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Vascular L-Type Ca^{2+} Channel Blocking Activity of Sulfur-Containing Indole Alkaloids from *Glycosmis petelotii*

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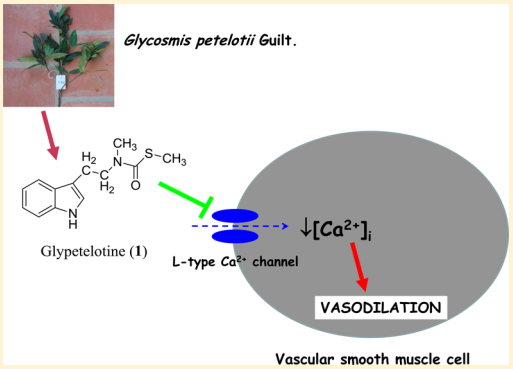
Supporting Information

ABSTRACT: In the search for novel natural compounds endowed with potential antihypertensive activity, a new sulfur-containing indole alkaloid, *N*-demethylglypetelotine (**2**), and its known analogue glypetelotine (**1**), were isolated from the leaves of *Glycosmis petelotii*. Their structures were established on the basis of spectroscopic evidence. The two alkaloids were assessed for vasorelaxing activity on rat aorta rings and for L-type Ba^{2+} current [$I_{Ba(L)}$] blocking activity on single myocytes isolated from rat tail artery. Both glypetelotine and *N*-demethylglypetelotine inhibited phenylephrine-induced contraction with IC_{50} values of 20 and 50 μM, respectively. The presence of endothelium did not modify their spasmolytic effect. Neither glypetelotine nor *N*-demethylglypetelotine affected Ca^{2+} release from the sarcoplasmic reticulum induced by phenylephrine. The spasmolytic effect of glypetelotine increased with membrane depolarization. In the presence of 60 mM K^+, both compounds inhibited, in a concentration-dependent manner, the contraction induced by cumulative addition of Ca^{2+}, this inhibition being inversely related to Ca^{2+} concentration. Glypetelotine and, less efficiently *N*-demethylglypetelotine, inhibited $I_{Ba(L)}$, the former compound also affecting $I_{Ba(L)}$ kinetics. In conclusion, glypetelotine is a novel vasorelaxing agent which antagonizes L-type Ca^{2+} channels.



Plants are a generous source of new molecules endowed with attractive chemical, biochemical, and pharmacological features.[1] In recent years, the pharmaceutical interest in plant products has increased, either as a result of the advances in mass spectrometry, NMR, and other spectroscopic techniques that have helped to overcome the challenges encountered in screening natural products libraries or as a consequence of frustration caused by numerous failures of the so-called lead generation strategy.[2] In the area of cancer, for example, more than 70% of the biologically active new chemical entities reported between 1940s and 2010 resulted from studies on natural products.[1] The chemical complexity renders natural products capable of interacting simultaneously with several pharmacologically relevant targets.[3] This aspect might explain their popular widespread use in human chronic-degenerative diseases (e.g., cancer, neurodegenerative diseases, and hypertension)[4] where monotarget drugs may prove of limited efficacy.

In Vietnam, the Rutaceae family comprises ca. 100 species grouped in 25 genera including *Clausena*, *Glycosmis*, and *Murraya*, distributed throughout the country and mostly concentrated in northern regions such as Hoa Binh (Cuc Phuong National Park), in limestone mountain regions, and in some regions in South Vietnam such as Quang Tri, Da Nang, Ha Tien, Phu Quoc.[5] In Vietnamese traditional medicine, decoction or extracts of *Glycosmis* plants are used for arthritis, jaundice, pain treatment and by external application for the cure of pimples, impetigo, snake bites, and scabies.[6] As part of our current investigation on bioactive compounds from Vietnamese medicinal plants aimed at the development of new therapeutic agents, we now report the isolation, structural elucidation, and vascular activity of two sulfur-containing indole alkaloids obtained from *Glycosmis* species. In particular, glypetelotine showed vasodilating activity partly attributable to blocking L-type Ca^{2+} channels.

RESULTS AND DISCUSSION

Isolation and Characterization of Glypetelotine and *N*-Demethylglypetelotine. From the leaf extract of *Glycosmis petelotii* collected in a mountain area of North Vietnam, two sulfur-containing indole alkaloids, glypetelotine (**1**) and *N*-demethylglypetelotine (**2**), have been isolated.

Compound **1** was isolated as white powder. The ^{1}H NMR (aceton-d_6, 500 MHz) spectrum of **1** featured six protons in the

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aromatic region (δ_H 6.99−8.20) and 10 protons in the aliphatic region (δ_H 2.36−3.70). Two doublets at δ_H 7.67 (H-4) and 7.39 (H-7) and two triplets at δ_H 7.04 (H-5) and 7.11 (H-6, J = 7.5 Hz) in the aromatic region were characteristic for a 1,2-disubstituted benzene ring. The broad signal at δ_H 10.05, exchangeable with D_2O, was assigned to an N−H group. The ^{13}C NMR and DEPT spectra showed 13 carbons, among which the signals of one aromatic methine at δ_C 123.4 (C-2) and three non-hydrogenated carbons, including δ_C 112.8 (C-3), 137.7 (C-8), and 128.5 (C-9), demonstrated the presence of an [1H]-indole nucleus in compound **1**. On the basis of these spectroscopic data, compound **1** was identified as the previously reported glypetelotine (Figure 1b).[7] Recently, this compound was totally synthesized from indole in six steps.[8]

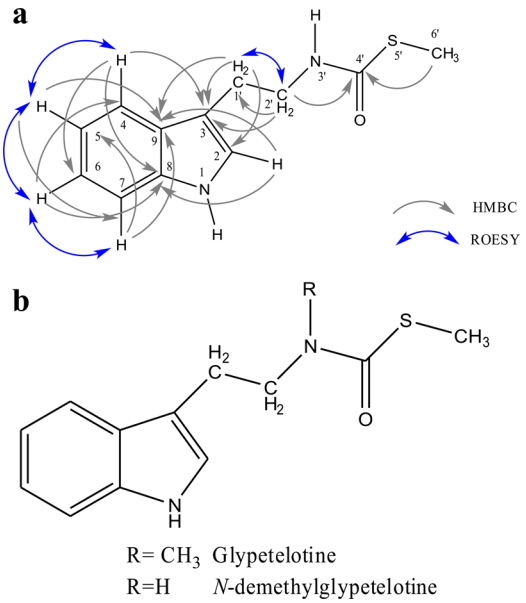

Figure 1. *N*-Demethylglypetelotine and glypetelotine. (a) Structure and HMBC/ROESY correlations in *N*-demethylglypetelotine. (b) Structures of the compounds.

Compound **2** was isolated as white needles and is a new natural product. The structure of compound **2** was investigated and confirmed by 2D-NMR spectra, including HSQC, HMBC, and ROESY (Table 1 and Figure 1a), and by IR and HR-MS data. The HR-MS spectrum of **2** showed a pseudomolecular ion at m/z 235.09017 ($[M + H]^+$), 14 Da less than that of compound **1**. The ^{13}C NMR and DEPT spectra of compound **2** showed only 12 carbon signals including those from one methyl carbon (δ_C 11.9), two methylene carbons (δ_C 26.4 and 42.7), five aromatic methine carbons, and four non-hydrogenated carbons. The ^1H NMR spectra of **2** led also to the presence of an [1H]-indole nucleus (C_8H_6N) and the side chain C_4H_8NOS. Two broad signals in the aromatic field at δ_H 10.02 (H-1) and 7.29 (H-3′), which were exchangeable with D_2O, were assigned to two N−H groups. The spectroscopic data of compound **2** showed it did not possess a methyl group attached to the *N*-amine of the side chain. This was also confirmed by IR spectroscopy where the broad peak at 3421 cm^{-1} represented two N−H groups (ν_{N-H} indol and ν_{N-H} side chain).

Similar to compound **1**, structure of **2** showed three spin systems, including one indole nucleus, two coupled methylenes, and one thiocarbamate group. The proton H-2 at δ_H 7.17 (broad signal) showed HMBC cross-peaks with carbons C-3 (δ_C 113.1), C-8 (137.7), and C-9 (128.5) of the indole nucleus. The methylene carbon C-1′ (δ_C 26.4) was attached directly to the indole ring at C-3 position, as confirmed by HMBC cross-peaks of methylene protons H-1′ to three indole carbons C-2 (δ_C 123.4), C-3, and C-9, whereas methylene protons H-2′ showed HMBC interaction with indole carbon C-3, methylene carbon C-1′, and thiocarbamate carbon C-4′ (δ_C 167.0). The presence of one thiocarbamate group was also shown by the HR-MS peak at m/z 187.08677 ($[M - CH_3SH + H]^+$). Thus, the structural sequence in compound **2** was determined as CH_3−S−CO−NH−CH_2CH_2−indole. From the above spectroscopic evidence, compound **2** was established as a new sulfur-containing indole alkaloid, named *N*-demethylglypetelotine (**2**) (Figure 1b).

Table 1. NMR Spectroscopic Data for Compound 2

position	δ_C, type	δ_H (J in Hz)	HMBC[a]	ROESY[b]
2	123.4, CH	7.17, br.s	3, 8, 9	
3	113.1, C			
4	119.2, CH	7.60, d (8.0)	3, 6, 8, 9	6
5	119.4, CH	7.02, t (7.5)	7, 9	
6	122.1, CH	7.09, t (7.5)	4, 8	4
7	112.1, CH	7.38, d (8.5)	5, 9	
8	137.7, C			
9	128.5, C			
1′	26.4, CH_2	2.97, t (7.5)	2, 3, 9, 2′	2′
2′	42.7, CH_2	3.56, dt (6.5, 6.5)	3, 1′, 4′	1′
4′	167.0, C			
6′	11.9, S-CH_3	2.27, s	4′	
NH (1)		10.02, br.s		
NH (3′)		7.29, br.s		

[a]HMBC correlations are from proton(s) stated to the indicated carbon. [b]ROESY correlations are from proton(s) stated to proton(s) attached to the indicated carbon.

Ca^{2+} Antagonistic Effects of Glypetelotine and *N*-Demethylglypetelotine. As shown in Figure 2, glypetelotine and *N*-demethylglypetelotine caused a concentration-dependent relaxation (spasmolytic effect) of endothelium-denuded rings contracted by 60 mM K^+, with IC_{50} values of 15.8 and 79.4 μM (Table 2), respectively. Glypetelotine, but not *N*-demethylglypetelotine, fully reverted the high K^+-induced

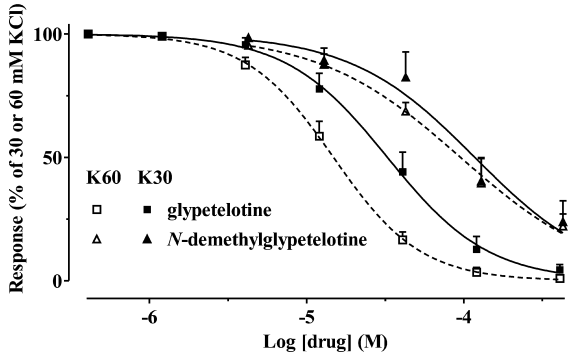

Figure 2. Effect of glypetelotine and *N*-demethylglypetelotine on high K^+-induced contraction of rat aorta rings. Effect of the compounds on endothelium-denuded rings depolarized with either 30 (K30) or 60 mM extracellular K^+ (K60). In the ordinate scale, relaxation is reported as a percentage of the initial tension induced by K30 or K60, taken as 100%. Data points are mean ± SE (n = 5−11).

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Table 2. Effect of Glypetelotine, *N*-Demethylglypetelotine, and Verapamil on Phenylephrine-, 30 mM- or 60 mM-K^+-Induced Contraction of Rat Aorta Rings

	60 mM K^+		30 mM K^+		phenylephrine			
	−endothelium		−endothelium		−endothelium		+endothelium	
drug	pIC_{50}	E_{max} (%)	pIC_{50}	E_{max} (%)	pIC_{50}	E_{max} (%)	pIC_{50}	E_{max} (%)
glypetelotine	4.8 ± 0.1[a]	99.0 ± 0.6	4.5 ± 0.1[b]	95.6 ± 2.1	4.7 ± 0.1[a]	94.8 ± 0.9	4.4 ± 0.1[a,c]	85.2 ± 5.2[d,e]
N-demethylglype	4.1 ± 0.1[a]	77.8 ± 5.0[a]	3.9 ± 0.2	76.1 ± 8.5	4.3 ± 0.2[a]	70.0 ± 7.5[a]	4.2 ± 0.1[a]	67.8 ± 5.0[a]
verapamil	7.1 ± 0.1	99.3 ± 0.7	N.C.	N.C.	6.4 ± 0.3	99.8 ± 0.2	5.8 ± 0.2	98.5 ± 1.0

Rings, endothelium-denuded (−endothelium) or -intact (+endothelium), were contracted either by 0.3 μM phenylephrine or by depolarization with 60 mM or 30 mM K^+. Potency (pIC_{50}) and efficacy (E_{max}) are reported as mean ± SE (n = 4−14). E_{max}, given as percentage of maximal relaxation, was evaluated at the highest concentration (403 μM for glypetelotine, 427 μM for *N*-demethylglypetelotine, and 100 μM for verapamil) of the drug assessed. N.C.: Not calculated. [c,d,e]$p < 0.05$, [a,b]$p < 0.01$, one-way ANOVA and Bonferroni or Dunnett's post test, or Student's t test for unpaired samples. [a,d] vs verapamil, [b,e] vs 60 mM K^+, [c] vs phenylephrine −endothelium.

contraction. Under the same experimental conditions, the Ca^{2+}-channel blocker verapamil[9] induced a concentration-dependent spasmolytic activity with an IC_{50} value of 79.4 nM.

When rings were depolarized with lower K^+ concentrations (e.g., 30 mM), the spasmolytic potency of glypetelotine decreased significantly while that of *N*-demethylglypetelotine was unaffected (Figure 2). In fact, glypetelotine showed a pIC_{50} value lower than that recorded in rings depolarized with 60 mM K^+ (Table 2).

Figure 3a shows the effects of the alkaloids on the contraction induced by cumulative additions of Ca^{2+} (0.03−3 mM) to endothelium-denuded rings depolarized with a 60 mM K^+-containing, Ca^{2+}-free solution. Both compounds reduced the Ca^{2+}-induced contraction in a concentration-dependent manner (antispasmodic effect; Table 3). A significant reduction in the maximum response was also observed, though only at the highest drug concentration tested (data not shown). It is also evident that the inhibition exerted by 40 μM glypetelotine and 128 μM *N*-demethylglypetelotine was inversely related to the extracellular Ca^{2+} concentration (from 100.0% and 98.8% at 300 μM Ca^{2+} to 74.0% and 73.9% at 3 mM Ca^{2+}, respectively). The reference compound verapamil (1 μM) blocked the Ca^{2+}-induced contraction (data not shown).

At the end of the assay, after the last addition of Ca^{2+}, any potential pharmacological interaction of either alkaloids with the Ca^{2+} channel agonist (*S*)-(−)-Bay K 8644[10] was assessed (Figure 3a). In rings pretreated with DMSO, 10 nM (*S*)-(−)-Bay K 8644 further stimulated vascular tone by about 40%. The inhibition exerted by glypetelotine and *N*-demethylglypetelotine was reversed by the addition of (*S*)-(−)-Bay K 8644 (Figure 3b).

The effects of glypetelotine and *N*-demethylglypetelotine on $I_{Ba(L)}$ recordings were assessed at a V_h of −50 mV. *N*-demethylglypetelotine was effective only at the highest concentrations tested (128 and 427 μM), reducing $I_{Ba(L)}$ to 75.9% and 53.4% of control value, and *N*-demethylglypetelotine was not further investigated. Glypetelotine antagonized $I_{Ba(L)}$ in a concentration-dependent manner (estimated IC_{50} value of 251 μM; Figure 4a) and, at the maximum concentration tested, significantly inhibited the current−voltage relationship without changing the apparent maximum and threshold values (Figure 4b). Under the same experimental conditions, verapamil induced a concentration-dependent block with an IC_{50} value of 481 ± 140 nM and an E_{max} value of 100% (n = 4).

Under control conditions, $I_{Ba(L)}$ evoked at 10 mV from a V_h of −50 mV was maximally activated within 5−10 ms after depolarization and then slowly inactivated (Figure 5, inset); its time course could be fitted by a two-exponential function.

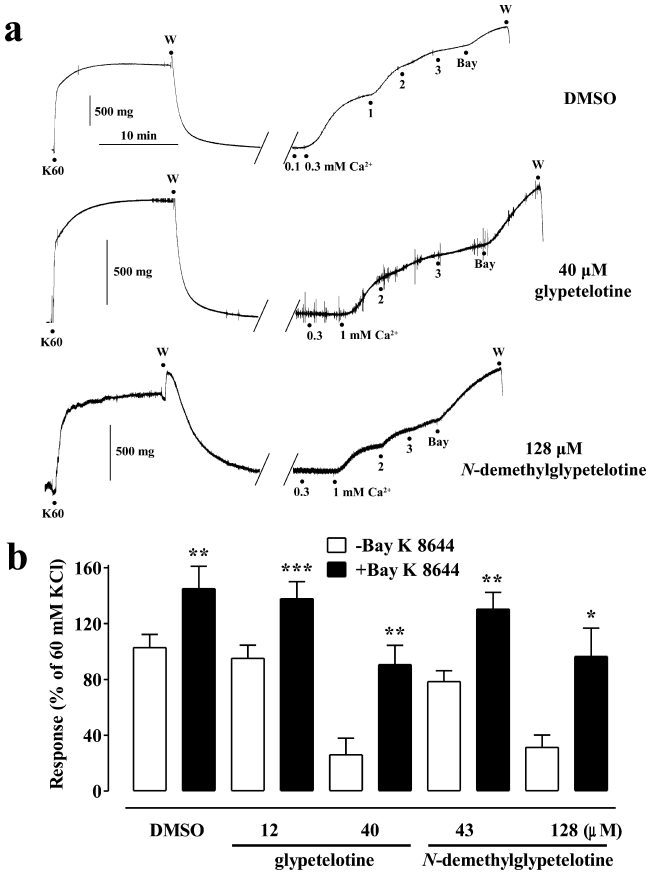

Figure 3. Effect of (*S*)-(−)-Bay K 8644 on glypetelotine and *N*-demethylglypetelotine inhibition of Ca^{2+}-induced contraction of rat aorta rings depolarized with high K^+. (a) Traces (representative of 4−17 similar experiments) of Ca^{2+}-induced contraction in endothelium-deprived rings depolarized with a Ca^{2+}-free, 60 mM K^+ PSS in the presence of DMSO (upper panel), 40 μM glypetelotine (mid panel), or 128 μM *N*-demethylglypetelotine (lower panel). At the end of the experiment, 10 nM (*S*)-(−)-Bay K 8644 was added to stimulate L-type Ca^{2+} channels. The effect of 60 mM K^+ recorded under control conditions is also shown (first part of the traces). (b) Effect of 10 nM (*S*)-(−)-Bay K 8644 on Ca^{2+}-induced vascular tone of depolarized rings treated with either glypetelotine or *N*-demethylglypetelotine. Columns are mean ± SE (n = 4−17) and represent the percentage of the response to 60 mM K^+, taken as 100%. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs −Bay K 8644, Student's t test for paired samples.

Glypetelotine accelerated both the τ of inactivation and that of activation in a concentration-dependent manner (Figure 5b).

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Table 3. Ca^{2+}-Induced Contraction in Rat Aorta Rings Depolarized with 60 mM K^+ in the Presence of Glypetelotine, *N*-Demethylglypetelotine, and Verapamil

drug	AUC	*n*
7 mM DMSO	82.7 ± 8.3	13
12 μM glypetelotine	68.8 ± 15.6	9
40 μM glypetelotine	12.7 ± 5.8[a]	5
43 μM *N*-demethylglypetelotine	57.6 ± 17.3	8
128 μM *N*-demethylglypetelotine	11.2 ± 3.6[a]	6
1 μM verapamil	0	6

[a]AUC (area under the curve) values calculated from Figure 3. Data represent mean ± ES $p < 0.01$ vs DMSO, one-way ANOVA and Dunnett's post-test.

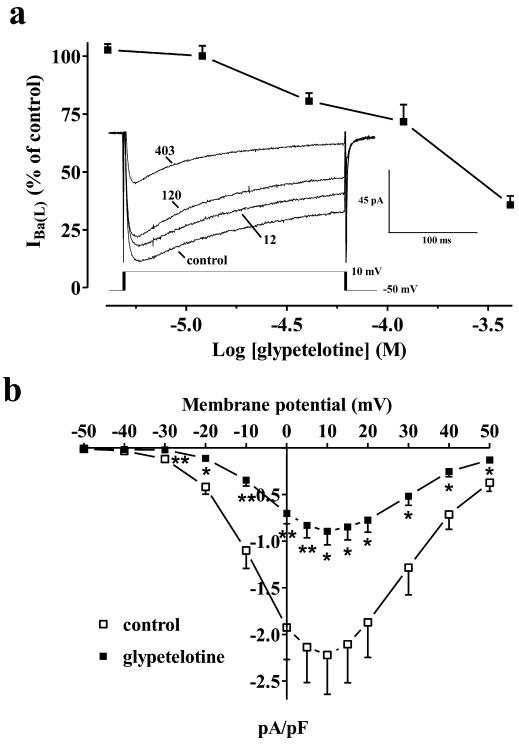

Figure 4. Glypetelotine inhibition of $I_{Ba(L)}$ of single rat tail artery myocytes. (a) Concentration-dependent effect of glypetelotine at the peak of $I_{Ba(L)}$ trace. On the ordinate scale, response is reported as percentage of control. Data points are mean ± SE (n = 5–6). Inset: average traces (recorded from five cells) of conventional whole-cell $I_{Ba(L)}$ elicited with 250 ms clamp pulses to 10 mV from a V_h of −50 mV (see schematic diagram), measured in the absence (control) or presence of various concentrations (μM) of glypetelotine. (b) Current–voltage relationships, recorded from a V_h of −50 mV, constructed prior to the addition (control) and in the presence of 403 μM glypetelotine. Data points are mean ± SE (n = 5). *$p < 0.05$, **$p < 0.01$ vs control, Student's t test for paired samples.

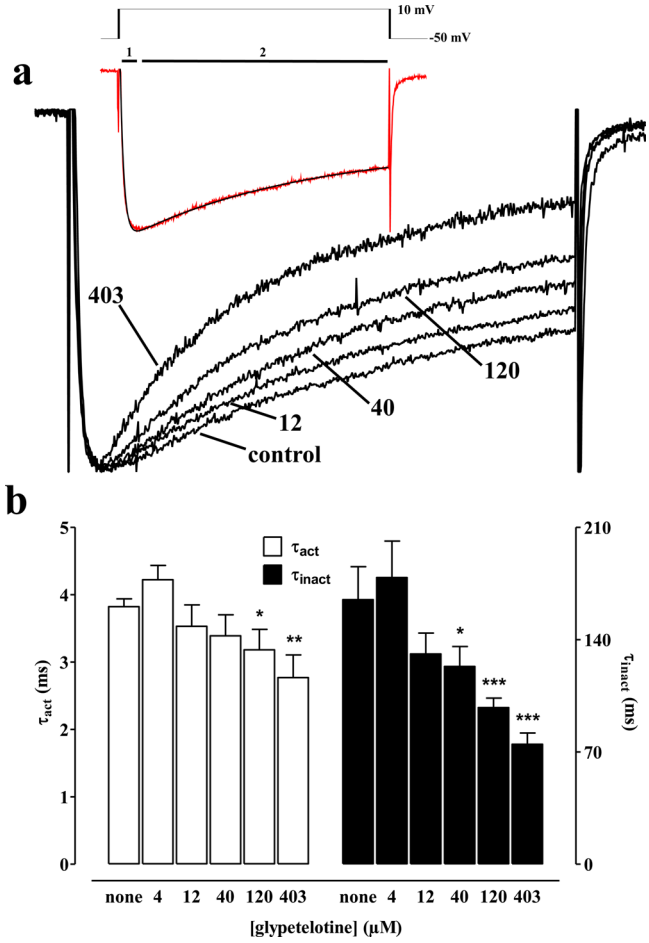

Figure 5. Effects of glypetelotine on $I_{Ba(L)}$ kinetics of single rat tail artery myocytes. (a) Traces of conventional whole-cell $I_{Ba(L)}$ elicited with 250 ms clamp pulses to 10 mV from a V_h of −50 mV, measured in the absence (control) or presence of various concentrations (μM) of glypetelotine. Traces recorded in the presence of glypetelotine were magnified so that the peak amplitude matched that of control. Inset: fitting curve to the control trace, as evidenced by the overlapping blue line. The horizontal lines indicate the fast activation (1) and slow inactivation (2) portion of the current, respectively. (b) Time constant for activation (τ_{act}) and for inactivation (τ_{inact}) measured in the absence (none) or presence of different concentrations of glypetelotine (μM). Columns represent mean ± SE (n = 4). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$, repeated measures ANOVA and Dunnett's post test.

The myorelaxation promoted by glypetelotine shared several basic features which characterize Ca^{2+} channel blockers such as verapamil.[11] First, the extent of the inhibition of high K^+-induced contraction by glypetelotine was inversely correlated to the external concentration of Ca^{2+}.[12] Second, glypetelotine potency improved as the membrane voltage (i.e., the concentration of extracellular K^+) increased,[13,14] thus suggesting that depolarization favored drug blockade of the channel. Third, inhibition of Ca^{2+}-induced contraction was reversed by the Ca^{2+} channel agonist (*S*)-(−)-Bay K 8644.[15] Fourth, glypetelotine antagonized $I_{Ba(L)}$ in a concentration-dependent manner.

Glypetelotine is 10–1000-fold less potent than verapamil. However, the Ca^{2+} antagonist drugs currently used for the treatment of hypertension display several unwanted side effects that represent the most important cause of noncompliance to therapy.[16] Therefore, it is conceivable that the sulfur-containing indole alkaloids may aid in the development of novel, safer antihypertensive agents.

Within the framework of the state-dependent pharmacology of the channel, two different mechanisms could explain glypetelotine Ca^{2+}-channel blocking activity: (i) a state-independent, tonic inhibition interpreted as a consequence of the drug binding to the resting channel; (ii) a state-dependent open channel inhibition leading to the faster L-type Ca^{2+} channel inactivation kinetics observed in the presence of the

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drug,[17] a phenomenon likely originating from the interaction of glypetelotine with the channel in the inactivated state. Finally, the partial antagonism of $I_{Ba(L)}$ exerted by glypetelotine suggested that other mechanisms beyond Ca^{2+} channel inhibition add to its myorelaxing activity. Opening of K^+ channels has to be ruled out, because the relaxant effect of K^+ channel openers becomes less pronounced as the extracellular K^+ concentration is raised.[18]

Effects of Glypetelotine and *N*-Demethylglypetelotine on Phenylephrine-Contracted Rings. As shown in Figure 6a, glypetelotine and *N*-demethylglypetelotine caused a concentration-dependent relaxation of endothelium-denuded rings contracted by 0.3 μM phenylephrine. Glypetelotine fully reverted phenylephrine-induced contractions (Table 2) with an IC_{50} value (20.0 μM) comparable to that recorded in preparations contracted by 60 mM K^+. Also *N*-demethylglypetelotine showed similar potency (IC_{50} value of 50 μM) and efficacy using the same protocol. Under the same experimental conditions, verapamil induced a concentration-dependent relaxation with an IC_{50} value of 398 nM.

In a second series of experiments, glypetelotine and *N*-demethylglypetelotine effects were tested on rings with intact endothelium contracted by 0.3 μM phenylephrine. Both compounds reverted phenylephrine-induced contraction with pIC_{50} values and maximum effects not significantly different from those recorded in preparations devoid of endothelium (Figure 6a and Table 2). Under the same experimental conditions, verapamil induced a concentration-dependent relaxation with an IC_{50} value of 1.6 μM.

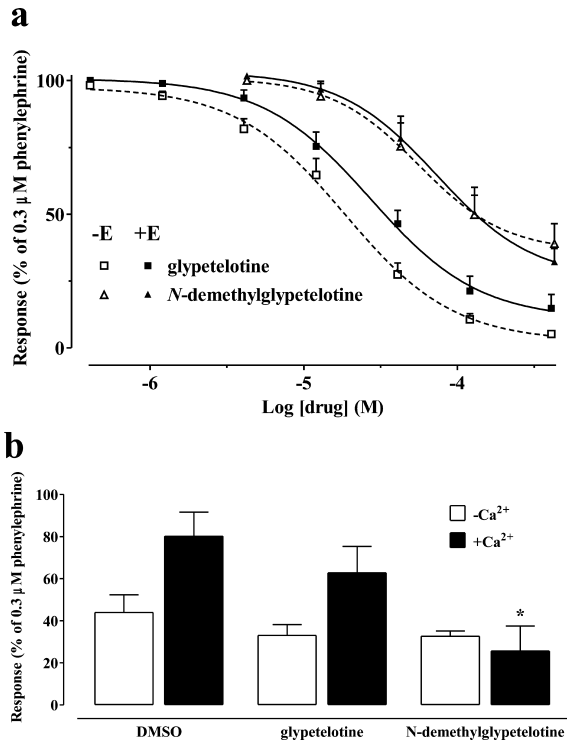

Figure 6. Effect of glypetelotine and *N*-demethylglypetelotine on phenylephrine-induced contraction of rat aorta rings. (a) Concentration–response curves for glypetelotine and *N*-demethylglypetelotine in endothelium-denuded (−E) or -intact (+E) rings precontracted by 0.3 μM phenylephrine. In the ordinate scale, relaxation is reported as percentage of the initial tension induced by phenylephrine, taken as 100%. Data points are mean ± SE (n = 5–14). (b) Columns represent 10 μM phenylephrine-induced contractions either in the absence ($-Ca^{2+}$) or in the presence ($+Ca^{2+}$) of extracellular Ca^{2+}, recorded in the presence of vehicle (DMSO), 40 μM glypetelotine, or 128 μM *N*-demethylglypetelotine. Columns are mean ± SE (n = 5–8) and represent the percentage of the response to 0.3 μM phenylephrine, taken as 100%. *p < 0.05 vs DMSO, one-way ANOVA and Dunnett's post test.

Vasorelaxing agents can antagonize phenylephrine promoted contraction by inhibiting phenylephrine-induced Ca^{2+} release from intracellular stores and/or extracellular Ca^{2+} influx. As shown in Figure 6b, pretreatment with submaximal concentrations of glypetelotine and *N*-demethylglypetelotine (see Figure 6a) did not affect the contraction elicited by 10 μM phenylephrine in Ca^{2+}-free medium. When the normal external Ca^{2+} concentration was restored, with phenylephrine still present, only *N*-demethylglypetelotine significantly inhibited the ensuing contraction (Figure 6b).

Findings obtained on aorta ring preparations stimulated with phenylephrine provided important information on the mechanism of action of both glypetelotine and *N*-demethylglypetelotine. First, endothelium-derived vasodilators (e.g., NO) are not involved in their effect, because either compound relaxed both endothelium-intact and endothelium-denuded rings with similar potency and efficacy. Second, depletion of phenylephrine-sensitive Ca^{2+} stores has to be ruled out, because contrary to the ryanodine receptor agonist ryanodine,[19] they failed to elicit contraction under conditions of passive tone imposed to the ring (data not shown). Third, glypetelotine was more effective under conditions of active than passive tone, that is, when key signaling systems that regulate the contraction/relaxation cycle are fully, rather than partially, on.[20]

The *N*-methyl group seemed a key determinant of glypetelotine vasorelaxing activity. *N*-Demethylglypetelotine was less potent and effective and, in contrast to the parent compound, showed moderate selectivity toward the mechanisms underlying phenylephrine-induced contraction. However, an α_1 adrenergic receptor blockade by *N*-demethylglypetelotine is unlikely: this mechanism, in fact, would have given rise to a reduced Ca^{2+} release from the intracellular stores induced by phenylephrine.

In conclusion, glypetelotine produces significant vasodilating effects, partly due to a negative modulation of L-type Ca^{2+} channel influx, although its novel *N*-demethylated analogue was less active. The Rutaceae family of Vietnamese plants might therefore represent a source of molecules active at the vascular level and/or useful for probing L-type Ca^{2+} channels.

EXPERIMENTAL SECTION

General Experimental Procedures. ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz), with tetramethylsilane as an internal standard, were performed on a Bruker Avance 500 MHz spectrometer, whereas the HR-MS analysis was done with a Varian FT-ESI-MS mass spectrometer; column chromatography was carried out on silica gel (230–400, 400–630 mesh, Merck). UV and IR spectra were recorded on JASCO V-630 and Impact 410 Nicolet FT-IR spectrometer, respectively. All solvents were redistilled before use. Precoated plates of silica gel 60 F_{254} were used for analytical purposes. Compounds were visualized by UV irradiation (254–365 nm) and by spraying plates with 10% H_2SO_4 followed by heating with a heat gun.

The physiological salt solution (PSS) was a modified Krebs–Henseleit saline solution containing (in mM): 118 NaCl, 4.75 KCl , 1.19 KH_2PO_4, 1.19 $MgSO_4{\cdot}7H_2O$, 25 $NaHCO_3$, 11.5

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glucose, 2.5 $CaCl_2 \cdot 2H_2O$; gassed with a 95% O_2/5% CO_2 gas mixture to create a pH of 7.4. PSS containing KCl at a concentration greater than 4.75 mM was prepared by replacing NaCl with equimolar KCl.

External solution contained (in mM): 130 NaCl, 5.6 KCl, 10 HEPES, 20 glucose, 1.2 $MgCl_2 \cdot 6\ H_2O$, and 5 Na-pyruvate; pH 7.4. The internal solution contained (in mM): 100 CsCl, 10 HEPES, 11 EGTA, 1 $CaCl_2$ (pCa 8.4), 2 $MgCl_2 \cdot 6\ H_2O$, 5 Na-pyruvate, 5 succinic acid, 5 oxalacetic acid, 3 Na_2-ATP, and 5 phosphocreatine; pH was adjusted to 7.4 with CsOH.

Phenylephrine, acetylcholine, collagenase (type XI), trypsin inhibitor, BSA, tetraethylammonium chloride, EGTA, HEPES, taurine, (*S*)-(−)-Bay K 8644, nifedipine, and verapamil were from Sigma Chimica (Milan, Italy); sodium nitroprusside was from Riedel-De Haën AG (Seelze-Hannover, Germany). Glypetelotine and demethylglypetelotine (100 mM stock solution) were dissolved directly in dimethyl sulfoxide; nifedipine and (*S*)-(−)-Bay K 8644 were dissolved in ethanol, and they were diluted at least 1000 times prior to use. All these solutions were stored at −20 °C and protected from light by wrapping the containers with aluminum foil. The resulting concentrations of dimethyl sulfoxide and ethanol (below 0.1%, v/v) failed to alter the response of the preparations. Phenylephrine was dissolved in 0.1 M HCl. Sodium nitroprusside was dissolved in distilled water. All other substances used were of analytical grade and used without further purification.

Plant Materials. The leaves and stems of *Glycosmis petelotii* were collected in March 2012 at Hoa Binh province, Vietnam. The plant was identified by the ethno-botanist Dr. Ngo Van Trai (Institute of Medicinal Materials, Hanoi). Herbarium specimens (C-451) were deposited in the herbarium of the Institute of Natural Products Chemistry, Vietnam Academy of Science and Technology, Hanoi, Vietnam.

Extraction and Isolation. Dried powder of leaves and stems of *Glycosmis petelotii* (10.4 kg) were extracted with MeOH (3 × 3.5 L) over a period of 5 days at room temperature; they were filtered and concentrated under decreased pressure to produce a crude MeOH extract (1.2 kg). This extract was suspended in a MeOH/H_2O mixture (1/1 v/v) and subsequently partitioned with *n*-hexane, chloroform, ethyl acetate, butanol, and water to obtain the respective *n*-hexane (GP-H, 231.2 g), chloroform (GP-C, 98.7 g), ethyl acetate (GP-E, 90.1 g), butanol (GP-B, 90.0 g), and water (GP-W) fractions. The chloroform fraction was subjected to CC on flash silica gel column (400−630 mesh) using a gradient of solvents (v/v) chloroform−EtOAc (19/1−1/1); chloroform−EtOAc−MeOH (1/1/0.1); and chloroform−MeOH (6/1−0/1) to afford 13 fractions (G1-G13). Fraction G1 was further rechromatographed on flash silica gel (230−400 mesh) (*n*-hexane−acetone 1/0−0/1 as eluent) to yield 12 subfractions (GT1−GT12). Subfraction GT5 was subjected to CC on a silica gel column (230−400 mesh) using *n*-hexane−acetone (10/1) as eluent to obtain compounds **1** as white powder (10.3 g, yield 0.10%) and **2** as white needles (0.095 g, yield 0.09%).

Glypetelotine (1). S-methyl-*N*,*N*-2-[(1H)-indol-3-ethyl]-methyl-thiocarbamate, $C_{13}H_{16}N_2OS$ (MW = 248); soluble in chloroform, acetone, and methanol; mp 92−93 °C. ^{1}H NMR (acetone-d_6, 500 MHz): δ 10.02 (1H, s, H-1), 7.67 (1H, d, J = 7.5 Hz, H-4), 7.39 (1H, d, J = 8.0 Hz, H-7), 7.19 (1H, br., H-2), 7.11 (1H, t, J = 7.5 Hz, H-6), 7.04 (1H, t, J = 7.5 Hz, H-5), 3.66 (2H, br., 1′−CH_2), 2.98 (2H, br., 2′−CH_2), 2.88 (3H, s, 3′-N-CH_3), 2.29 (3H, s, 6′−S−CH_3). ^{13}C NMR (acetone-d_6, 125 MHz): δ 168.2 (C, S-CO−N), 137.7 (C, C-8), 128.5 (C, C-9), 123.4 (CH, C-2), 122.2 (CH, C-6), 119.5 (CH, C-5), 119.2 (CH, C-4), 112.8 (C, C-3), 112.2 (CH, C-7), 51.5 (CH_2, C-1′), 24.2 (CH_2, C-2′), 12.8 (CH_3, 6′- S-CH_3), 35.2 (CH_3, 7′-N-CH_3); ESI-MS m/z: $[M + Na]^+$ 271.1.

***N*-Demethylglypetelotine (2).** S-methyl *N*,*N*-2-[(1H)-indol-3-ethyl]-thiocarbamate, $C_{12}H_{14}N_2OS$ (MW = 234); soluble in chloroform, acetone and methanol; mp 90 °C. UV λ_{EtOH}, (nm log ϵ): 282 (3.98), 223 (4.60). IR (KBr, cm^{-1}): 3421 (br.) (ν_{N-H} indol and ν_{N-H} side chain), 3057 (aromatic $\nu_{=CH}$), 1678 ($\nu_{C=O}$), 1590 ($\delta_{N-H\ (amide)}$), 1467 (δ_{CH}), 1104 (ν_{CO-S}), 730 (ν_{C-S}) and 2222. ^{1}H NMR (acetone-d_6, 500 MHz): δ 10.02 (1H, s (br.), H-1), 7.60 (1H, d, J = 8.0 Hz, H-4), 7.38 (1H, d, J = 8.5 Hz, H-7), 7.29 (1H, s (br.), H-3′), 7.17 (1H, s (br.), H-2), 7.09 (1H, t, J = 7.5 Hz, H-6), 7.02 (1H, t, J = 7.5 Hz, H-5), 3.55 (2H, dt, J = 6.5, 6.5 Hz, 2′−CH_2), 2.97 (2H, t, J = 7.5 Hz,1′−CH_2), 2.27 (3H, s, 6′−CH_3). ^{13}C NMR (acetone-d_6 125 MHz): δ 167.0 (C, S-CO−N), 137.7 (C, C-8), 128.5 (C, C-9), 123.4 (CH, C-2), 122.1 (CH, C-6), 119.4 (CH, C-5), 119.2 (CH, C-4), 113.1 (C, C-3), 112.1 (CH, C-7), 42.7 (CH_2, 2′- CH_2), 26.4 (CH_2, 1′-CH_2), 11.9 (CH_3, 6′- S-CH_3). HR-ESI-MS m/z: $[M + H]^+$ calcd for $C_{12}H_{15}N_2OS$, 235.09050; found, 235.09017. HR-ESI-MS m/z: $[M + Na]^+$ calcd for $C_{12}H_{14}N_2OSNa$, 257.07245; found, 257.07261. HR-ESI-MS m/z: $[M + Na]^+$ calcd for $C_{11}H_{11}N_2O$, 187.08713; found, 187.08677.

Aorta Ring Preparation. All animal care and experimental procedures complied with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH publication no. 85-23, revised 1996) and were approved by the Animal Care and Ethics Committee of the Università di Siena, Italy (08-02-2012). Aorta rings (2 mm wide), either endothelium-intact or -denuded, were prepared from male Wistar rats (350−400 g; Charles River Italia, Calco, Italy), anaesthetized (ip) with a mixture of Ketavet (30 mg/kg ketamine; Intervet, Aprilia, Italy) and Xilor (8 mg/kg xylazine; Bio 98, San Lazzaro, Italy), decapitated, and exsanguinated as described elsewhere.[21] The endothelium was removed by gently rubbing the lumen of the ring with the curved tips of a pair of forceps. Each arterial ring was mounted over two rigid parallel, L-shaped stainless steel bars, one fixed in place and the other attached to an isometric transducer (Fort 25, WPI, Berlin, Germany). Contractile tension was recorded with a digital PowerLab data acquisition system (PowerLab 8/30; ADInstruments, Castle Hill, Australia) and analyzed by using LabChart 7.3.7 Pro (Power Lab; ADInstruments). The preparations were allowed to equilibrate for 60 min in order to overcome mechanical stress. During equilibration, PSS was renewed every 15 min and tension, if diminished by more than 25% within the first 15 min, was restored to its initial value. The presence of functional endothelium was assessed in all preparations by testing the ability of acetylcholine (10 μM) to reduce the 0.3 μM phenylephrine-induced contraction by a value ≥75%; on the contrary, a relaxation <10% was considered representative of the lack of the endothelial layer. Experiments were mostly conducted on endothelium-denuded rings unless otherwise indicated. Control preparations were treated with the drug vehicle only.

Spasmolytic Effect of the Indole Alkaloids on Aorta Rings Depolarized with High K^+ Concentrations. Steady tension was evoked in endothelium-deprived rings by either 30 mM or 60 mM K^+ (K30 or K60); thereafter, the drug under investigation was added cumulatively. At the end of each experiment, 10 μM nifedipine followed by 100 μM sodium

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nitroprusside were added to test muscle functional integrity. Spasmolysis was evaluated as a percentage of the initial response to K^+, taken as 100%.

Effect of the Indole Alkaloids on the Concentration−Response Curve for Ca^{2+}. Rings were stimulated with K60 for 15 min and then washed for 90 min with a Ca^{2+}-free PSS containing 1 mM EGTA. The preparations were then challenged with 0.3 μM phenylephrine to empty the intracellular Ca^{2+} stores. The antispasmogenic response to Ca^{2+} (0.03−3 mM) was assayed on rings depolarized with Ca^{2+}-free K60 by constructing cumulative concentration−response curves. The test substance or vehicle was present for 30 min before, as well as throughout the concentration−response curve procedure. At the end of each experiment, 10 nM (*S*)-(−)-Bay K 8644 followed by 100 μM sodium nitroprusside were added to test L-type Ca^{2+} channels as well as smooth muscle functional integrity. The antispasmodic effect was evaluated as a percentage of the initial response to K60, taken as 100%.

Myorelaxant Effect of the Indole Alkaloids on Aorta Rings Contracted by Phenylephrine. Steady tension was evoked in rings, either endothelium-intact or -deprived, by 0.3 μM phenylephrine; thereafter the drug under investigation was added cumulatively. At the end of each experiment, 100 μM sodium nitroprusside was added to test muscle functional integrity. Spasmolysis was evaluated as a percentage of the initial response to phenylephrine, taken as 100%.

Effect of the Indole Alkaloids on both Ca^{2+} Release from Intracellular Stores and Extracellular Ca^{2+} Influx Triggered by Phenylephrine. A Ca^{2+}-free solution containing 1 mM EGTA replaced PSS. Rings were exposed to this solution for 15 min[22] and then stimulated with 10 μM phenylephrine, which gave rise to a contraction due solely to the release of Ca^{2+} from the sarcoplasmic reticulum. The subsequent addition of Ca^{2+} (3.5 mM) to the solution, still in the presence of phenylephrine, caused a further contraction that was taken as an index of Ca^{2+} influx from the extracellular space triggered by the α_1-adrenoceptor agonist. Phenylephrine-elicited contractions were obtained after 30 min incubation with vehicle only or with the alkaloids. Rings were then washed for 45 min with PSS, which was renewed every 15 min. The entire procedure was repeated in the presence of a higher concentration of the drug. Responses were measured as a percentage of the contraction induced by 0.3 μM phenylephrine in PSS, taken as 100%.

Smooth Muscle Cell Isolation Procedure and Whole-Cell Patch Clamp Recordings. Smooth muscle cells were freshly isolated from the tail main artery, as previously described.[23] In brief, the artery was incubated at 37 °C in 2 mL of 0.1 mM Ca^{2+} external solution containing 20 mM taurine (prepared by replacing NaCl with equimolar taurine), 1.35 mg/mL collagenase (type XI), 1 mg/mL soybean trypsin inhibitor, and 1 mg/mL BSA, gently bubbled with a 95% O_2/5% CO_2 gas mixture After 45 min of incubation, the tissue was carefully washed with Ca^{2+}-free external solution, and individual smooth muscle cells were then obtained by gentle agitation with a Pasteur pipet, until the solution became cloudy. Cells were continuously superfused with an external solution containing 0.1 mM Ca^{2+} and 30 mM tetraethylammonium using a peristaltic pump (LKB 2132, Bromma, Sweden), at a flow rate of 400 μL/min.

The conventional whole-cell patch-clamp method[24] was employed to voltage-clamp smooth muscle cells. Recording electrodes were pulled from borosilicate glass capillaries (WPI, Berlin, Germany) and fire-polished to obtain a pipet resistance of 2−5 MΩ when filled with the internal solution. An Axopatch 200B patch-clamp amplifier (Molecular Devices Corporation, Sunnyvale, CA, U.S.A.) was used to generate and apply voltage pulses to the clamped cells and record the corresponding membrane currents. At the beginning of each experiment, the junction potential between the pipet and bath solution was electronically adjusted to zero. Current signals, after compensation for whole-cell capacitance and series resistance (between 70 and 75%), were low-pass filtered at 1 kHz and digitized at 3 kHz prior to being stored on the computer hard disk. Electrophysiological responses were tested at room temperature (20−22 °C).

The L-type Ba^{2+} current [$I_{Ba(L)}$] was always recorded in an external solution containing 30 mM tetraethylammonium and 5 mM Ba^{2+}. $I_{Ba(L)}$ was elicited with 250 ms clamp pulses (0.067 Hz) to 10 mV from a V_h of −50 mV. Data were collected once the $I_{Ba(L)}$ amplitude had been stabilized (usually 7−10 min after the whole-cell configuration had been obtained). $I_{Ba(L)}$ did not run down during the following 25−30 min under these conditions.[25]

K^+ currents were blocked with 30 mM tetraethylammonium in the external solution and Cs^+ in the internal solution. Current values were corrected for leakage using 10 μM nifedipine, which completely blocked $I_{Ba(L)}$.

The osmolarity of the 30 mM tetraethylammonium- and 5 mM Ba^{2+}-containing external solution (320 mosmol) and that of the internal solution (290 mosmol[26]) was measured with an osmometer (Osmostat OM 6020, Menarini Diagnostics, Florence, Italy).

Statistical Analysis. Analysis of data was accomplished by using GraphPad Prism version 5.04 (GraphPad Software, Inc., San Diego, CA, U.S.A.). Data are reported as mean ± SE; *n* is the number of rings or cells processed (indicated in parentheses), isolated from at least three animals. Statistical analysis and significance as measured by either one-way ordinary or repeated measures ANOVA (followed by Dunnett's post test) or Student's *t* test for paired samples (two tailed) were obtained using GraphPad InStat version 3.06 (GraphPad Software, Inc., U.S.A.). In all comparisons, $p < 0.05$ was considered significant.

The pharmacological response to each substance was given as either pIC_{50} or IC_{50}.

Time constants (τ) of $I_{Ba(L)}$ activation and inactivation were obtained by a fit from the $I_{Ba(L)}$ value at the beginning to that at the end of the voltage pulse by a two-exponential function using pCLAMP 9.2.1.9 (Molecular Devices Corporation, Sunnyvale, CA, U.S.A.). All fits showed a correlation coefficient >0.98.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

One-dimensional NMR (^1H, ^{13}C, DEPT), HMBC, HSQC, and ROESY spectra for *N*-demethylglypetelotine (**2**) are available online and freely accessible at http://pubs.acs.org.This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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