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NOTE

N-acetyl-3,4-dihydroxy-L-phenylalanine, a second identified bioactive metabolite produced by *Streptomyces* sp. 8812

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The emergence of multi-drug-resistant bacteria and the lack of new antibiotics in the antibiotic drug development pipeline is a major health concern.[1] Therefore, there is a constant need to search for novel antibacterial compounds. *Streptomyces* species are the richest source of bioactive metabolites, including antibacterial compounds.[2]

In this paper, we report the isolation of a second bioactive metabolite, N-acetyl-3,4-dihydroxy-L-phenylalanine, named JS-2 (Figure 1), from the supernatant of a *Streptomyces* sp. 8812 culture.[3,4] In the course of our screening program for antimicrobial compounds from actinomycetal metabolites for specific inhibitors of DD-peptidases (EC 3.4.16.4), we used exocellular DD-carboxypeptidase/transpeptidase (DD-peptidase) 64–575 II from *Saccharopolyspora erythraea* 64–575 II.[5] JS-2 exhibited slight inhibitory properties on the DD-peptidases 64–575 II and R39 from *Actinomadura* R39 (formerly *Streptomyces* R39).[6] It showed antimicrobial activity against *Bordetella bronchiseptica* (*B. bronchiseptica*), *Staphylococcus epidermidis* (*S. epidermidis*) and *S. aureus* (methicillin-resistant (MRSA) or vancomycin resistant (VRSA).

Streptomyces sp. 8812 fermentation culture was conducted as reported previously.[3] Isolation and purification of JS-2 was performed, guided by DD-peptidase 64–575 II assay.[3,7] Purification steps were done as follows: anion exchange resin IRA-400 (AcO^-), Backerbond SPE (PolarPlus C_{18} column, J.T. Baker, Phillipsburg, NJ, USA) and HPLC (Atlantis, dC18, Waters, Milford, MA, USA) (Supplementary Figure 1S; Supplementary Purification of JS-2).

The structure of JS-2 compound was determined using 1- and 2D NMR spectroscopy, UV, IR and MS measurements. Physico-chemical properties of the compound are shown in Table 1.

The molecular formula of JS-2 was determined to be $C_{11}H_{13}NO_5$ on the basis of HRESI-MS.

NMR data are shown in Table 1 and in the Supplementary materials (Supplementary Figures 2S, 3S, 4aS, 4bS). NMR spectra were recorded at 298 K on Varian INOVA 500 MHz spectrometer (Varian, Palo Alto, CA, USA) operated at 499.8 MHz for ^1H. The 2D experiments were run by using the standard Varian software, except the HMBC experiment.[8,9]

The ^1H NMR spectrum consists of seven signals: an ABM system in the aromatic part at 6.85, 6.70 and 6.79 p.p.m. (3H); an ABX aliphatic system at 2.80, 3.06 and 4.38 p.p.m. (3H); and a singlet from the CH_3 group at 1.93 p.p.m. (3H). Dynamic O**H**, COO**H** and N**H** protons are in fast exchange with bulk water and are not observed in the ^1H NMR spectrum. The assignment of protons was accomplished following the general rule of chemical-shift dispersion, using the proton–proton coupling pattern and the COSY spectrum.

Carbon resonances were obtained from the ^1H-{^{13}C} HSQC (Supplementary Figures 4aS, 4bS) (six signals of proton-bearing carbon atoms) and ^1H {^{13}C} HMBC (five signals of the quaternary carbon atoms) spectra. The ^1H {^{13}C} HMBC spectrum was used also as a final tool to make assignment of carbon resonance and to confirm the structure of JS-2.

IR absorption at 3366 and 1724–1608 cm^{-1} suggested the presence of hydroxyl and carbonyl groups in the structure (Supplementary Figure 5S). The UV spectrum showed a typical pattern for an aromatic compound. Strong absorption at 280 nm confirmed a phenolic moiety (Supplementary Figure 6S). According to the interpretation of these data, compound JS-2 presents the structure of N-acetyl-3,4-dihydroxy-L-phenylalanine.

Unequivocal confirmation of the JS-2 structure has been obtained by its comparison with a synthetic sample prepared by acetylation of

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L-Dopa with excess of acetic anhydride in hot water. The analytical sample was obtained by preparative HPLC (RPC18, 5% acetonitrile in 0.1% aq. AcOH). The spectral data of the obtained product fully agreed with the above analysis.

Under the procedures described previously,[10–12] the inhibitory effect of DD-carboxypeptidases activity was measured. Thioester C_6H_5-CO-NH-(D)CH(CH_3)-CO-S-CH_2-COOH was used as a substrate[10,12] (Supplementary Bioassay). JS-2 and structurally related compounds, 3,4-dihydroxy-L-phenylalanine (Sigma, St Louis, MO, USA), 3,4-dihydroxy-D-phenylalanine (Sigma) and N-acetyl-3,4-dihydroxy-D-phenylalanine (synthesized analogously as L- form) were examined. The IC_{50} values of these compounds for DD-peptidases 64–575 II and R39 are presented in Table 2.

Results showed that IC_{50} values ranged from 3.96 to 17.79 mM. The IC_{50} values of JS-2 for DD-peptidase 64–575 II and for DD-peptidase R39 were compared with the IC_{50} values of L-Dopa, D-Dopa and N-acetyl-D-Dopa for these enzymes. The IC_{50} values of JS-2 for DD-peptidases 64-575 II and R39 were the lowest. In each case, L-isomer of the compound inhibited both DD-peptidases in lower concentration than the D-isomer. Furthermore, the inhibitory activity of evaluated compounds was weaker for DD-peptidase 64–575 II than for DD-peptidase R39.

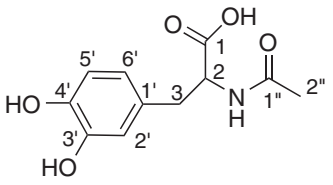

Figure 1 Chemical structure of JS-2.

The IC_{50} values of β-lactam antibiotics for DD-peptidase 64–575 II and R39 were compared with the IC_{50} values of JS-2 for both enzymes.[5,13] IC_{50} values of many β-lactam antibiotics (ranging from 1.6 mM to 0.015 μM) for DD-peptidase 64–575 II, as well as for DD-peptidase R39 (ranging from 5 to 0.03 μM) were lower than the IC_{50} value of the investigated compounds for these enzymes. JS-2 exhibited weak inhibitory effects on the DD-peptidases 64–575 II and R39. However, so far, no protoalkaloid inhibitors of DD-peptidases have been described.

To examine the specificity spectrum of the JS-2 compound, inhibition assays were performed for a range of common proteolytic enzymes: pancreatic elastase (type IV) from porcine pancreas, carboxypeptidase A from bovine pancreas, trypsin from porcine pancreas, and papain from Papaya latex, according to the procedures described in our previous paper,[3] and leucine aminopeptidase (type IV-S) from porcine kidney microsomes as reported by Cahan *et al.*[14] It was determined that at a concentration of 1.4 mM, the JS-2 compound causes 50% inhibition of carboxypeptidase A, whereas the rest of the enzymes remained active at higher concentration, 1.84 mM.

Antibacterial activity of JS-2 was tested by liquid microdilution method (Spectrostar Omega, BMG Labtech, Offenburg, Germany), under standard conditions according to the CLSI references.[15]

A panel of Gram-positive and Gram-negative bacterial strains was used (Supplementary Bioassay). The JS-2 compound showed weak antibacterial activity against *B. bronchiseptica*, *S. aureus* (MRSA and VRSA) and *S. epidermidis* (Table 2). Moreover, JS-2 showed a 4-h delay of growth of *S. aureus* (MSSA) at 256 μg ml^{-1}, a 2.5-h delay of bacterial growth at concentration 128 μg ml^{-1}, and a 2-h delay of bacterial growth at 64 μg ml^{-1}, although the general MIC value for

Table 1 (a) Physico-chemical properties and (b) ^{1}H and ^{13}C NMR data of JS-2

Appearance	*White-cream crystal*
(a)	
Molecular weight	239
Molecular formula	$C_{11}H_{13}NO_5$
HRESI-MS (m/z)	
Calcd	238.07210 $(M-H)^-$
Found	238.07226 $(M-H)^-$
UV (MeOH) λ_{max} nm (ε)	231 (2850), 281 (2290)
$[\alpha]_D^{24}$ +41.1^O	(c=1.11, H_2O)
IR (KBr) ν_{max} (cm^{-1})	3366, 1724, 1608, 1527, 1445, 1375, 1286, 1115, 814, 589

Position	δ_C[a]	δ_H[a] (*J in Hz*)	^{1}H-^{13}C *correlations*	δ_N[a]
(b)				
1	180.9			
2	59.0	4.38 (dd, 8.7, 4.9)	C1', C3	
3	39.6	2.80 (dd, 14.0, 8.7)	C1', C6', C2', C2, C1	
		3.05 (dd, 14.0, 4.9)	C1', C6', C2', C2, C1	
1′	133.2			
2′	119.5	6.79 (d, 2.1)	C4', C6', C3, C3'	
3′	146.3			
4′	145.2			
5′	118.8	6.85 (d, 8.1)	C3', C1', C4', C6'	
6′	124.2	6.70 (dd, 2.1, 8.1)	C4', C2'	
1″	175.9			
2″	24.5	1.93 (s)	C1"	
NH				−251

[a]The ^{1}H and ^{13}C chemical shifts, δ, given in p.p.m., were referenced against the internal reference TSPA-d_4, solvent H_2O/D_2O (9:1).

Table 2 Biological activity of JS-2 and structurally related compounds: (a) inhibitory effect on DD-peptidases and (b) *in vitro* antibacterial activity (MIC μg ml^{-1})

	Inhibition, IC_{50} (mM)			
DD-peptidases	*JS-2 (N-Ac-L-Dopa)*	*L-Dopa*	*N-Ac-D-Dopa*	*D-Dopa*
(a)				
DD-peptidase 64-575 II	10.97	13.84	11.85	17.79
DD-peptidase R39	3.95	5.11	4.92	6.05

	(MIC μg ml^{-1})			
Microorganism	*JS-2*	*L-Dopa*	*N-Ac-D-Dopa*	*D-Dopa*
(b)				
S. aureus (MSSA)	>256	128	640	80
S. aureus (MRSA)	256	128	640	40
S. aureus (VRSA)	256	128	320	40
S. epidermisis	64	32	320	40
B. bronchiseptica	40	20	320	40
Acinetobacter baumanii	>640	80	>640	640
Proteus vulgaris	>640	160	640	160
P. mirabilis	>640	160	>640	160
Burkholderia cepacia	>640	640	>640	320
Pseudomonas aeruginosa	>640	640	>640	640
Stenotrophomonas maltophilia	>640	160	640	40

this bacteria was higher than 256 μg ml^{-1} (Supplementary Figure 7S). Additionally, for comparison, the antimicrobial activity of compounds: 3,4-dihydroxy-L-phenylalanine, 3,4-dihydroxy-D-phenylalanine and N-acetyl-3,4-dihydroxy-D-phenylalanine were examined. These compounds showed a slightly higher activity than JS-2 (Table 2).

Moreover, other activities of JS-2 were also examined, including genotoxicity and hemolytic properties. For assessment of genotoxicity, two genetically modified *Bacillus subtilis* strains, M45 rec$^-$ and H17 rec$^+$, obtained from Dr Yoshito Sadaie (Department of Induced Mutation, National Institute of Genetics, Shizuoka, Japan) were examined by the disc-diffusion method according to Kada's procedure.[3,16] The JS-2 compound did not inhibit the growth of the tested *B. subtilis* strains; hence, it was determined as nongenotoxic.

The hemolysis test was carried out according to a method described in literature.[3] No hemolytic activity of JS-2 was observed on human erythrocytes.

In conclusion, N-acetyl-3,4-dihydroxy-L-phenylalanine (JS-2) had been identified earlier as a *Streptomyces akiyoshiensis* metabolite[17] and obtained by semisynthesis using microorganisms; for example, *Pseudomonas striafaciens* and *Vibrio tyrosinaticus*.[18,19] Only its antitumour properties were studied so far.[17,20] DD-peptidase inhibitory activity and antimicrobial properties of JS-2 have not been previously defined. JS-2 compound belongs to a group of protoalkaloids, β-phenylethylamines and -amides isolated usually as plant metabolites.[21] Results of this study demonstrate that β-phenylethylamides, like JS-2, may show antimicrobial activities and inhibitory properties on some closely related proteinases (for example DD-peptidases).

Natural compounds themselves are highly important, as they constitute also the basis for developing synthetic drugs. In the last 10 years among the launched antibiotics, there are more natural product-derivative new antibiotic templates compared with those synthetically derived.[1] Protoalkaloid JS-2 shows weak antibacterial activity; however, due to the lack of hemolytic properties and genotoxicity, it can be considered as a candidate for chemical modifications that can increase its antibacterial activity.

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Supplementary Information accompanies the paper on The Journal of Antibiotics website (http://www.nature.com/ja)