS were listed in Table 2. The cytotoxicities *in vitro* against murine leukaemia P388 cells, human lung adenocarcinoma epithelial cell lines A549, human liver hepatocellular carcinoma cell lines HepG2, human pancreatic adenocarcinoma cell lines SW1990, human cervical cancer HeLa cells were measured by the methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay.[12] PJS exhibited activities with IC_{50} values of 103.6, 70.7, 39.5, 34.2, and 4.0 μM, separatively.

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