ORIGINAL ARTICLE

A novel isoquinoline alkaloid, DD-carboxypeptidase inhibitor, with antibacterial activity isolated from *Streptomyces* sp. 8812. Part II: Physicochemical properties and structure elucidation

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A novel antimicrobial agent labeled JS-1, being a member of isoquinoline alkaloids, of molecular formula $C_{10}H_9NO_4$ was isolated from the culture broth of *Streptomyces* sp. 8812. In this study, we present the structure based on physicochemical and spectroscopic NMR investigations and on quantum chemical structure modeling. The structure of a molecule suggests the biosynthetic path starting from 3'-hydroxy tyrosine. The synthesis was undertaken and it resulted in NMR data that fully agree with the presented analysis.

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INTRODUCTION

The increasing bacterial resistance to antibiotics causes a great therapeutic problem because of the widespread presence of multiresistant pathogenic bacteria. The solution of this problem involves screening for microorganisms producing novel antimicrobial drugs. Streptomycetes are well-known producers of antibiotic and other bioactive metabolites.¹ The purpose of this work was the investigation of novel antimicrobial agents, inhibitors of DD-carboxypeptidase/transpeptidase (DD-peptidase). In our screening program for new inhibitors of DD-peptidase 64–575 II (EC 3.4.16.4)^{2–4} from microbial secondary metabolites, a novel isoquinoline alkaloid with documented antibacterial activity, JS-1, was isolated from the culture broth of *Streptomyces* sp. 8812.⁵ In a recent paper,⁶ we have described the taxonomy, fermentation of producing strain and isolation, as well as the purification and biological activity of novel isoquinoline alkaloid. In this study, we present the structure based on physicochemical and spectroscopic NMR investigations, quantum chemical structure modeling and synthesis protocol, which confirmed an established structure and chirality.

RESULTS AND DISCUSSION

The physicochemical properties of a compound are shown in Table 1.

JS-1 is soluble in water (phosphate buffer pH 7.2), and insoluble in $CHCl_3$ or acetonitrile. It is soluble, but degrading, in methanol and dimethylsulfoxide.

The molecular formula of JS-1 was determined to be $C_{10}H_9NO_4$ on a basis of high resolution ESI-MS $[(M+H)^+]$, 208.06043 m/z (-0.03 mmu error), thus requiring 7 degrees of unsaturation. The IR spectrum of JS-1 showed a broad hydroxyl band at 3436 cm^{-1} , and the multiple bands in a region of $1600\text{--}1700\text{ cm}^{-1}$ may indicate the presence of an aromatic carbonyl group, a $C=N$ double bond at 1681 cm^{-1} , a carboxylate group as well as a carbonyl group conjugated to a double bond, or an enamine at the $C-N$ bond at 1636 cm^{-1} . The UV absorption maximum at 321 nm suggests the presence of a conjugated system.

JS led us to suggest an isoquinoline structure shown in Figure 1.

It is essential to know which tautomeric form has the biological activity and therefore all spectroscopic investigations were carried out in physiological conditions, that is, pH 7.2 in phosphate buffer. The tautomeric equilibrium, if present, is fast in the NMR timescale and therefore only one set of sharp signals is observed at room temperature in buffered water solution. In Table 2, NMR data are presented that allow the confirmation of a structure.

A deceptively simple ^1H NMR spectrum, consisting of only three singlets, at 6.53, 7.04 and 8.23 p.p.m., and an ABM system, at 4.39, 3.29 and 3.18 p.p.m., with 5 heteroatoms present makes structure assignment ambiguous and requires a detailed recourse to $^1H\sim^1H$ and $^1H\sim^{13}C$ correlations in heteronuclear single quantum coherence (HSQC), heteronuclear multiple bond correlation (HMBC) and

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double pulsed field gradient spin-echo NOE,⁷ respectively. In addition, dynamic OH, COOH and NH protons are in fast exchange with bulk water, thus making impossible the assignment of nitrogen atom functionality in the latter case, by means of running the ¹H-¹⁵N HSQC spectrum or drastically less sensitive ¹H-¹⁵N HMBC correlation.

¹³C Chemical shifts of proton-bearing carbon atoms were established using the HSQC spectrum. It proved useful to distinguish two carbon atoms of the same chemical shifts, that is, C-5 and C-8, 117.1 p.p.m., and to assign the two geminal protons to carbon atom C-4 at 3.29 and 3.18 p.p.m. This latter observation brings about the important conclusion that the molecule has a stable stereogenic center giving rise to the nonequivalence of the geminal protons. The ³J (H, H) coupling constants in the ABM system of 7.4 and 9.2 Hz indicate that the stereogenic center is in vicinal arrangement with the geminal protons. Figure 2 presents key correlations split for clarity into two projections. Only one tautomer, ketone, is shown for simplicity. The HMBC correlations in a left projection prove the connection of an AMX aliphatic system to aromatic carbon atom C-5. The NOE

contacts also prove the spatial vicinity of 1-H and 8-H, both being distant from 5-H. Comparable NOE effects in both directions point to the isolated two-spin system, in agreement with the structural environment. The NOE contacts in the right projection in Figure 2 confirm the connectivity of 5-H with the ABM system. In addition, nearly equal NOEs from H-5 to H^{4a} and H-4^b suggest that they are nearly equidistant to 5-H. Three-bond HMBC correlations from 8-H to C-1 and C-6 and from 1-H to C-3 allow for the unambiguous assignment of δ_C 159.5 and 168.8 p.p.m. to their positions in a structure, C-1 and C-6, respectively. Our experience in an enamine carbon chemical shifts suggests that the same assignment as=C-N carbon atom with a formally single bond in a conjugated system of α,β -unsaturated ketones gives resonances up to 155 p.p.m. for an alkyl disubstituted nitrogen atom.⁸

In contrast, the C=N bonds in imines give carbon resonances at approximately 160 p.p.m.^{9,10}

It is commonly recognized that aromatization leads to enhanced stability and defines the thermodynamically more stable species in equilibrium.

To address in more depth the position of tautomeric equilibrium, we have performed the theoretical calculations of the ¹H, ¹³C chemical shift and the geometry of all possible tautomers (see Supplementary Figure 2S) using the PCM protocol in the H₂O box at the B3LYP/6-31/+6(2d₁₈) basis set level. The chemical shift data are compiled in Supplementary Tables 1S–3S in the Supplementary materials along with the structures. Scarce data regarding the ¹³C chemical shifts in a similar system are available in the literature.¹²

The calculated chemical shifts of conformers suggested for the equilibrium are shown in Figure 3. The protonated dihydroxy imine tautomer **4a** can also be considered as a species present in equilibrium.

Table 1 Physicochemical properties of JS-1

Appearance	Yellow powder
$[\alpha]_D^{23}$	21.1° (c=0.185, H ₂ O)
Molecular formula	C ₁₀ H ₉ NO ₄
LR ESI-MS (m/z)	
Positive	208 (M+H) ⁺
Negative	206 (M–H) [–]
HRESI-MS (m/z)	
Calcd	208.06043
Found (M+H) ⁺	208.06040
UV λ_{max} nm (ϵ) (H ₂ O)	397.92 (3475), 321.33 (943), 266.38 (2225)
IR (KBr) ν_{max} cm ^{–1}	3436, 1681, 1636, 1131, 1075, 986, 984, 804, 724

Figure 1 Postulated structure of JS-1.

Figure 2 Key HMBC correlations and NOE¹¹ contacts in JS-1. For clarity, only one tautomer is shown.

Table 2 ¹³C (176 MHz) and ¹H (500 MHz) NMR data for JS-1 in buffered water solution (pH 7.2)

Proton spectral parameters, scalar, ¹ H– ¹³ C, and dipolar, ¹ H– ¹ H, correlations				
C	δ_C	δ_H	HMBC correlations	DPFGSE NOE contacts (%)
1	159.5	8.21(s)	C-3, C-4a, C-8, C8a	8-H (2.9)
3	55.8	4.39(dd, 7.4, 9.2)	C-1, C-4, C-4a, C=O	4-H ^a (1.7); 4-H ^b (1.1)
4	29.2	3.29(dd, 7.4, 16.7) 4-H ^a 3.18(dd, 9.2, 16.7) 4-H ^b	C-3, C-4a, C-8a, C=O C-3, C-4a	3-H(1.9); 5-H(1.5)*
4a	135.0			
5	117.1	6.53(s)	C-4, C-7, C-8a	4-H ^a (1.1); 4-H ^b (1.2)
6	168.8			
7	146.5			
8	117.1	7.04(s)		1-H(2.2)
8 ^a	110.5			

Abbreviations: DPGSE NOE, double pulsed field gradient spin-echo NOE; HMBC, heteronuclear multiple bond correlation.

*These values represent the effect of simultaneous irradiation of both 4-H^a and 4-H^b protons, which cannot be selectively irradiated due to strong coupling.

Figure 3 Comparison of calculated, *in vacuo*, ^1H and ^{13}C chemical shift in tautomers **1a** and **4a** of JS-1 with experimental ones, $^1\text{H}, ^{13}\text{C}_{\text{exp}}$, assigned to specific carbon atoms using heteronuclear multiple bond correlation and double pulsed field gradient spin-echo NOE data. The hypothetical structures **2a** and **3a** are also shown (see text).

Figure 4 Proposed equilibrium for JS-1 at basic conditions.

The predominance of the enamine tautomer in solution is further confirmed in ^{15}N NMR, which gives the δ (^{15}N) = -229.0 p.p.m. (versus external CH_3NO_2), at pH 7.2. This chemical shift is representative both for nitrogen atoms in enamine, **1a** or **1b**, and protonated imine (zwitter ion) **4a**. A small amount of the form **2a** can be allowed in equilibrium with **1a**. The **3a** form cannot be considered on the basis of the observed ^{15}N chemical shift as its nitrogen resonance appears at approximately -300 p.p.m. from nitromethane. The situation above applies to experimental conditions close to the isoelectric point.

Under more basic conditions, the carboxylate is deprotonated and the equilibrium shown in Figure 4 applies. As discussed above, the ^{15}N chemical shift allows a small amount of imine in equilibrium.

It is worth noting that near the isoelectric point, $\text{pI}=6.0$, for this compound (see to Supplementary Materials), the molecule can be both neutral and ionized and therefore the species discussed in Figures 3 and 4 should be considered.

Figure 5 presents the possible species in tautomeric equilibrium in solution. Structures **1a** and **1b** are the conformers having planar ring B in **1a** and twisted in **1b**.

The calculations carried out also allow comparison of the geometry of both tautomers, relating it to experimental observables. The geometry of the $\text{N}-\text{C}_{\text{sp}^3}-\text{C}_{\text{sp}^3}$ motif is expected to be most sensitive to tautomerization because of the changes in nitrogen hybridization and bond length around it. The ABM system of investigated molecule offers an experimental check of the calculated geometry along the C4–C3 bond in both conformers shown in Figure 5.

It is shown in Figure 5 that the $\text{H}-4^{\text{a}}$ and $\text{H}-4^{\text{b}}$ protons in **1b** are both in synclinal positions with respect to the H-3 proton, having torsion angles of -69.9° and $+47.0^\circ$, respectively. This spatial arrangement leads to $^3\text{J}(\text{H}-3, \text{H}-4^{\text{a}})$ and $^3\text{J}(\text{H}-3, \text{H}-4^{\text{b}})$ vicinal coupling constants 1.6 and 7.7 Hz, in disagreement with observed experimental observation, 7.4 and 9.2 Hz. On the contrary, in **1a**, $\text{H}-4^{\text{b}}$ is in synclinal and $\text{H}-4^{\text{a}}$ in anti-periplanar position to H-3 with torsion angles of -51.0° and -167.0° leading to 4.5 and 13.7 Hz, respectively, again in disagreement with experimental values. Averaging of the respective coupling constants in both forms results in agreement with the experiment and this fact gives strong evidence to the predominance of this tautomeric form in equilibrium in solution.

With respect to the above discussion, Lakhli *et al.*¹³ report the ^1H NMR spectrum of closely related 3,4-dihydro-6,7-dimethoxy-3-methylcarboxylate-isoquinoline. Unfortunately, the geminal nonequivalence of $\text{H}-4^{\text{a}}$ and $\text{H}-4^{\text{b}}$ was not observed and the reported splitting in the A_2M system is 10.2 Hz.

Figure 5 Established structure of JS-1 based on a comparison of calculated and experimental ^1H and ^{13}C chemical shifts. Projection along the C4–C3 bond in both conformers 1a, 1b calculated using the PCM protocol.

Scheme 1

Finally, the configuration of the C-3 carbon atom should be addressed. The structure of a molecule suggests the biosynthetic path starting from the 3'-hydroxy tyrosine. This defines the (S) C-3 configuration of natural L-amino acid. The verification of this hypothesis was performed by comparison of a specific rotation with the synthetic product.

The synthesis was undertaken and its product yielded NMR data that fully agree with the above analysis (Scheme 1). Spectrum 1S (Supplementary Information, Figure 1S) presents the heteronuclear multiple bond correlation trace of a synthetic product which verifies proper structure elucidation of JS-1.

EXPERIMENTAL SECTION

IR spectrum was obtained on an FT-IR-1600 Perkin-Elmer spectrophotometer (Perkin-Elmer, Ramsey, MN, USA). UV spectrum was recorded on a Jasco V-630 UV-Visible spectrophotometer (Jasco, Tokyo, Japan). The pI was established using this facility for UV measurements and the Orion pH meter (Orion, Boston, MA, USA).

The optical rotation was measured with a Jasco P-2000 digital polarimeter. A low-resolution mass spectrum was recorded on an HPLC-MS (Hewlett-Packard 1100; API 365, PESCIEX) mass spectrometer (Hewlett-Packard, Santa Clara, CA, USA). A high-resolution mass spectrum was recorded on a Bruker APEX-Q (9.4T) spectrometer (ESI, positive-ion mode (Bruker, Leipzig, Germany)).

The NMR spectra were recorded at 303 K on a Varian INOVA 500 spectrometer (Varian, Palo Alto, CA, USA) operated at 499.8, 125.7 and 50.51 MHz for ^1H and ^{13}C , and ^{15}N , respectively. A 0.1 mg amount of compound was dissolved in 0.7 ml of $\text{H}_2\text{O}/\text{D}_2\text{O}$ (9:1), pH 7.2 and transferred to a 5 mm NMR tube. Chemical shifts, δ given in p.p.m., were referenced against internal

reference (trimethylsilyl)propanoic acid. The spectrometer was equipped with an inverse $^1\text{H}\{^{31}\text{P}-^{15}\text{N}\}$, the 5 mm Nalorac probe with an actively shielded z-gradient coil (ID-pulsed field gradients).

^1H NMR was run using the standard method of presaturation of residual water implemented in Varian software.

HSQCAD spectra:¹⁴ The Echo-antiecho phase-sensitive ^1H - ^{13}C HSQC (adiabatic version) NMR spectra were obtained with a spectral width of 6000 Hz, 2048 points in the ^1H dimension and 20 100 Hz, 400 increments in the ^{13}C dimension; 128 transients per t_1 increment, with a relaxation delay of 1.0 s and $^1\text{J}(\text{C},\text{H})=135$ Hz. The data were linearly predicted to 1600 points and zero-filled to 8192 points in F_1 before Fourier transformation.

The $^1\text{H}/^{13}\text{C}$ -HMQC (heteronuclear multiple quantum coherence) technique with pulsed field gradients coherence selection using two pulsed field gradient pulses of relative amplitudes of $+(\gamma\text{I}+\gamma\text{S})$: $-(\gamma\text{I}-\gamma\text{S})$ and $-(\gamma\text{I}-\gamma\text{S})$: $+(\gamma\text{I}+\gamma\text{S})$, for heteronuclear echo and antiecho, respectively was used. The S-spin 90 degree pulse low-pass filter was used to suppress one-bond correlation. Such implementation enabled us to obtain a nearly pure absorption line shape along F_1 , combined with an absolute value mode in F_2 dimension.^{15,16} The $^1\text{H}\{^{13}\text{C}\}$ HMQC spectra were recorded with the following experimental conditions: an acquisition time of 0.2 s, spectral windows of 5000 (F_2) and 25500 (F_1) Hz, 2048 data points in the ^1H dimension, 256 increments in the ^{13}C dimension, $^1\text{J}(\text{C},\text{H})=8$ Hz, a 1.3 s relaxation delay and 512 transients per increment. The data were zero filled to 4096 points in F_1 and processed using sine bell multiplication in t_2 and cosine window function in t_1 dimensions before Fourier transformation. The proton and carbon $\pi/2$ pulse lengths were 8.0 and 11.5 μs , respectively.

$^1\text{H}/^{15}\text{N}$ -HMQC ^{15}N parameters: F_2 , 5000 Hz; F_1 , 10000; number of increments, 80; 512 scans/increment; delay, 120 ms. A low-pass filter (see above) was not used.

Double pulsed field gradient spin-echo NOE experiments were run using a pulse sequence published by Stott *et al.*¹⁷ using shaped selective pi soft pulses generated by a standard Varian program. The rsnob pi pulse was calibrated for each individual multiplet.

(S)-6,7-dihydroxy-3,4-dihydroisoquinolin-3-carboxylic acid synthesis: To a warm solution of *N*-formyl-(L)-DOPA¹⁸ (70 mg, 0.31 mmol) in acetonitrile (13 ml) was added phosphorus oxychloride (342 mg, 2.24 mmol) and the mixture was heated under reflux for 1 h. The precipitate was separated and the filtrate was evaporated to give brown oil. The product was purified by preparative HPLC on an RP C18 column (Nucleosil, Duren, Germany) using first 0.1% TFA in water as eluent and then 10% acetonitrile in 0.1% aqueous TFA. Spectral data is identical as for JS-1.

HRESI-MS calculated for $C_{10}H_{10}NO_4$ (M+H)⁺, 208.06043. Found 208.06093.

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