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[1-8-NαC]-Zanriorb A1, a Proapoptotic Orbitide from Leaves of *Zanthoxylum riedelianum*

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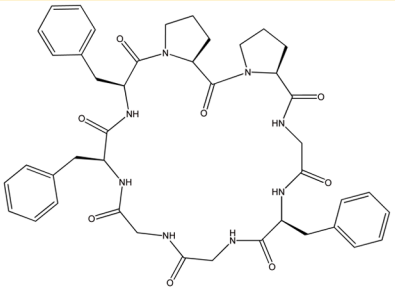
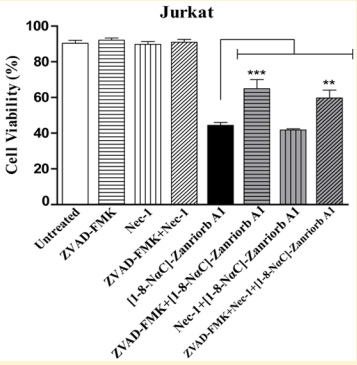
Supporting Information



[1-8-NαC]-zanriorb A1 **(1)**



ABSTRACT: A new orbitide named [1-8-NαC]-zanriorb A1 (**1**) was isolated and characterized from the leaves of *Zanthoxylum riedelianum* using NMR and mass spectrometry. The absolute configuration of the amino acids was determined using Marfey's method on the acid hydrolysates. Compound **1** induced cell death by apoptosis in Jurkat leukemia T cells (IC_{50} 218 nM).

The genus *Zanthoxylum* (Rutaceae), comprising approximately 250 species, is well-known for its diverse ethnobotanical uses.[1] The plants belonging to this genus are sources of biologically active compounds, such as alkaloids, aliphatic and aromatic amides, coumarins, and lignans.[2]

Zanthoxylum riedelianum Engl. is known popularly as "pig's nipple" and, in Brazil, as "mamica de porca" due to the particular shape of its thorn. It has been used as an analgesic for toothache. The confirmation of the anti-inflammatory and antinociceptive activities of the leaves and stem bark corroborated the reputed utility of this species in folk medicine.[3] Previous studies on *Z. riedelianum* have described the chemical constituents of the essential oil from the leaves,[4] as well as alkaloids from the roots[5] and lignans from the stem bark.[3] There is only one study that reported on cyclized peptides in the genus *Zanthoxylum*.[6] This class of compounds, recently redefined as orbitides,[7] has been found in Rutaceae species such as *Citrus*,[8] *Evodia*,[9] and *Clausena*.[10,11] Published studies on orbitides have described important biological activities such as cytotoxic activity against several human cancer cell lines,[12−15] making them a promising class of bioactive molecules.

Herein, we report on the structural elucidation as well as the cytotoxic and apoptotic activities of a novel orbitide, [1-8-NαC]-zanriorb A1 (**1**), isolated from the leaves of *Z. riedelianum*. This study is a part of an ongoing research program to discover bioactive compounds in the Brazilian flora.

Compound **1** was isolated as a white, amorphous powder (16 mg, 0.53% dry weight) from the leaves of *Z. riedelianum* through repeated purification steps. The IR absorptions at 3308 and 1659 cm^{-1} were attributed to amino and amide carbonyl groups, respectively. The molecular formula was established as $C_{43}H_{51}N_8O_8$, based on the HRESIMS (observed $[M + H]^+$ m/z 807.3832; calcd m/z 807.3824), 1D and 2D ^1H and ^{13}C NMR spectroscopic data, and amino acid analysis.

The ^1H NMR spectrum (measured in DMSO-d_6) exhibited resonances typical of a peptide, including amide proton signals at δ_H 7.45 to 9.17, multiplets of α-protons at δ_H 3.71 to 4.67, and numerous overlapping signals between δ_H 1.32 and 3.63. The DEPTQ-NMR spectrum was consistent with this observation, showing signals attributable to amide carbonyls (δ_C 169.0 to 173.9), aromatic groups (δ_C 126.8 to 130.0), methines (δ_C 52.8 to 60.6), and methylene carbons (δ_C 21.7 to 47.6). The ^{13}C and ^1H NMR chemical shift assignments for the amino acid residues of **1** in DMSO-d_6 are listed in Table 1.

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Table 1. NMR Spectroscopic Data (500 MHz, DMSO-d_6) for [1-8-NαC]-Zanriorb A1 (1)

	position		δ_C, type	δ_H, (J in Hz)	NOESY	HMBC (H→C)	COSY
Pro_1	1	α	60.6, CH	4.28, dd (4.0, 10.5)	2, 37	2, 3, 5, 36	2
	2	β	28.4, CH_2	2.10, m	1, 3	1, 3, 4	1, 3, 4
	3	γ	24.6, CH_2	1.32, m[a]	1, 2, 4	1, 2, 4	2, 4
	4	δ	47.2, CH_2	3.42, m[a]	3, 6	3, 5	3
	5	CO	172.0, C				
Pro_2	6	α	58.9, CH	4.42, dd (4.5, 10.9)	4, 7	5, 7	7
	7	β	31.4, CH_2	3.63, m	6, 8	5, 6, 8,	6, 8
	8	γ	21.7, CH_2	1.32, m[a]	7, 9	7, 9	7, 9
	9	δ	47.6, CH_2	3.42, m[a]	8, 11	7, 8, 10	8
	10	CO	172.1, C				
Phe_3	11	α	55.3, CH	4.59, m	9, 12, 16	10, 12, 13, 15	12, 16
	12	β	37.2, CH_2	3.18, m	11	10, 11, 13, 14	11
	13	1′	138.8, C				
	14	2′−6′	126.8−130.0, CH	7.30, m[a]		12	14
	15	CO	170.7, C				
	16	NH		8.10, d (8.2)	11, 17	10, 11, 15	11
Phe_4	17	α	52.8, CH	4.67, m	16, 18, 22	15, 21	18, 22
	18	β	36.7, CH_2	2.96, m	17	17, 19, 20	17
	19	1′	138.1, C				
	20	2′−6′	126.8−130.0, CH	7.30, m[a]		18	20
	21	CO	170.3, C				
	22	NH		7.45, d (8.2)	16, 17, 23, 25	15, 17, 21	11
Gly_5	23	α	42.3, CH_2	3.71, m[a]	22, 25	21, 24	25
	24	CO	169.0, C				
	25	NH		7.92, t (6.0)	22, 23, 28, 34	21, 24, 26	23
Gly_6	26	α	43.8, CH_2	3.71, m[a]	25, 28	24, 27	28
	27	CO	169.2, C				
	28	NH		9.17, t (6.0)	25, 26, 29, 34	24, 27, 29	26
Phe_7	29	α	56.3, CH	4.38, m	28, 30, 34	27, 30, 33	30, 34
	30	β	36.4, CH_2	3.96, m	29	27, 29, 31, 32	29
	31	1′	137.6, C				
	32	2′-6′	126.8−130.0, CH	7.30, m[a]		30	32
	33	CO	173.9, C				
	34	NH		7.72, d (8.2)	25, 29, 35, 37	27, 29, 33	29
Gly_8	35	α	43.2, CH_2	3.75, m	34, 37	33, 36	37
	36	CO	171.8, C				
	37	NH		8.03, t (6.2)	1, 34, 35	1, 33, 35, 36	35

[a]Mutiplicity due to overlapping.

The correlations in the HSQC spectrum displayed signals of α-protons linked to sequential methylene carbons, confirming the presence of three glycine residues (δ_H 3.71, 3.75 to δ_C 42.3, 43.2, 43.8). Furthermore, the correlations from δ_H 1.32 to δ_C 21.7 and 24.6 and the signal at δ_H 3.42 to δ_C 47.2 and 47.6, attributed to the γ- and δ-CH_2 systems, respectively, gave evidence for the presence of two proline residues (Figure S3, Supporting Information). On the basis of correlations observed in the COSY spectrum, spin systems were identified for the couplings between multiplets at δ_H 7.30 of the aromatic rings and between the signals δ_H 4.59/3.18, δ_H 4.67/2.96, and δ_H 4.38/3.96, which were related to the α- and β-protons, indicating three phenylalanine residues (Figure S4, Supporting Information). The HSQC spectrum also confirmed the phenylalanine residues from evident correlations in the aromatic rings [δ_H 7.30 (m)/δ_C 126.8−130.0], β-protons to sequential methylene carbons (δ_H 2.96, 3.18, 3.96/δ_C 36.4, 36.7, 37.2), and finally α-protons to the methine carbons (δ_H 4.38, 4.59, 4.67/δ_C 52.8, 55.3, 56.3). These detected values were compared with literature data of peptides containing Phe, Pro, and Gly residues.[6,16]

Further analysis of the NOEDIFF, NOESY, and HMBC data of **1** confirmed the proximity of the amino acid residues of the peptide. The NOE correlations Pro_1-Hα/Pro_2-Hα, Pro_1-Hγ/Pro_2-Hα, Phe_3-NH/Phe_4-NH, Phe_4-NH/Gly_5-NH, Gly_5-NH/Gly_6-NH, Gly_6-NH/αH-Phe_7, and Phe_7-NH/Gly_8-NH were used to establish two fragments, Pro_1-Pro_2 and Phe_3-Phe_4-Gly_5-Gly_6-Phe_7-Gly_8 (Figure S5, Supporting Information). Additionally, for sequencing purposes, NOESY analysis showed correlations between Gly_8-NH/Pro_1-Hα as well as Pro_2-Hδ/Phe_3-Hα, indicating the proximity of these fragments, and revealed a sequence (-Pro_1-Pro_2-Phe_3-Phe_4-Gly_5-Gly_6-Phe_7-Gly_8-) in the cyclic system of **1** (Figure S6, Supporting Information). Other clear correlations also were defined from the HMBC spectrum as a result of the couplings of the eight carboxylic groups with neighboring protons according to ^3J correlations between Phe_4-NH/Phe_3-CO, Gly_5-NH/Phe_4-CO, Gly_6-NH/Gly_5-CO, Phe_7-NH/Gly_6-CO, Gly_8-NH/Phe_7-CO, Pro_1-αH/Gly_8-CO, Phe_3-αH/Pro_2-CO, and finally Pro_2-αH/Pro_1-CO (Figure S7, Supporting Information).

Analysis by MS/MS allowed the observation of m/z values consistent with the loss of sequential amino acid residues from

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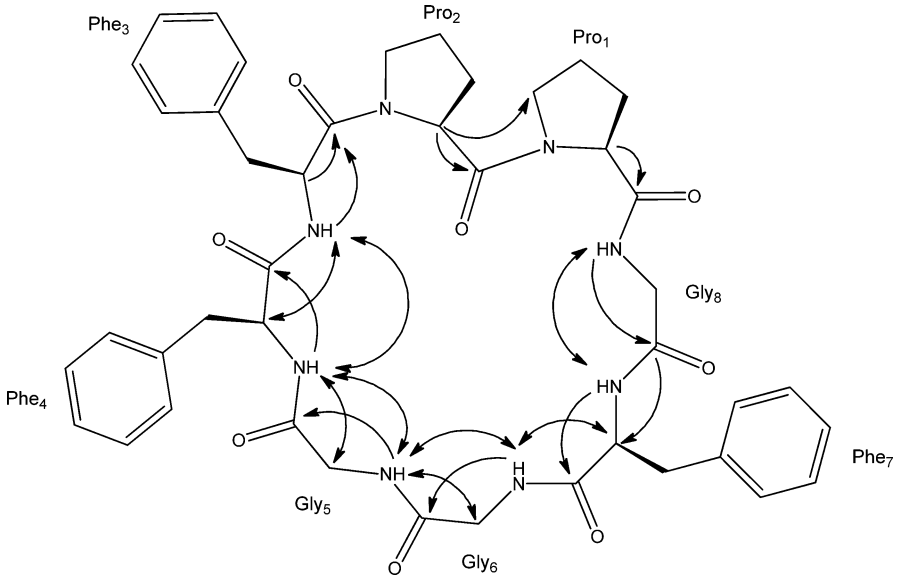

1

Figure 1. Some HMBC (→) and NOE correlations (↔) for [1-8-NαC]-zanriorb A1 (**1**).

m/z 807.3832, corroborating the proposed sequence: *m/z* 710.3302 ($[M + H]^+ - Pro_1$), *m/z* 660.3143 ($[M + H]^+ - Phe_3$), *m/z* 603.2922 ($[M + H]^+ - Phe_4Gly_5$), *m/z* 563.2611 ($[M + H]^+ - Pro_2Phe_3$), *m/z* 546.2705 ($[M + H]^+ - Phe_4Gly_5Gly_6$), *m/z* 399.2025 ($[M + H]^+ - Phe_3Phe_4Gly_5Gly_6$), *m/z* 342.1811 ($[M + H]^+ - Phe_4Gly_5Gly_6Phe_7Gly_8$), *m/z* 302.1497 ($[M + H]^+ - Pro_2Phe_3Phe_4Gly_5Gly_6$), and *m/z* 245.1211 ($[M + H]^+ - Phe_4Gly_5Gly_6Phe_7Gly_8Pro_1$), respectively (Figure S8, Supporting Information).

The absolute configurations of the amino acids present in **1** were determined by a modified version of Marfey's method.[17,18] After hydrolysis of **1** and reaction with 1-fluoro-2,4-dinitrophenyl-5-L-alaninamide, the derivatives were analyzed by HPLC. The results showed the presence of three types of amino acids in the mixture, which were identified as L-phenylalanine, L-proline, and glycine by comparison with authentic samples. No D-amino acid derivative peaks were detected.

A combination of HRESIMS spectrometric data, DEPT-Q, COSY, HMBC, HSQC, NOESY, NOEDIFF NMR spectra interpretation (see Supporting Information), and amino acid hydrolysis was used to define the structure of compound **1**, which, according to Shim, Young, Arnison, Gilding, and Reaney,[19] can be designated as [1-8-NαC]-zanriorb A1.

When evaluated against three cancer cells lines, K562 (chronic myeloid leukemia), Kasumi-1 (acute myeloid leukemia), and Jurkat (T cell leukemia), and also using normal mononuclear cells from human healthy donors, compound **1** showed cytotoxic activity against Jurkat cells with a low IC_{50} value of 218 nM. A higher concentration of **1** (100 μM) did not reduce the viability of the other leukemia types or normal mononuclear cells by more than 20% (Figure S9a, Supporting Information). On the basis of the information that annexin-V is a protein that binds specifically to phosphatidylserine and can be used to detect apoptotic cells, and propidium iodide (PI) is a nonvital dye that enters only cells that lose their cellular membrane integrity such as late apoptotic or necrotic cells,[20] it was observed that compound **1** induced the formation of annexin-V^+PI^+ (Figure S9b, Supporting Information). In order to determine if the annexin-V^+PI^+ cells induced by compound **1** were necrotic or apoptotic, the total DNA content was labeled with vital dye Hoechst 33342. Annexin-V^+ exhibited a low DNA content typically observed by the formation of apoptotic bodies (Figure S9c–e, Supporting Information). Moreover, cell death induced by **1** was partially inhibited by ZVAD-FMK, a caspase inhibitor. Necrostatin-1, a RIP-1 inhibitor involved in necroptosis, did not block cell death induced by **1** (Figure S10, Supporting Information). Considering that caspase-3 is an important protease regulating apoptotic events and that loss of Ψ_{mit} is a hallmark of apoptosis, compound **1** was found to activate caspase-3 after 12 h of treatment (Figure S11a and b, Supporting Information). In addition, Ψ_{mit} was drastically reduced (Figure S12, Supporting Information). These results confirm that annexin-V^+PI^+ cells induced by **1** were representative of late apoptosis. Staurosporine and CCP, two apoptotic agents, were used as positive controls in these experiments.[21,22] Bioactive orbitides represented by "proline-rich" compounds could be potential candidates for the development of novel anticancer drugs.[16] The proline units have an important role in defining the conformation and provide a mechanism for cytotoxicity,[16,23] enabling an interaction with the cell membrane of Jurkat cells.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured on a JASCO P-2000 polarimeter (Easton, MD, USA). IR spectra were recorded on a Vertex 70 Bruker spectrometer (Karlsruhe, Germany) using KBr pellets. ^1H (500 MHz), DEPT-Q (125 MHz), NOEDIFF, COSY, HSQC, and HMBC spectra were acquired at room temperature on a Bruker TopSpin 500 NMR spectrometer (Rheinstetten, Germany) with DMSO-d_6 from Sigma-Aldrich (St. Louis, MO, USA) as the solvent. Spectra were referenced to residual solvent signals with resonances at δ_H 2.51 and δ_C 39.8 and tetramethylsilane as the internal standards. High-resolution ESIMS was carried out on an UHR-QqTOF Bruker Impact HD mass spectrometer (Bremen, Germany). The sample was dissolved in 1 mL of water–acetonitrile (50:50) with 0.1% formic acid, infused into the HD mass spectrometer at 3 μL/min, and used (N_2) at 10 eV for MS and 45 eV for MS/MS in the positive-ion mode. Analytical HPLC was performed on a Shimadzu Prominence LC-20AT instrument (Kyoto,

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J. Nat. Prod. XXXX, XXX, XXX–XXX

Japan) equipped with a diode array detector using a Betasil-Thermo-Scientific (Waltham, MA, USA) C_{18} column (4.6 × 250 mm, 100 Å pore size, 5.0 μm particle size, 1 mL/min). Column chromatography was performed with silica gel (70−230 mesh) from Merck (Darmstadt, Germany) and Sephadex LH-20 gel from GE Healthcare (Uppsala, Sweden). Fractions were monitored by silica gel plates (60 F254; Macherey-Nagel (Düren, Germany)). The solvents used for extraction and chromatography procedures were of ACS grade from Vetec (Rio de Janeiro, Brazil) and for HPLC analysis were of spectroscopic grade from Tedia (Fairfield, OH, USA).

Plant Material. The leaves of *Zanthoxylum riedelianum* were collected in Poconé, Mato Grosso, Brazil (16°18′50″ S/56°33′23″ W) in September 2011. A voucher specimen (Arnildo Pott, no. 33228) is stored at the Central Herbarium of the Universidade Federal do Mato Grosso do Sul, Campo Grande-MS, Brazil.

Extraction and Isolation. Dried leaves (3 kg) of *Z. riedelianum* were powdered and extracted with methanol (8 L) by maceration at room temperature, performed in seven cycles of 5 days. The macerates were concentrated under reduced pressure to yield a crude MeOH extract (408 g). The MeOH extract was partitioned with hexane (4 L), $CHCl_3$ (7 L), EtOAc (8 L), and MeOH (10 L), sequentially. The solvents were removed under reduced pressure, and the $CHCl_3$ residue (8.94 g) was fractionated by silica gel column chromatography (160 g, 70−230 mesh) using gradient mixtures of $CHCl_3$−EtOAc (7:3, 1:1, and 3:7, v/v) and EtOAc−MeOH (8:2, 7:3, 1:1, and 3:7, v/v) as eluents, to produce 97 fractions. Fractions 70 to 86 (75 mg) were combined after TLC analysis and further chromatographed over Sephadex LH-20 (30 g), eluted with MeOH, affording a white, amorphous powder, corresponding to compound **1** (16 mg).

[1-8-NαC]-Zanriorb A1 (1): white, amorphous solid; $[\alpha]^{25}_{D}$ −68.4 (*c* 0.05, MeOH); IR (KBr) ν_{max} 3308, 2965, 1659, 1553, 1295, 757 cm^{-1}; ^{1}H NMR and ^{13}C NMR (see Table 1); HRESIMS *m/z* 807.3832 ($[M + H]^+$) (calcd for $C_{43}H_{51}N_8O_8$ *m/z* 807.3824); MS/MS peaks are mentioned in the Results and Discussion (see Figure S8, Supporting Information).

Acid Hydrolysis of 1. Compound **1** (1 mg) was dissolved in 6 N HCl (1 mL) and heated at 110 °C for 14 h in a reaction vial (2 mL). After cooling, the solution was evaporated to dryness under reduced pressure and redissolved in 100 μL of H_2O.

Preparation and Analysis of Marfey Derivatives. A 100 μL aliquot of a 1% (w/v) solution of *N*-α-(2,4-dinitro-5-fluorophenyl)-L-alaninamide [L-FDAA, Marfey's reagent] (Sigma-Aldrich) in acetone was added to 50 μL of a 50 mM solution of an amino acid (or 50 μL of the acid hydrolysate solution). Then, 1 M $NaHCO_3$ (20 μL) was added, and the mixture was heated at 40 °C for 1 h. The reaction was stopped by the addition of 10 μL of 2 N HCl solution. The solvents were evaporated to dryness, and the residue was redissolved in MeOH. An aliquot of 20 μL was analyzed by analytical HPLC with a linear gradient of 30% CH_3CN (A) and 70% H_2O with 1% acetic acid (B) (pH 3.1) (linear gradient for 40 min), from 35% to 60% A over 35 min; 30 °C, flow 1 mL/min and detection at 340 nm. Peaks obtained from the chromatogram were identified by comparing the retention times values with those of the L-FDAA derivatives of authentic amino acids. The standard derivatives gave the following retention time values (min): 7.9 min (Gly-L-FDAA); 25.8 min (L-Phe-L-FDAA), 29.1 min (D-Phe-L-FDAA), 10.5 min (L-Pro-L-FDAA), and 12.2 min (D-Pro-L-FDAA). The L-FDAA derivatives of the released amino acids from **1** showed peaks at 8.1 min (Gly-L-FDAA), 25.9 min (L-Phe-L-FDAA), and 10.5 min (L-Pro-L-FDAA).

Cell Culture. Kasumi-1, Jurkat, and K562 cells were obtained from the Cell Bank of Rio de Janeiro (BCRJ). After thawing, the cells were maintained and grown as suspensions in RPMI-1640 medium (Sigma-Aldrich), pH 7.4, supplemented with 10% fetal bovine serum from Gibco FBS, Invitrogen (Grand Island, NY, USA), 100 U/mL penicillin, and 100 mg/mL streptomycin. Cells were maintained in culture flasks at 37 °C in a humidified incubator in an atmosphere of 5% CO_2.

Isolation of Peripheral Blood Mononuclear Cells. Peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors after obtaining informed patient consent, as approved by the local Ethical Committee of the Universidade Federal de São Paulo (document number 0225/10). A gauge needle was used to collect PBMCs into a tube containing heparin. Following collection, the cells were manipulated in a sterile environment. To separate the fraction of mononuclear cells, Ficoll-Hypaque (density 1.077 g/mL) centrifugation was used (400*g* for 30 min).

Annexin-V-FITC/Propidium Iodide Double-Staining and Flow Cytometric Analysis. Cell lines and PBMCs were seeded (1×10^5/mL) in 96-well plates (200 μL) and incubated with **1** and staurosporine (Sigma-Aldrich) at different concentrations for 24 h. The cells were harvested and resuspended in 200 μL of annexin-V buffer [(10 mM HEPES pH 7.4), 140 mM NaCl, and 2.5 mM $CaCl_2$] and then incubated at room temperature with 1 μL of annexin-V-FITC (Becton Dickinson, San Diego, CA, USA) and 1 μg/mL of PI (Sigma-Aldrich), for 30 min at 37 °C. Sample analysis was performed using a BD C6 Accuri flow cytometer (Becton Dickinson), with the acquisition of 5000 events. Hoechst 33342 (10 μg/mL) from Life Technologies (Eugene, OR, USA) was added to some samples to evaluate the DNA content, which were analyzed using a FACSAria IIII flow cytometer (Becton Dickinson), with the acquisition of 20 000 events. In some experiments, inhibitors were preincubated for 1 h prior to peptide addition: 10 μM ZVAD-FMK from Tocris (Minneapolis, MN, USA); 5 μM necrostatin-1 (Tocris).

Caspase-3 Activation. Caspase-3 activity was measured by flow cytometry. Jurkat cells (1×10^5 cells/mL) were treated with 218 nM **1** or 1 μM staurosporine for 12 h. Then, cells were fixed and permeabilized with BD Cytofix/Cytoperm solution (Becton Dickinson) for 20 min. Cells were subsequently stained with rabbit anticleaved caspase-3 antibody conjugated with Alexa Fluor 488 from Cell Signaling Technology (Danvers, MA, USA) according to the manufacturer's instructions. Cells were analyzed using a BD C6 Accuri flow cytometer (Becton Dickinson) with 15 000 events collected per sample.

Determination of Mitochondrial Membrane Potential (Ψ_{mit}) by Flow Cytometry. The Ψ_{mit} was measured by flow cytometry using the lipophilic cationic dye JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolecarbocyanine iodide (Life Technologies). JC-1 is a cationic dye that exhibits potential-dependent accumulation in mitochondria by a fluorescence emission shift from green (B520 nm) to red (B590 nm). Consequently, mitochondrial depolarization is indicated by a decrease in the red−green fluorescence intensity ratio.[24] Jurkat cells (1×10^5 cells/mL) were incubated for 24 h with 218 nM **1** or 50 μM carbonyl cyanide 3-chlorophenylhydrazone (CCCP) from Tocris. Then, 5 μg/mL of JC-1 was added for 30 min at room temperature. Fluorescence was analyzed using a BD C6 Accuri flow cytometer, with 15 000 events collected per sample.

Statistical Analysis. The results of parametric tests are expressed as means ± SEM. One-way analysis of variance (ANOVA) was used for the comparison of more than two means followed by Dunnett's multiple comparison post hoc test; p values < 0.05 were considered significant. GraphPad Prism 5 software version 5.01 (San Diego, CA, USA) was used for data analysis.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.5b00177.

1D and 2D NMR spectra and MS/MS data of [1-8-NαC]-zanriorb A1 (**1**) and experimental details on the activity against leukemia cell lines (PDF)

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Notes

The authors declare no competing financial interest.

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J. Nat. Prod. XXXX, XXX, XXX−XXX

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