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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 C₁₈ 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 C₁₁H₁₃NO₅ 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 CH₃ group at 1.93 p.p.m. (3H). Dynamic OH, COOH and NH 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-¹³C HSQC (Supplementary Figures 4aS, 4bS) (six signals of proton-bearing carbon atoms) and ¹H ¹³C HMBC (five signals of the quaternary carbon atoms) spectra. The ¹H ¹³C 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 C₆H₅-CO-NH-(D)CH(CH₃)-CO-S-CH₂-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 IC₅₀ values of these compounds for DD-peptidases 64–575 II and R39 are presented in Table 2.

Results showed that IC₅₀ values ranged from 3.96 to 17.79 mM. The IC₅₀ values of JS-2 for DD-peptidase 64–575 II and for DD-peptidase R39 were compared with the IC₅₀ values of L-Dopa, D-Dopa and N-acetyl-D-Dopa for these enzymes. The IC₅₀ 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.

The IC₅₀ values of β-lactam antibiotics for DD-peptidase 64–575 II and R39 were compared with the IC₅₀ values of JS-2 for both enzymes.^{5,13} IC₅₀ 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 IC₅₀ 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

Figure 1 Chemical structure of JS-2.

Table 1 (a) Physico-chemical properties and (b) ¹H and ¹³C 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)			
[α] _D ²⁴ +41.1°	(c=1.11, H ₂ O)			
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

^aThe ¹H and ¹³C chemical shifts, δ, given in p.p.m., were referenced against the internal reference TSP-d₄, solvent H₂O/D₂O (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 $\mu\text{g ml}^{-1}$)

	Inhibition, IC_{50} (mM)			
	JS-2 (N-Ac-L-Dopa)	L-Dopa	N-Ac-D-Dopa	D-Dopa
<i>DD-peptidases</i>				
(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 $\mu\text{g ml}^{-1}$)			
	JS-2	L-Dopa	N-Ac-D-Dopa	D-Dopa
<i>Microorganism</i>				
(b)				
<i>S. aureus</i> (MSSA)	> 256	128	640	80
<i>S. aureus</i> (MRSA)	256	128	640	40
<i>S. aureus</i> (VRSA)	256	128	320	40
<i>S. epidermidis</i>	64	32	320	40
<i>B. bronchiseptica</i>	40	20	320	40
<i>Acinetobacter baumannii</i>	> 640	80	> 640	640
<i>Proteus vulgaris</i>	> 640	160	640	160
<i>P. mirabilis</i>	> 640	160	> 640	160
<i>Burkholderia cepacia</i>	> 640	640	> 640	320
<i>Pseudomonas aeruginosa</i>	> 640	640	> 640	640
<i>Stenotrophomonas maltophilia</i>	> 640	160	640	40

this bacteria was higher than $256 \mu\text{g ml}^{-1}$ (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* metabolite¹⁷ 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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