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Rubiyunnanins A and B, two novel cyclic hexapeptides from *Rubia yunnanensis*

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ABSTRACT

Two novel cyclic hexapeptides, named rubiyunnanins A (**1**) and B (**2**), were isolated from the roots of *Rubia yunnanensis* (Franch.) Diels. Their structures were elucidated extensively by spectroscopic analysis and theoretical computation. Possible biosynthetic pathways for **RAs**, **1**, and **2** were proposed. Compound **2** showed moderate cytotoxicities against 11 cancer cell lines and inhibited nitric oxide (NO) production in LPS and IFN-γ-induced RAW 264.7 murine macrophages.

The genus *Rubia* is a prolific source of rubiaceae-type bicyclic hexapeptides, anthraquinones, and arborinane-type triterpenoids.[1] Among them, rubiaceae-type bicyclic hexapeptides (RA-series cyclopeptides, RAs) attracted great interest for their distinctive bicyclic structural feature and potential antitumor activities in vivo and in vitro.[2,3] Regular RAs are homodicyclohexapeptides mainly composed of one D-α-alanine, one L-α-alanine, three modified *N*-methyl-L-α-tyrosines, and another L-α-amino acid. The most unusual feature is a 14-membered ring formed by a phenolic oxygen linkage between two adjacent tyrosines with a *cis* peptide bond, and the 14-membered ring is fused to the 18-membered cyclic hexapeptide ring. Up to now, 30 RAs have been isolated only from four Rubiaceae plants, that is, *Bouvardia ternifolia*, *Rubia cordifolia*, *R. akane*, and *R. yunnanensis*.[4–7] *R. yunnanensis* is native to Yunnan province, China, and its roots are widely used as a Chinese traditional medicine for the treatment of tuberculosis, menoxenia, rheumatism, contusion, hematemesis, anemia, and lipoma.[8] Previously, one new RA (RY-II) and two known RAs (RA-V and RA-XII) were reported from *R. yunnanensis*.[4,9,10] In our search for structurally and pharmacologically interesting cyclopeptides from Chinese medicinal plants, two new cyclic hexapeptides, rubiyunnanins A (**1**) and B (**2**), with the unique skeletons, were isolated from the titled plant roots (Fig. 1). Herein, we describe their isolation, structural elucidation, possible biosynthetic pathways, and bioactivities.

Rubiyunnanin A (**1**)[11] was obtained as an amorphous solid. Its molecular formula was determined to be $C_{40}H_{50}N_6O_9$ by positive HRESIMS (*m/z* 781.3545, $[M+Na]^+$, calcd 781.3536), indicating 19 degrees of unsaturation. Its UV spectrum showed the existence of phenyl groups based on the absorptions at 204 and 285 nm. The IR spectrum exhibited absorption bands at 3440, 3385, and 1656 cm^{-1} ascribable to OH, NH, and CO groups. The ^1H and ^{13}C NMR spectra of **1** in methanol-d_4 (Table 1) displayed some characteristics of RAs and demonstrated the presence of two conformers in a ratio of 90:10.[12,13] Further analysis of 1D and 2D NMR spectral data of **1** displayed the major conformer's signals for three methyls, three amide *N*-methyl signals, one *O*-methyl signal, three methylenes, six α-amino methines, one 1,4-disubstituted benzene ring, one 1,2,4-trisubstituted benzene ring, and six amide carbonyls, which allowed five amino acid residues to be established, that is, three alanines and two *N*-methyl tyrosines. Except for the signals mentioned above, two oxymethines (δ_H/δ_C 4.28/64.5, 4.87/85.3), one methine (δ_H/δ_C 3.67/44.3), one methylene (δ_H/δ_C 2.20, 2.40/28.9), and two olefinic carbons (δ_H/δ_C 5.58/120.0, 136.1) remained unknown.

An extensive comparison of 1D and 2D NMR data of **1** with those of RA-XII (**3**)[14] strongly suggested a similar structure for the 18-membered ring of both compounds, that is, D-Ala[1], Ala[2], *N*-Me Tyr[3], and Ala[4], which were deduced from the similar carbon and proton chemical shifts, proton coupling constants, and NOE correlations. Sequencing the amino acid residues in **1** was mainly accomplished by HMBC correlations of the NCH_3 proton or NH proton of one amino acid residue with the carbonyl carbon of the neighboring

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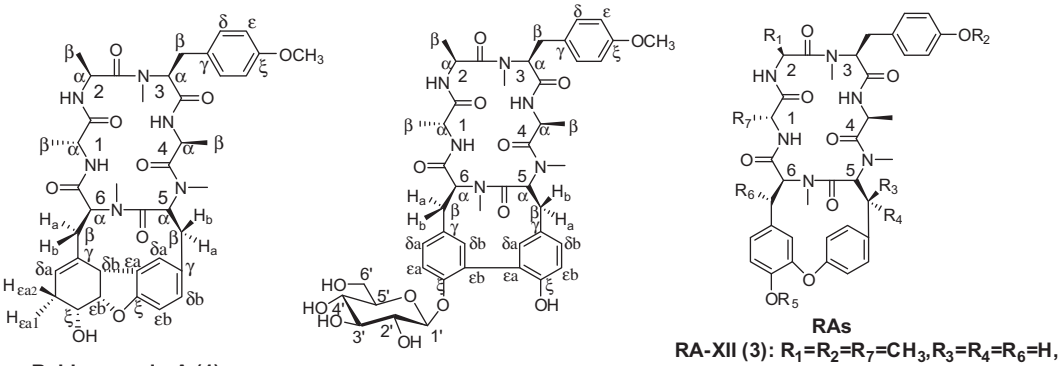

Figure 1. Structures of **1**, **2**, **3**, and **RAs.**

Table 1
^{1}H and ^{13}C NMR data of compounds **1** and **2** in methanol-*d*4 (δ in ppm, *J* in Hz)

Position	**1**[a]		**2**[b]	
	δ_H	δ_C	δ_H	δ_C
D-Ala[1]				
α	4.09 (q, 6.8)	50.5 d	4.48 (q, 7.0)	48.5 d
β	1.39 (d, 6.8)	19.9 q	1.32 (d, 7.0)	20.6 q
C=O		174.2 s		174.0 s
Ala[2]				
α	4.47 (q, 6.8)	46.7 d	4.58 (m)	46.3 d
β	1.29 (d, 6.8)	16.1 q	1.30 (d, 7.0)	16.1 q
C=O		174.7 s		174.7 s
Tyr[3]				
α	3.87 (dd, 10.0, 6.0)	68.7 d	3.83 (dd, 9.0, 7.0)	68.6 d
β	3.21 (m)	33.8 t	3.22 (m)	33.8 t
γ		131.5 s		131.5 s
δ 2	7.06 (d, 8.4)	131.5 d	7.05 (d, 8.5)	131.5 d
ε 2	6.85 (d, 8.4)	115.0 d	6.85 (d, 8.5)	115.0 d
ζ		160.0 s		160.0 s
C=O		171.5 s		171.3 s
NMe	2.86 (s)	40.5 q	2.86 (s)	40.5 q
OMe	3.75 (s)	55.6 q	3.75 (s)	55.6 q
Ala[4]				
α	4.92 (overlap)	47.7 d	4.85 (m)	47.6 d
β	1.00 (d, 6.8)	18.1 q	0.99 (d, 6.5)	18.9 q
C=O		174.0 s		172.4 s
Tyr[5]				
α	4.61 (dd, 12.0, 5.6)	58.7 d	5.71 (d, 9.5)	55.6 d
βa	3.08 (dd, 12.0, 5.6)	36.3 t	2.29 (d, 14.5)	35.2 t
βb	3.15 (overlap)		4.02 (dd, 14.5, 9.5)	
γ		127.7 s		130.4 s
δa	7.20 (s)	128.9 d	6.30 (d, 2.0)	141.2 d
δb	7.17 (d, 8.0)	130.2 d	6.96 (dd, 8.5, 2.0)	129.8 d
εa		130.6 s		128.7 s
εb	6.64 (d, 8.0)	110.1 d	6.68 (d, 8.5)	116.4 d
ζ		160.1 s		153.5 s
C=O		175.0 s		173.3 s
NMe	3.20 (s)	32.6 q	2.99 (s)	31.2 q
Residue[6]				
α	3.69 (d, 11.2)	70.1 d	5.39 (dd, 11.5, 4.0)	61.6 d
βa	3.02 (d, 15.6)	29.4 t	3.47 (overlap)	36.3 t
βb	2.59 (dd, 15.6, 11.2)		3.11 (dd, 16.0, 11.5)	
γ		136.1 s		129.9 s
δa	5.58 (d, 5.2)	120.0 d	7.16 (overlap)	130.4 d
δb	3.67 (d, 7.2)	44.3 d	6.72 (br s)	141.8 d
εa_1	2.20 (m)	28.9 t	7.16 (overlap)	117.0 d
εa_2	2.40 (br d, 17.6)			
εb	4.87 (overlap)	85.3 d		131.3 s
ζ	4.28 (br s)	64.5 d		155.3 s
C=O		171.2 s		171.7 s
NMe	2.24 (s)	39.9 q	2.78 (s)	31.7 q
Glc				
1′			5.02 (d, 7.0)	103.8 d
2′			3.49 (overlap)	75.0 d
3′			3.49 (overlap)	77.6 d
4′			3.38 (m)	71.3 d
5′			3.49 (overlap)	78.4 d
6′			3.70 (dd, 12.0, 6.0)	62.5 t
			3.91 (dd, 12.0, 2.0)	

[a] ^{1}H NMR recorded at 400 MHz, ^{13}C NMR recorded at 100 MHz.
[b] ^{1}H NMR recorded at 500 MHz, ^{13}C NMR recorded at 100 MHz.

residue. In the HMBC spectrum, cross-peaks of Tyr^3-NMe/Ala^2-CO, Tyr^5-NMe/Ala^4-CO, and Residue^6-NMe/Tyr^5-CO demonstrated the connectivities of the Ala^2-Tyr^3 and Ala^4-Tyr^5-Residue^6. Then NMR experiments were reperformed in DMSO-d_6 due to the absence of amide-NH signals in methanol-d_4 (see Supplementary data). The HMBC correlations of Ala^1-NH/Residue^6-CO, Ala^2-NH/Ala^1-CO, and Ala^4-NH/Tyr^3-CO were observed. So all obtained HMBC correlations confirmed the 18-membered ring. Then study of the ^{1}H–^{1}H COSY spectrum starting from the olefinic proton at δ_H 5.58 (H-6δa) revealed the presence of a spin-system H-6δa/H-6εa/H-6ζ/H-6εb/H-6δb (=CH–CH$_2$–CH–CH–CH–). This information coupled with the HMBC correlations of H-6δa/C-6β, C-6δb, C-6ζ, and H-6δb/C-6δa, C-6γ indicated the existence of a substituted cyclohexene ring. Furthermore, C-6ζ (δ_C 64.5) and C-6εb (δ_C 85.3) presented at low field ascribed as bearing hydroxyl and phenolic oxygen groups, respectively. Considering the 19 degrees of unsaturation of **1**, one more ring was required. The crucial HMBC correlations of H-6δb/C-5ζ, C-5εa, and H-5δa/C-6δb gave another rigid oxygen-containing five-membered ring. Thus, the planar structure of **1** was determined.

The amino acid analysis of the hydrolyzate of **1** showed that it contained D-Ala and L-Ala in the ratio of 1:2 according to the Marfey's method,[15] which confirmed the D(*R*), L(*S*), and L(*S*) configurations of Ala^1, Ala^2, and Ala^4 similar to those of RAs. In the ROESY spectrum, the presence of NOE correlations of Tyr^3-NMe with H-2α and H-3α indicated the L(*S*)-configuration of Tyr^3. Interestingly, unlike regular RAs, no NOE correlations were seen between H-5α and H-6α whereas correlations of Residue^6-NMe with H-5α and H-6α were observed, respectively, which suggested that the *N*-methyl peptide bond between Tyr^5 and Residue^6 was *trans*. Furthermore, in order to confirm the absolute configuration of Tyr^5 and Residue^6, two isomers (L-Tyr^5/L-Residue^6 and D-Tyr^5/D-Resi-

due[6]) of **1** were optimized at the B3LYP/6-31G* level using GAUSSIAN 03 program package.[16] Our calculation results indicated that the L-Tyr[5]/L-Residue[6] isomer was in accordance with the observed NOE correlations (see Supplementary data). For the absolute stereochemistry at C-6δb, C-6εb, C-6ζ, firstly, H-6βb was suggested to be β-oriented based on the coupling constant of H-6βb/H-6α (dd, J = 15.6, 11.2 Hz). Then considering the NOE correlations of H-6βb/H-6δb and H-6εb/H-6δb and the rigidity of oxygen-containing five-membered ring, H-6δb and H-6εb were placed at β orientation. However, the ROESY spectrum in methanol-d_4 could not identify the absolute configuration at C-6ζ. Fortunately, NOE correlation of H-6ζ with H-6δb was clearly observed in DMSO-d_6, which implied that H-6ζ was also β-oriented. So the absolute configurations of C-6δb, C-6εb, and C-6ζ were assigned as *R*, *S*, and *R*, respectively. Therefore, through the above-mentioned NMR and DFT analysis, the structure of **1** was determined as shown in Figure 2.

Rubiyunnanin B (**2**)[17] was obtained as an amorphous solid and possessed the molecular formula $C_{46}H_{58}N_6O_{14}$ by HRESIMS (m/z 941.3917, $[M+Na]^+$, calcd 941.3908), with 21 degrees of unsaturation. The ^1H and ^{13}C NMR spectra of **2** in methanol-d_4 (Table 1) also showed some characteristics of RAs and demonstrated the presence of two conformers in a ratio of 84:16. An extensive comparison of 1D and 2D NMR data of **2** with those of **1** and RA-XII (**3**) also established the presence of the 18-membered ring, that is, D-Ala[1], Ala[2], *N*-Me Tyr[3], and Ala[4]. The absolute configurations of Ala[1], Ala[2], and Ala[4] were also identified as D(*R*), L(*S*), and L(*S*), respectively, by application of the Marfey's method. Furthermore, it possessed two 1,2,4-trisubstituted benzene rings and a set of additional signals arising from a glucopyranosyl moiety.

HMBC cross-peaks of Tyr[5]-NMe/Ala[4]-CO and Tyr[6]-NMe/Tyr[5]-CO revealed the sequence of the two tyrosines. Then crucial HMBC correlations of H-5δa/C-6εb and H-6δb/C-5εa, together with NOE correlations of H-5δa/H-6δb indicated the linkage of C-5εa and C-6εb. As for the glucopyranosyl moiety of **2**, HMBC correlation of the anomeric proton (δ_H 5.02, d, J = 7.0 Hz) (β-form) with C-6ζ indicated that the sugar group was ligated to ζ-position of Tyr-6. Enzymatic hydrolysis of **2** with β-glucosidase yielded D-glucose, which was detected by TLC and optical rotation, $[\alpha]_D^{19}$ +103.7 (*c* 0.10 H_2O).

In the ROESY spectrum, NOE correlation that was apparently observed between H-5α and H-6α suggested that the *N*-methyl peptide bond between Tyr[5] and Tyr[6] was *cis*. Similarly, two geometries (L-Tyr[5]/L-Tyr[6] and D-Tyr[5]/D-Tyr[6]) of **2** were optimized, and the calculation results supported the L-Tyr[5]/L-Tyr[6] structure (see Supplementary data). Moreover, H-5δa exhibited NOE correlation with Tyr[6]-NMe, which indicated that the benzene ring of Tyr[5] was at the left angle of the benzene ring of Tyr[6]. Consequently, based on the above-mentioned NMR and DFT analysis, the structure of **2** was determined as shown in Figure 2.

Comparing with all known 30 RAs, both **1** and **2** have unique skeletons. Except for the 18-membered ring, compound **1** has extra two rings formed between Tyr[5] and Residue[6] via a phenolic oxygen linkage and a new carbon–carbon bond. In addition, compound **2** is discovered for the first time that it lacks the typical phenolic oxygen linkage, but rather has a carbon–carbon bond at the *ortho*-positions of the hydroxyl groups of Tyr[5] and Tyr[6].

The biosynthetic mechanism for plant cyclopeptides has not been well established, except for the cyclotides.[18] The hexapeptide precursor of RAs could be synthesized via a non-ribosomal peptide biosynthetic mechanism.[4,19] To form a mature RA, several modification steps are expected to take place, among which is the phenolic coupling step that leads to the most characteristic structural feature of RAs. It is well established that the coupling is via a free radical mechanism, typically at *ortho–ortho*, *ortho–para,* or *para–para* position. The hydroxyl of Tyr[5] and the *ortho*-carbon of Tyr[6] can generate free radicals. An oxygen–carbon coupling would lead to the 14-membered cycloisodityrosine moiety that is found in all RAs. For compound **2**, the coupling is between two *ortho*-carbons. To form the very unusual three-ring system of compound **1**, the aromatic ring of Tyr[6] of a RA precursor needs to be reduced first. This sets a stage for an electrophilic aromatic substitution-like reaction, using the *meta*-carbon of Tyr[6] as the electrophile to add to the *ortho*-carbon of Tyr[5]. This step could be facilitated by a protonation on Tyr[6] ring and the lone-pair electrons on the oxygen bridge between the two tyrosine residues. An elimination of the proton on Tyr[5] ring driven by the restoration of the aromatic ring of Tyr[5] would lead to the formation of the new carbon–carbon bond of **1**. Possible biosynthetic pathways for RAs, **1** and **2** are shown in Figure 3.

Cytotoxicities of **1** and **2** against 11 cancer cell lines (HepG2, BEL-7402, SMMC-7721, MDA-MB-231, DU-145, PC-3, A549, BGC-823, Hela, U251, and B16) were measured by SRB assay with the maximum concentration of 50 μg/mL.[20] Only compound **2** exhibited moderate cytotoxicities with IC_{50} values of 31.3, 13.2, 33.7, 7.3, 16.3, 21.9, 4.8, 5.6, 21.3, 6.5, and 3.6 μg/mL, respectively. Furthermore, the inhibitory effects of **1** and **2** on NO production in LPS and IFN-γ-induced RAW 264.7 murine macrophages were examined. Only compound **2** was found to inhibit NO production with IC_{50} value of 10.7 μg/mL.

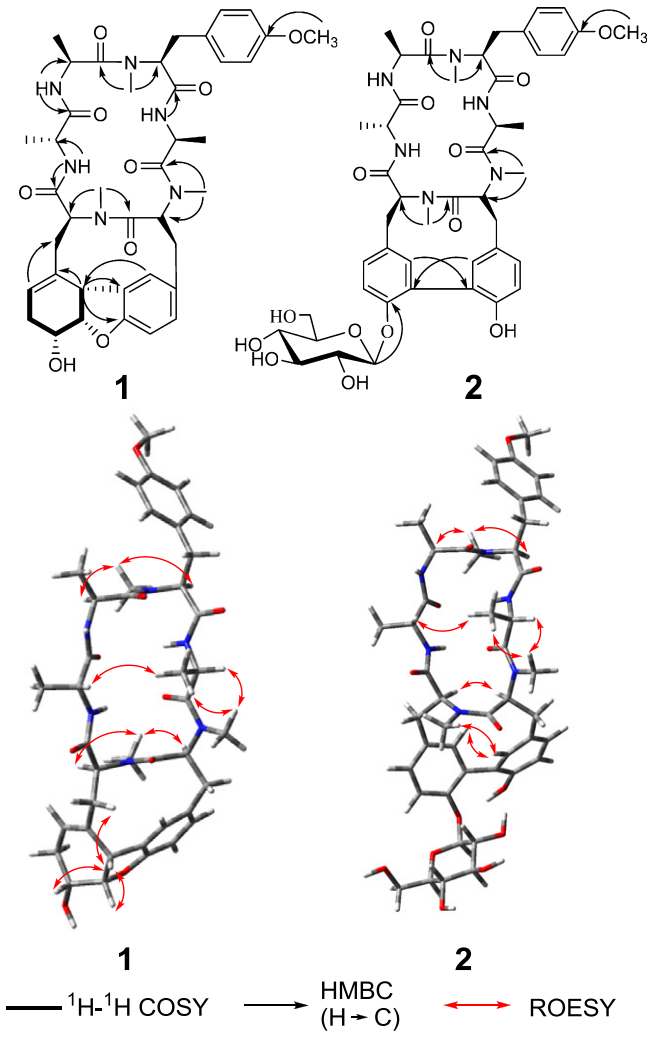

Figure 2. Selected 2D NMR correlations of **1** and **2.**

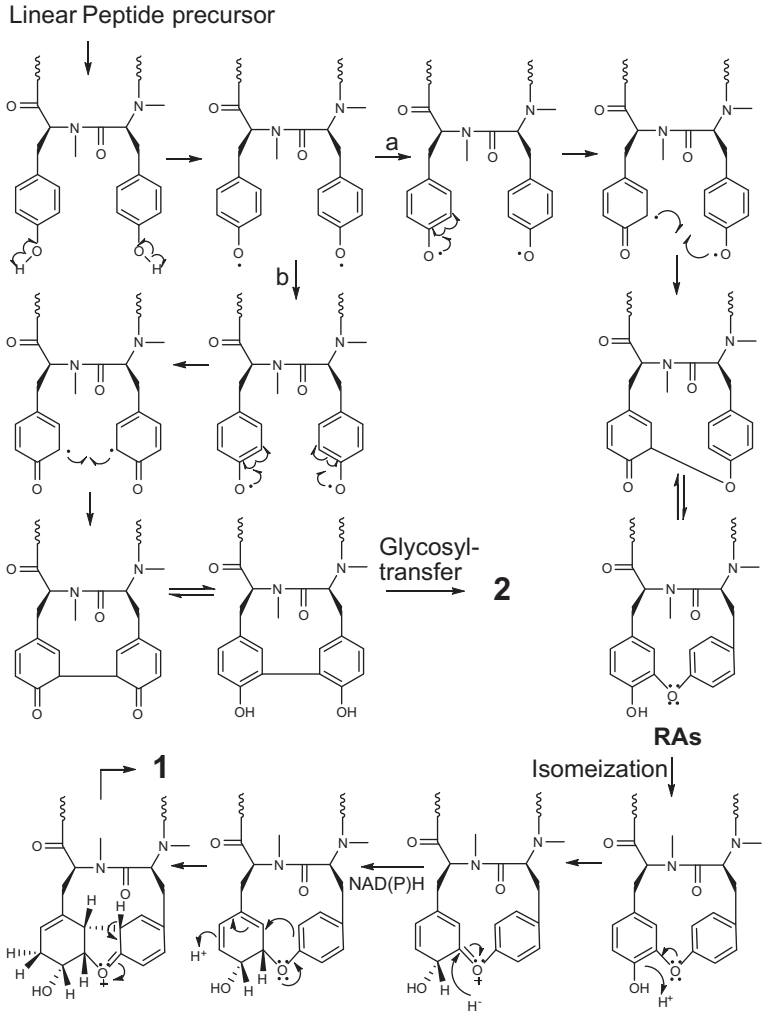

Figure 3. Proposed biosynthetic pathways for **RAs**, **1**, and **2.**

Acknowledgments

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Supplementary data

Supplementary data (detailed experimental section, DFT computational results, copies of NMR and MS spectra of **1** and **2**) associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2010.07.066.

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11. Rubiyunnanin A (**1**): $[\alpha]_D^{28}$ −115.8 (*c* 0.11, MeOH); UV (MeOH) λ_{max} (log ε) 204 (4.75), 285 (3.73) nm; IR (KBr) ν_{max} 3440, 3385, 2932, 1656, 1512, 1488, 1242 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; positive FABMS *m*/*z* $[M+H]^+$ 759 (100), 164 (30), 121 (11); HRESIMS *m*/*z* 781.3545 $[M+Na]^+$ (calcd for $C_{40}H_{50}N_6O_9Na$, 781.3536).
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17. Rubiyunnanin B (**2**): $[\alpha]_D^{24}$ −205.8 (*c* 0.30 MeOH); UV (MeOH) λ_{max} (log ε) 203 (4.74), 284 (3.78) nm; IR (KBr) ν_{max} 3427, 2932, 1639, 1512, 1246, 1075 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; positive FABMS *m*/*z* $[M+H]^+$ 919 (100), 757 (11), 593 (7), 501 (8), 409 (18); HRESIMS *m*/*z* 941.3917 $[M+Na]^+$ (calcd for $C_{46}H_{58}N_6O_{14}Na$, 941.3908).
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