JOURNAL OF NATURAL PRODUCTS

Article

pubs.acs.org/jnp

Semisynthetic Analogues of Toxiferine I and Their Pharmacological Properties at α7 nAChRs, Muscle-Type nAChRs, and the Allosteric Binding Site of Muscarinic M_2 Receptors

Darius P. Zlotos,*[,†] Christian Tränkle,[‡] Ulrike Holzgrabe,[§] Daniela Gündisch,[⊥] and Anders A. Jensen[∥]

[†]Department of Pharmaceutical Chemistry, The German University in Cairo, New Cairo City, 11835 Cairo, Egypt

[‡]Pharmacology and Toxicology Section, Institute of Pharmacy, Rheinische Friedrich-Wilhelms-University, D-53121 Bonn, Germany

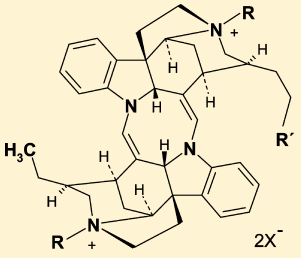
[§]Institute of Pharmacy and Food Chemistry, University of Würzburg, 97074 Würzburg, Germany

[⊥]Department of Pharmaceutical Sciences, Daniel K. Inouye College of Pharmacy, University of Hawai'i at Hilo, Hilo, Hawaii 96720, United States

[∥]Department of Drug Design and Pharmacology, Faculty of Health and Medical Sciences, University of Copenhagen, DK-2100 Copenhagen, Denmark

Supporting Information

ABSTRACT: A new series of analogues of the calabash curare alkaloid toxiferine I was prepared and pharmacologically evaluated at α7 and muscle-type nAChRs and the allosteric site of muscarinic M_2 receptors. The new ligands differ from toxiferine I by the absence of one (**2a**−**c**) or two (**3a**−**c**) hydroxy groups, saturation of the exocyclic double bonds, and various N-substituents (methyl, allyl, 4-nitrobenzyl). At the muscle-type nAChRs, most ligands showed similar binding to the muscle relaxant alcuronium, indicating neuromuscular blocking activity, with the nonhydroxylated analogues **3b** (K_i = 75 nM) and **3c** (K_i = 82 nM) displaying the highest affinity. At α7 nAChRs, all ligands showed a moderate to low antagonistic effect, suggesting that the alcoholic functions are not necessary for antagonistic action. Compound **3c** exerted the highest preference for the muscle-type nAChRs (K_i = 82 nM) over α7 (IC_{50} = 21 μM). As for the allosteric site of M_2 receptors, binding was found to be dependent on N-substitution rather than on the nature of the side chains. The most potent ligands were the *N*-allyl analogues **2b** and **3b** ($EC_{0.5,diss}$ = 12 and 36 nM) and the *N*-nitrobenzyl derivatives **2c** and **3c** ($EC_{0.5,diss}$ = 32 and 49 nM). The present findings should help delineate the structural requirements for activity at different types of AChRs and for the design of novel selective ligands.



	R	R´	X
2a	CH_3	OH	I
2b	allyl	OH	Br
2c	pNO_2Bn	OH	Br
3a	CH_3	H	I
3b	allyl	H	Br
3c	pNO_2Bn	H	Br

The neurotransmitter acetylcholine (ACh) exerts its effects in the central and peripheral nervous systems through two distinct families of receptors, namely, the muscarinic ACh receptors (mAChRs) and the nicotinic ACh receptors (nAChRs). Whereas the mAChRs are G-protein-coupled receptors, the nAChRs belong to the family of ligand-gated ion channels. The nAChRs are pentameric receptor complexes, and they can be divided into the muscle-type receptors, made up of α1, β1, δ, and γ/ε subunits, and neuronal receptors composed of α2−α10 and β2−β4 subunits. The neuronal nAChRs exist as homomeric receptors composed of α7 or α9 subunits or as heteromeric receptors made up of various combinations of α2−α6 and β2−β4 subunits or of α9 and α10 subunits.[1] The most abundant nAChR subtypes in the CNS are the α4β2 and the α7 receptors, whereas the α3β4 subtype is predominant at ganglionic synapses.[2] The neuronal nAChRs are involved in a wide range of physiological and pathophysiological processes, and they have been proposed as potential therapeutic targets in a number of neurodegenerative and psychiatric disorders, in various forms of pain, and in nicotine addiction.[1,3,4] Since augmentation of nAChR signaling seems to hold therapeutic potential for most of these indications, the medicinal chemistry efforts in the nAChR field have been focused predominantly on the development of agonists and positive allosteric modulators of nAChRs.[1,4−6] However, despite considerable efforts to find new therapeutic strategies based on nAChRs, apart from nicotine, the only nicotinic agonists approved for nicotine replacement therapies are varenicline and cytisine.[7,8] nAChR antagonists offer therapeutic prospects as antidepressants and smoking cessation aids.[1,9,10] Moreover, antagonists selective for one of the nAChR subtypes could be used as pharmacological tools for determining the physiological functions mediated by the different receptor subtypes.[4]

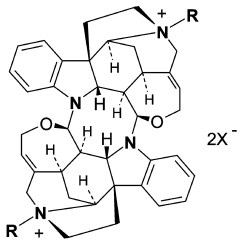
Most of the nAChR ligands are natural products or are derived from the latter. For example, the curare alkaloids, (+)-tubocurarine and toxiferine I, are antagonists at the muscle-type nAChRs, the frog alkaloid (+)-epibatidine is an α4β2

Received: March 21, 2014
Published: September 5, 2014

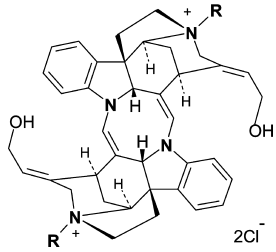
agonist, while the larkspur diterpenoid alkaloid methyllycaconitine (MLA) is an α7-selective antagonist. Several ligands selectively targeting α7 nAChRs may take advantage of the presence of a hydroxy group. For instance, choline bearing a hydroxy group instead of the acetate function of acetylcholine is able to activate α7 receptors, while it is not active at heteromeric nAChRs.[11] Other examples are the α7-selective agonist quinuclidol, obtained by addition of a hydroxy group to the nonselective quinuclidine,[12] and the 4-hydroxy metabolite of the α7-selective partial agonist 3-(2,4-dimethoxybenzylidene)anabasine, which shows greater efficacy for human α7 receptors than the parent compound.[13]

In the course of studies on *Strychnos* alkaloids, our group has reported that bisquaternary analogues of caracurine V (**1**), the main alkaloid of the stem bark of *Strychnos toxifera*, are competitive antagonists at human α7 receptors and do not display activities at heteromeric neuronal nAChRs.[14] The most potent analogues at α7 receptors were compounds **1b** and **1c**, substituted with allyl and 4-nitrobenzyl groups, respectively (Chart 1). The caracurine V ring system is formally obtained from the bisnortoxiferine I scaffold of the major bisquaternary alkaloid of calabash curare toxiferine I (R = CH_3) and of the clinically used muscle relaxant alcuronium (R = allyl), by an intramolecular addition of both allyl alcohol side chains to the enamine double bonds of the central eight-membered ring (Chart 1). Interestingly, dimethyl- and diallylcaracurine V analogues **1a** and **1b**, respectively, were found to show considerably lower affinity for the muscle-type nAChRs than the equally N,N′-substituted neuromuscular-blocking agents toxiferine I and alcuronium.[15]

Chart 1. Structural Formulas of the Previously Reported Caracurine V and Bisnortoxiferine I Analogues[14,17]



caracurine V analogues
1 R = H
1a R = Me
1b R = allyl
1c R = 4-nitrobenzyl



bisnortoxiferine I R = H
toxiferine I R = Me
alcuronium R = allyl

Bisquaternary caracurine V and bisnortoxiferine analogues were reported also to be potent allosteric enhancers of binding of the antagonist *N*-methylscopolamine to muscarinic M_2 receptors.[16,17] However, the binding affinity for the allosteric site located at the extracellular vestibule of M_2 receptors seems to be similar for both ring systems, as indicated by the similar allosteric potency of diallylcaracurine V (**1b**) and alcuronium.

In this paper, the synthesis and pharmacological evaluation of novel bisnortoxiferine I analogues with structurally modified side chains are described. In particular, a series of nonsymmetrical derivatives lacking only one alcohol group (**2**, **2a**−**c**) and of symmetrical compounds lacking both alcohol functions (**3**, **3a**−**c**) have been examined (Scheme 1). The findings could help delineate the structural requirements for the action at different (sub)types of AChRs. The results could be particularly useful for the design of urgently needed ligands selective for α7 nAChRs in terms of clarification as to whether the hydroxy group, known to be beneficial for receptor activation, is also important for antagonistic action.

It is worth mentioning that the structures of compound **3** and its *N*,*N*′-dimethyl analogue **3a** are very similar to those of the naturally occurring *Strychnos* alkaloid bisnordihydrotoxiferine[18,19] and calabash curare alkaloid dihydrotoxiferine,[20,21] respectively. While bisnordihydrotoxiferine and dihydrotoxiferine possess double bonds at positions C-19−C-20 and C-19′−C-20′, compounds **3** and **3a** have single bonds at these positions.

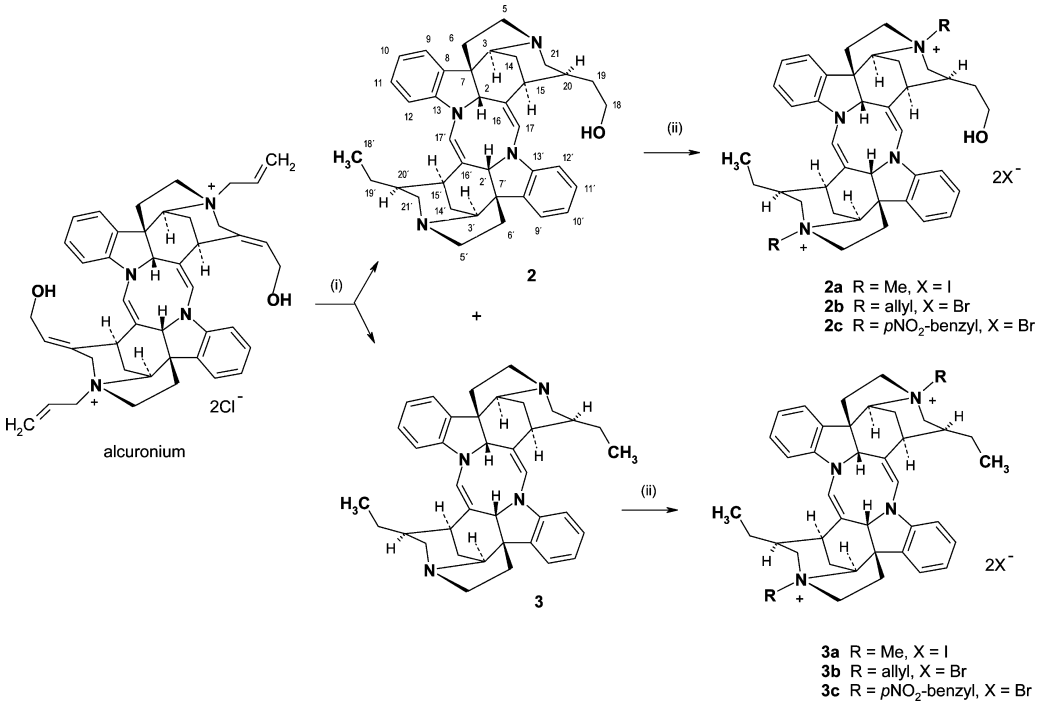
RESULTS AND DISCUSSION

The ideal starting material for the modification of the side chains of the toxiferine I scaffold is the bistertiary amine bisnortoxiferine I (Chart 1). However, because no synthesis of the latter has been reported to date and previous attempts to prepare it from its easily available cyclization product caracurine V failed,[22,23] it was decided to use alcuronium chloride as a starting material. In the course of catalytic hydrogenation of alcuronium chloride using Pd/C 10% and H_2 (15 bar), both *N*-allyl groups as well as both exocyclic double bonds were saturated. Moreover, one or two alcoholic groups were eliminated, leading after hydrogenation of the resulting double bonds to terminal ethyl substituents. The crude hydrogenation product was subjected to Hofmann elimination using KOH in DMF. In the course of the latter, both *N*-propyl groups were eliminated as propene, to give a mixture of the bistertiary amines **2** and **3**, which could be separated by silica gel chromatography. The double quaternization of **2** and **3** using methyl iodide, allyl bromide, and *p*-NO_2-benzyl bromide to yield **2a**−**c** and **3a**−**c** readily proceeded in a chloroform solution at room temperature (Scheme 1).

In the course of hydrogenation of bisnortoxiferine I, both exocyclic double bonds C-19−C-20 and C-19′−C-20′ were saturated, generating two novel stereogenic centers, C-20 and C-20′. ^{1}H and ^{13}C NMR spectra of the hydrogenation products **2** and **3** showed single sets of signals indicating a stereoselective course of the hydrogen addition that took place from the less sterically hindered side of the double bonds. The absolute configuration of C-20 and C-20′ was determined by a 600 MHz NOESY experiment for compound **3** (see Supporting Information). Strong NOEs between H-20 (and H-20′) and H-14a (H-14a′) adopting an axial orientation in the piperidine ring revealed the axial position of H-20 (and H-20′). The resulting *S*-configuration of C-20 (and C-20′) is in agreement with the stereochemistry of the corresponding atoms in the related tetrahydrocaracurine V ring system.[24] The essential

dx.doi.org/10.1021/np500259j | J. Nat. Prod. 2014, 77, 2006−2013

Scheme 1[a]



[a]Reagents and conditions: (i) 1. Pd/C, H_2 (15 bar), EtOH, 48 h, 2. KOH, DMF, reflux, 2 h; (ii) $CHCl_3$, MeI (**2a**, **3a**) or CH_2=CH−CH_2Br (**2b**, **3b**) or *p*NO_2-benzylbromide (**2c**, **3c**), rt.

NOEs of H-20 confirming its axial orientation in the piperidine ring are displayed in Figure 1.

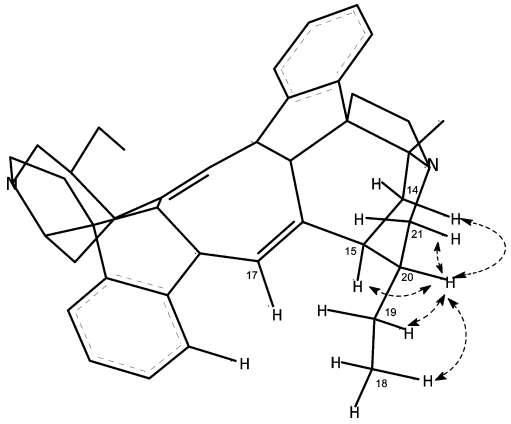


Figure 1. Essential NOEs of compound **3**. The 3D structure of **3** was built starting from the 3D structure of toxiferine I[24] by removal of the *N*-methyl groups and exchange of the allyl alcohol side chains with ethyl groups. The structure was optimized using the Trident semiempirical PM3 calculation.[25]

The antagonistic potency at the α7 nAChRs was assessed as the ability to inhibit by 50% (IC_{50}) the effect of 20 μM ACh in ha7-GH3 cells using a Ca^{2+}/Fluo-4 functional assay.[14] The equilibrium inhibition constants K_i of ligand binding to the muscle-type nAChRs were determined according to a previously developed assay in membrane fractions of the *Torpedo californica* electric organ using (±)-[^{3}H]epibatidine as radioligand.[15] As a measure of test compound affinity at the allosteric binding site of the muscarinic M_2 receptor, their potency to inhibit the dissociation of the orthosteric antagonist [^{3}H]*N*-methylscopolamine from the receptors in homogenates of porcine heart ventricles was determined and expressed as $EC_{0.5,diss}$.[26] The results are presented in Table 1.

All compounds synthesized in the present study displayed a moderate to low antagonistic effect at α7 nAChR. Most ligands were less potent than the equally substituted ring-closed caracurine V analogues.[14] These findings confirmed that the caracurine V ring system is more favorable for binding at α7 nAChRs than the spatially different bisnortoxiferine I skeleton present in ligands **2a**−**c** and **3a**−**c**. The most potent α7 antagonists among the new series were found to be the *N,N′*-dimethyl analogues **2a** and **3a**, bearing one or no hydroxy groups, respectively. These compounds showed similar IC_{50} values at the α7 nACh receptor (590 and 820 nM, respectively) and were 10−15 times more potent than toxiferine I, having two hydroxy groups. These results indicated that the alcohol functions are not necessary for antagonistic action at the α7 nAChRs and may in fact impair the binding properties of these compounds to the receptor. The latter conclusion is supported by the fact that the highly potent α7 nAChR ligands from a previously reported caracurine V series (**1a**−**c**) also lack OH groups.[14]

At the muscle-type nAChRs, all ligands showed significantly reduced binding when compared to the strong neuromuscular blocking agent toxiferine I (K_i = 14 nM), but an affinity similar to alcuronium (K_i = 234 nM).[15] However, in contrast to the action at the α7 nAChRs, the new compounds displayed considerably higher binding for the muscle-type nAChRs than the caracurine V analogues, indicating stronger neuromuscular blocking potential. The binding affinity in the monodeoxy (**2a**−**c**) and dideoxy (**3a**−**c**) series seems to be less dependent on the N-substituent than for the bisnortoxiferine analogues toxiferine I and alcuronium. For example, while the *N*-methyl-substituted toxiferine I displayed a 17-fold higher

dx.doi.org/10.1021/np500259j | *J. Nat. Prod.* 2014, 77, 2006−2013

Table 1. Pharmacological Characterization of the Compounds at α7 and Muscle-Type nAChRs and the Allosteric Binding Site of Muscarinic M_2 Receptors

	α7 nAChR[a]	muscle-type nAChR[b]	allosteric site of M_2 receptors[c]
	IC_{50} [nM] pIC_{50} ± SEM	K_i ± SEM [nM]	$EC_{0.5,diss}$ [nM] $pEC_{0.5,diss}$ ± SEM
toxiferine I	9500[14]	14[15]	96[17]
alcuronium	4100[14]	234[15]	2[17]
1	1600[14]	>100 000[15]	436[17]
1a	1500[14]	5200[15]	8[17]
1b	280[14]	1500[15]	11[17]
1c	370[14]	820[15]	339[17]
2	2200 [5.63 ± 0.07]	390.1 ± 34.0	410* [6.39 ± 0.03]
2a	590 [6.22 ± 0.05]	455.0 ± 55.1	320 [6.49 ± 0.09]
2b	3100 [5.50 ± 0.01]	250.0 ± 33.8	12 [7.91 ± 0.11]
2c	4200 [5.38 ± 0.03]	290.5 ± 33.8	32 [7.50 ± 0.05]
3	3300 [5.48 ± 0.04]	668.8 ± 76.2	500 [6.30 ± 0.13]
3a	820 [6.09 ± 0.06]	150.0 ± 23.2	260 [6.58 ± 0.11]
3b		75.1 ± 34.2	36 [7.44 ± 0.07]
3c	21 000 [4.67 ± 0.07]	81.9 ± 16.8	49* [7.31 ± 0.12]

[a]Functional properties at the human α7 nAChR. The IC_{50} values were obtained using the Ca^{2+}/Fluo-4 assay at the hα7-GH3 cell line. An assay concentration of 20 μM ACh was used as agonist in the antagonist experiments. The EC_{50} value for ACh is 3.3 μM [pEC_{50} = 5.48 ± 0.02]. The IC_{50} values for the antagonists are given in nM with pIC_{50} ± SEM values in brackets, respectively, and represent the means of 3 or 4 experiments performed in duplicate. [b]Inhibition constants (K_i values in nM) were obtained in radioligand binding assays using (±)-[^{3}H]epibatidine and membrane fraction from the *Torpedo californica* electric organ. Data represent means ± SEM obtained from 3 to 5 experiments. [c]$pEC_{0.5,diss}$, −log equilibrium dissociation constant of allosteric agent binding to [^{3}H]NMS-bound M_2 receptors: concentration causing a half-maximal reduction of the observed rate constant k_{obs} of [^{3}H]NMS dissociation from porcine M_2 receptors in the absence of test compound; * the slope factor n of the curves of **2** (n = −2.08) and **3c** (n = −2.29) was different from n = −1.0 (F-test, $p < 0.05$). Given are means ± SEM of 3 to 4 experiments. For further details see text.

affinity than the *N*-allyl analogue alcuronium (K_i = 234 nM), the equivalently substituted **2a** and **2b** showed very similar binding constants (K_i = 455 and 250 nM, respectively). The highest affinity was observed for the nonhydroxylated analogues **3b** and **3c**, with K_i = 75 and 82 nM, respectively, indicating that the hydroxy groups are not essential for binding.

Since toxiferine I, alcuronium, and the caracurine derivatives bind to the allosteric site of the muscarinic M_2 receptor,[17] the new compounds were also tested for their ability to inhibit the dissociation of the antagonist *N*-methylscopolamine. With regard to the allosteric M_2 receptor site, the binding is dependent on N-substitution rather than on the nature of the side chains. For example, all the *N*-methyl-substituted analogues, toxiferine I, **2a**, and **3a**, displayed similar $EC_{0.5,diss}$ values in the three-digit nanomolar range of concentration. In accordance with the data for the previously reported series of bisquaternary caracurine V derivatives, **1** and **1a**−**1c**,[17] the highest affinity was observed for the *N*-allyl derivatives alcuronium (2 nM), **2b** (12 nM), and **3b** (36 nM). The di(4-nitrobenzyl) analogues **3c** and **4c** displayed slightly reduced affinities. Interestingly, the curve slopes n of **2** and **3c** observed in the current study (cf. legend of Table 1) were significantly larger compared to the value reported earlier for the typical muscarinic allosteric modulator alcuronium[26] and may point to an atypical allosteric binding mode.[27] It should be mentioned that a direct interassay comparison of the M_2 data was hampered for three reasons. First, the affinity measure, $EC_{0.5,diss}$, was obtained in orthosterically (i.e., at the ACh binding site) occupied (with NMS) M_2 receptors, whereas pIC_{50} and K_i (cf. Table 1) were obtained in orthosterically unoccupied nicotinic acetylcholine receptors. Second, the allosteric potency measure $EC_{0.5,diss}$ was determined in a low ionic strength, i.e., high affinity, buffer, known to facilitate a direct labeling of the allosteric site[28] and an elucidation of allosteric agent binding mechanisms.[27] Third, the allosteric action, i.e., $EC_{0.5,diss}$, depends on the structure of the orthosteric ligand applied.[29]

Comparing the ring systems, the activities of the caracurine V analogues **1a**−**c** at the receptors under investigation were found to be different from those observed for the new compounds **2a**−**c** and **3a**−**c**. While the ring-closed caracurine V ligands **1a**−**c** showed high antagonistic potency at α7 nAChRs and high affinity to the allosteric site of M_2 receptors, their affinity for the muscle-type nAChRs proved to be low. In contrast, the new compounds showed low antagonistic potency at α7 nAChRs, moderate affinity for the allosteric site of M_2 receptors, and high binding to the muscle-type nAChRs. These differences can be explained by the diverse geometries of the ring-closed caracurine-V skeleton and the ring-opened bisnortoxiferine I ring system.[24] Interestingly, the α7 nAChR is far more sensitive to these conformational changes than the other two receptors.

In summary, even though receptors of very different structures and functionalities were examined, general structure−activity relationships were derived for the different series of compounds investigated. Whereas the caracurine V skeleton is a suitable lead structure for α7 nAChR antagonists, the N-substituted bisnortoxiferine I ring system is a lead for the muscle-type nAChR ligands. Since the substituents attached to the nitrogen atoms seem to play a pivotal role, further variations with regard to substituents of different electronic, lipophilic, and hydrogen-bonding properties should be studied. As the allosteric binding site of the M_2 acetylcholine receptor is located at the extracellular vestibule of the M_2 receptor[30] and is more flexible than orthosteric binding sites, each compound was found to exert an optimal binding mode, with the *N*-allyl-substituted analogues alcuronium, **1b**, **2b**, and **3b** showing the highest affinity.

dx.doi.org/10.1021/np500259j | J. Nat. Prod. 2014, 77, 2006−2013

When taken together, the new compounds derived from the natural products caracurine V and toxiferine I have been shown to be excellent leads for the development of new ligands for nicotinic and muscarinic receptors.

EXPERIMENTAL SECTION

General Experimental Procedures. Melting points were determined using a capillary melting point apparatus (Gallenkamp, Sanyo) and are uncorrected. Bruker AV400 and Bruker AV600 NMR spectrometers were used to obtain ^{1}H NMR and ^{13}C NMR spectra, respectively. ^{1}H NMR chemical shifts are referred to $CHCl_3$ (7.26 ppm) and DMSO-d_6 (2.50 ppm). ^{13}C NMR chemical shifts are referred to $CDCl_3$ (77.26 ppm) and DMSO-d_6 (39.52 ppm). The NMR resonances were assigned by means of COSY and HMQC experiments. EIMS were determined on a Finnigan MAT 90. MALDIMS were run on a Bruker Daltonic MALDI-TOF spectrometer. Elemental analyses were performed by the microanalytical section of the Institute of Inorganic Chemistry, University of Würzburg. All reactions were carried out under an argon atmosphere. Column chromatography was carried out on silica gel 60 (0.063−0.200 mm) obtained from Merck. Alcuronium chloride was synthesized in pure form from Wieland-Gumlich aldehyde as previously reported.[31]

19,20,19′,20′-Tetrahydro-18′-deoxybisnortoxiferine I (2) and 19,20,19′,20′-Tetrahydro-18,18′-dideoxybisnortoxiferine I (3). Pd/C 10% (500 mg) was added to a solution of alcuronium chloride (500 mg, 0.678 mmol) in EtOH (100 mL), and the reaction mixture was hydrogenated at 15 bar H_2 at rt for 48 h. The catalyst was filtered over Celite and washed with EtOH, and the combined filtrates were concentrated in vacuo to give a crude product (400 mg), which was subjected to Hofmann elimination without purification. Crude products resulting from two catalytic hydrogenations were combined (800 mg), absolute DMF (50 mL) and KOH pellets (650 mg) were added, and the reaction mixture was heated under reflux for 2 h. Water (300 mL) was added, and the products were extracted with EtOAc (3 × 70 mL). The combined organic layers were washed with water (4 × 20 mL) and dried over Na_2SO_4, and the solvent was removed in vacuo. The residue was subjected to column chromatography on silica gel ($CHCl_3$−MeOH−25%NH_3, 100:10:1) to give **2** (120 mg, 16%) and **3** (180 mg, 25%) as colorless solids.

Compound 2. ^{1}H NMR ($CDCl_3$, 600 MHz) δ 7.15−7.10 (2H, m, H-11, H-11′), 7.07 (2H, d, J = 7.3 Hz, H-9, H-9′), 6.82−6.76 (2H, m, H-10, H-10′), 6.43−6.38 (2H, m, H-12, H-12′), 6.10 (1H, s, H-17), 6.05 (1H, s, H-17′), 5.15 (1H, s, H-2′), 5.13 (1H, s, H-2), 3.80 (2H, t, J = 6.5 Hz, CH_2-18), 3.13 (2H, ddd, J = 10.7, 9.5, 4.1 Hz, H-5a, H-5a′), 3.03 (2H, dd, J = 6.0, 3.1 Hz, H-3, H-3′), 2.98−2.93 (2H, m, H-5b, H-5b′), 2.77 (1H, dd, J = 11.6, 3.5 Hz, H-21a), 2.73 (1H, dd, J = 11.6, 3.7 Hz, H-21a′), 2.66 (1H, s, H-15′), 2.63 (1H, s, H-15), 2.39−2.31 (2H, m, H-6a, H-6a′), 2.30−2.22 (2H, m, H-6b, H-6b′), 2.15 (1H, t, J = 11.6 Hz, H-21b), 2.05 (1H, t, J = 11.6 Hz, H-21b′), 2.03−1.97 (2H, m, H-20), 1.93 (1H, m, H-14a), 1.89 (1H, m, H-14a′), 1.80 (1H, m, H-19a), 1.72−1.67 (2H, m, H-14b, H-14b′), 1.69−1.63 (1H, m, H-20′), 1.58 (1H, m, H-19b), 1.48 (1H, m, H-19a′), 1.37 (1H, m, H-19b′), 1.04 (3H, t, J = 7.4 Hz, CH_3-18′); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 146.9 (C, C-13), 146.8 (C, C-13′), 137.9 (C, C-8′), 137.7 (C, C-8), 131.0 (CH, C-17), 130.6 (CH, C-17′), 128.28 (CH, C-11), 128.25 (CH, C-11′), 122.37 (CH, C-9′), 122.34 (CH, C-9), 119.40 (CH, C-10), 119.33 (CH, C-10′), 117.9 (C, C-16), 117.6 (C, C-16′), 107.83 (CH, C-12), 107.78 (CH, C-12′), 73.22 (CH, C-2), 73.15 (CH, C-2), 67.95 (CH, C-3)′, 67.70 (CH, C-3), 60.7 (CH_2, C-18), 55.20 (CH_2, C-5′), 55.14 (CH_2, C-5), 53.92 (C, C-7), 53.88 (C, C-7′), 51.39 (CH_2, C-21), 51.32 (CH_2, C-21′), 43.9 (CH, C-20′), 43.36 (CH_2, C-6), 43.23 (CH_2, C-6′), 38.7 (CH, C-20), 35.6 (CH_2, C-19), 33.2 (CH, C-15) 32.2 (CH, C-15′), 26.52 (CH_2, C-14), 26.48 (CH_2, C-14′), 25.0 (CH_2, C-19′), 11.9 (CH_3, C-18′); EIMS m/z 573 $[M]^+$ (45), 572 (100), 571 (43), 286 (8); *anal.* C 78.99, H 7.38, N 9.93%, calcd for $C_{38}H_{44}N_4O$, C 79.68, H 7.74, N 9.78%.

Compound 3. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.12 (2H, m, H-11, H-11′), 7.08 (2H, dd, J = 7.3, 1.0 Hz, H-9, H-9′), 6.78 (2H, m, H-10, H-10′), 6.39 (2H, d, J = 7.3 Hz, H-12, H-12′), 6.05 (2H, s, H-17, H-17′), 5.16 (2H, s, H-2, H-2′), 3.12 (2H, m, H-5a, H-5a′), 3.00 (2H, dd, J = 3.8, 2.3 Hz, H-3, H-3′), 2.97 (2H, m, H-5b, H-5b′), 2.71 (2H, dd, J = 10.6, 3.5 Hz, H-21a, H-21a′), 2.67 (2H, m, H-15, H-15′), 2.33 (2H, m, H-6a, H-6a′), 2.25 (2H, m, H-6b, H-6b′), 2.25 (2H, m, H-6b, H-6b′), 2.06 (2H, t, J = 11.3 Hz, H-21b, H-21b′), 1.89 (2H, m, H-14a, H-14a′), 1.70 (2H, m, H-14b, H-14b′), 1.64 (2H, m, H-20, H-20′), 1.50 (2H, m, H-19a, H-19a′), 1.38 (2H, m, H-19b, H-19b′), 1.05 (6H, t, J = 7.3 Hz, H-18, H-18′); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 146.8 (C, C-13,C-13′), 137.9 (C, C-8, C-8′), 130.5 (CH, C-17, C-17′), 128.2 (CH, C-11, C-11′), 122.4 (CH, C-9, C-9′), 119.2 (CH, C-10, C-10′), 117.7 (C, C-16, C-16′), 107.6 (CH, C-12, C-12′), 73.1 (CH, C-2, C-2′), 68.1 (CH, C-3, C-3′), 55.2 (CH_2, C-5, C-5′), 53.8 (C, C-7, C-7′), 51.2 (CH_2, C-21, C-21′), 43.9 (CH, C-20, C-20′), 43.1 (CH_2, C-6, C-6′), 32.0 (CH, C-15, C-15′), 26.4 (CH_2, C-14, C-14′), 25.0 (CH_2, C-19, C-19′), 11.9 (CH_3, C-18, C-18′); EIMS m/z 557 $[M]^+$ (38), 556 (100), 555 (32), 456 (14), 278 (18); *anal.* C 81.74, H 7.39, N 10.06%, calcd for $C_{38}H_{44}N_4$, C 81.97, H 7.96, N 9.56%.

General Double Quaternization Procedure of the Tertiary Bases 2 and 3. The respective halide was added dropwise to a solution of **2** or **3** in $CHCl_3$ (10 mL). After being stirred at room temperature for 2 h, the crystallized ammonium salt was isolated by filtration. If no crystallization occurred, the product was precipitated by adding Et_2O. The collected ammonium salt was washed with $CHCl_3$ or with a $CHCl_3$−Et_2O mixture (1:1) and dried in vacuo (0.001 mbar) at 50 °C. No further purification was necessary, as indicated by the ^{1}H NMR spectra measured.

4,4′-Dimethyl-19,20,19′,20′-tetrahydro-18′-deoxybisnortoxiferinium I Diiodide (2a). Compound **2a** (110 mg, 81%) was obtained from **2** (90 mg, 0.157 mmol) and methyl iodide (0.2 mL) as a white solid: ^{1}H NMR (DMSO-d_6, 400 MHz) δ 7.48 (2H, d, J = 7.3 Hz, H-9, H-9′), 7.22 (2H, m, H-11, H-11′), 6.95 (2H, m, H-10, H-10′), 6.69−6.65 (2H, m, H-12, H-12′), 6.22 (1H, s, H-17), 6.20 (1H, s, H-17′), 5.23 (2H, s, H-2, H-2′), 4.57 (1H, t, J = 4.7 Hz, OH), 4.22 (2H, br s, H-3, H-3′), 3.90−3.82 (4H, m, CH_2-5, CH_2-5′), 3.53−3.42 (4H, m, CH_2-18, H-21a, H-21a′), 3.41 (6H, s, N^+-CH_3, N'^+-CH_3), 3.40−3.30 (2H, m, H-21b, H-21b′), 2.54−2.45 (4H, m, H-15, H-15′, H-6a, H-6a′), 2.36−2.22 (5H, m, H-6b, H-6b′, H-14a, H-14a′, H-20), 2.10 (1H, m, H-20′), 1.60 (2H, m, H-14b, H-14b′), 1.48 (1H, m, H-19a), 1.38−1.26 (2H, m, H-19b, H-19a′), 1.21 (1H, m, H-19b′), 0.89 (3H, t, J = 7.3 Hz, H-18′); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 147.1 (C, C-13,C-13′), 132.9 (CH, C-17), 132.8 (CH,C-17′), 132.6 (C, C-8, C-8′), 129.6 (CH, C-11, C-11′), 122.9 (CH, C-9, C-9′), 121.1 (CH, C-10, C-10′), 112.9 (C, C-16), 112.8 (C, C-16′), 110.4 (CH; C-12), 110.3 (CH, C-12′), 72.0 (CH, C-3′), 71.9 (CH, C-3), 69.9 (CH, C-2, C-2′), 64.7 (CH_2, C-5, C-5′), 59.5 (CH_2, C-21), 59.4 (CH_2, C-21′), 58.0 (CH_2, C-18), 52.99 (C, C-7′), 52.97 (C, C-7), 47.8 (CH_3, N^+-CH_3, N'^+-CH_3), 39.3 (CH_2, C-6, C-6′), 37.4 (CH, C-20′), 34.4 (CH, C-20), 33.8 (CH, C-15′), 33.4 (CH_2, C-19), 32.9 (CH, C-15), 23.0 (CH_2, C-19′), 22.6 (CH_2, C-14, C-14′), 11.0 (CH_3, C-18′); MALDIMS (matrix, 2,5-dihydroxybenzoic acid in MeOH−H_2O, 1:3) m/z 739.5 $[M - I + Na + 3H]^+$, 713.4 $[M - I]^+$ (100%); *anal.* C 54.51, H 5.95, N 6.12%, calcd for $C_{40}H_{50}I_2N_4O{\cdot}H_2O$, C 54.93, H 5.99, N 6.41%.

4,4′-Diallyl-19,20,19′,20′-tetrahydro-18′-deoxybisnortoxiferinium I Dibromide (2b). Compound **2b** (80 mg, 56%) was obtained from **2** (100 mg, 0.175 mmol) and allyl bromide (0.5 mL) as a white solid: ^{1}H NMR (DMSO-d_6, 400 MHz) δ 7.49 (2H, d, J = 7.3 Hz, H-9, H-9′), 7.22 (2H, m, H-11, H-11′), 6.94 (2H, m, H-10, H-10′), 6.70−6.65 (2H, m, H-12, H-12′), 6.30−6.16 (2H, m, 2 × −CH_2−C<u>H</u>=CH_2), 6.24 (1H, s, H-17), 6.21 (1H, s, H-17′), 5.81−5.68 (4H, m, 2 × −CH_2−CH=C<u>H_2</u>), 5.53 (2H, s, H-2, H-2′), 4.58−4.50 (3H, m, OH, 2 × −C<u>H</u>H−CH=CH_2), 4.30−4.20 (2H, m, 2 × −CH<u>H</u>−CH=CH_2), 4.23 (2H, br s, H-3, H-3′), 4.00−3.90 (2H, m, H-5a, H-5a′), 3.66−3.26 (8H, m, H-5b, H-5b′, CH_2-18, H-21a, H-21a′, H-21b, H-21b′), 2.60 (2H, m, H-6a, H-6a′), 2.47 (2H, br s, H-15, H-15′), 2.44−2.22 (5H, m, H-20, H-6b, H-6b′, H-14a, H-14a′), 2.12 (1H, m, H-20′), 1.59 (2H, m, H-14b, H-14b′), 1.49 (1H, m, H-19a), 1.41 (1H, m, H-19b), 1.37−1.21 (2H, m, H-19a′, H-19b′), 0.85 (3H, t, J = 7.3 Hz, H-18′); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 147.2 (C, C-13,C-13′), 132.8 (C, C-8, C-8′), 132.7 (CH, C-17), 132.5 (CH,C-17′),

dx.doi.org/10.1021/np500259j | J. Nat. Prod. 2014, 77, 2006−2013

129.6 (CH, C-11, C-11′), 127.7 (CH_2, $-CH_2-CH=\underline{C}H_2$), 127.4 ($CH_2$, $-CH_2'-CH'=\underline{C}H_2'$), 126.8 (CH, $-CH_2-\underline{C}H=CH_2$), 126.6 ($-CH_2'-\underline{C}H'=CH_2'$), 122.8 (CH, C-9, C-9′), 121.0 (CH, C-10, C-10′), 112.8 (C, C-16′), 112.6 (C, C-16), 110.22 (CH, C-12), 110.16 (CH, C-12′), 71.68 (CH, C-3), 71.62 (CH, C-3′), 69.3 (CH, C-2′), 69.2 (CH, C-2), 61.5 (CH_2, C-5, C-5′), 60.56 (CH_2, $-CH_2'-CH'=\underline{C}H_2'$), 60.49 ($CH_2$, $-CH_2-CH=\underline{C}H_2$), 58.2 ($CH_2$, C-18), 55.6 ($CH_2$, C-21, C-21′), 52.7 (C, C-7, C-7′), 38.9 (CH_2, C-6, C-6′), 36.9 (CH, C-20′), 34.5 (CH, C-15′), 33.9 (CH, C-15), 33.2 (CH_2, C-19), 32.6 (CH, C-20), 22.9 (CH_2, C-19′), 22.6 (CH_2, C-14, C-14′), 10.9 (CH_3, C-18′); MALDIMS (matrix, α-cyano-4-hydroxycinnamic acid in MeOH−MeCN−H_2O, 2:1:1) *m/z* 734.5 $[M - Br]^+$, 692.39 $[M - Br - allyl]^+$ (100%); *anal.* C 62.08, H 6.50, N 6.34%, calcd for $C_{44}H_{54}Br_2N_4O{\cdot}2H_2O$ C 62.12, H 6.87, N 6.59%.

4,4′-Di(4-Nitrobenzyl)-19,20,19′,20′-tetrahydro-18′-deoxybisnortoxiferinium I Dibromide (2c). Compound **2c** (95 mg, 54%) was obtained from **2** (100 mg, 0.175 mmol) and 4-nitrobenzyl bromide (200 mg, 0.92 mmol) as a yellow solid; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 8.44−8.38 (4H, m), 7.90−7.96 (4H, m), 7.51 (1H, d, *J* = 7.3 Hz, H-9,) 7.48 (2H, d, *J* = 7.3 Hz, H-9′), 7.22−7.28 (2H, m, H-11, H-11′), 6.92−6.98 (2H, m, H-10, H-10′), 6.64 (1H, d, *J* = 7.5 Hz, H-12′), 6.61 (1H, d, *J* = 7.5 Hz, H-12), 6.33 (1H, s, H-17), 6.27 (1H, s, H-17′), 5.67 (2H, s, H-2, H-2′), 5.31−5.22 (2H, m, 2 × $N^+-C\underline{H}H-C_6H_4-NO_2$), 4.93−4.84 (2H, m, 2 × $N^+-CH\underline{H}-C_6H_4-NO_2$), 4.75 (1H, t, *J* = 4.7 Hz, OH), 4.32−4.22 (2H, m, H-5a, H-5a′), 4.18 (2H, br s, H-3, H-3′), 3.59−3.45 (2H, m, CH_2-18), 3.38−3.17 (4H, m, H-5b, H-5b′, H-21a, H-21a′), 3.06 (1H, m, H-21b′), 2.89 (1H, m, H-21b), 2.72−2.58 (2H, m, H-6a, H-6a′), 2.48−2.31 (7H, m, H-15, H-15′, H-20, H-6b, H-6b′, H-14a, H-14a′), 1.70 (2H, m, H-14b, H-14b′), 1.63 (1H, m, H-20′), 1.51−1.40 (2H, H-19a, H-19a′), 1.34−1.21 (2H, H-19b, H-19b′), 0.93 (3H, t, *J* = 7.3 Hz, H-18′); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 148.76 (C, C-NO_2), 148.69 (C, C-NO_2), 146.6 (C, C-13, C-13′), 135.7 (C, 2 × C1 benzyl), 134.1 (CH, C2 benzyl), 133.8 (CH, C2 benzyl), 133.3 (CH, C-17, C-17′), 132.7 (C, C-8, C-8′), 129.6 (CH, C-11, C-11′), 124.2 (CH, C3 benzyl), 124.0 (CH, C3 benzyl), 123.0 (CH, C-9, C-9′), 120.5 (CH, C-10, C-10′), 112.3 (C, C-16′), 112.0 (C, C-16), 109.5 (CH; C-12, C-12′), 74.1 (CH, C-3), 73.9 (CH, C-3′), 68.7 (CH, C-2), 68.6 (C-2′), 61.5 (CH_2, C-5, C-5′), 59.6 (CH_2, 2 × CH_2 benzyl), 58.1 (CH_2, C-18), 54.3 (CH_2, C-21′), 53.4 (CH_2, C-21), 52.31 (C, C-7′), 52.24 (C, C-7), 38.4 (CH_2, C-6, C-6′), 36.7 (CH, C-20′), 35.6 (CH, C-15′), 33.6 (CH, C-15), 33.1 (CH_2, C-19), 32.8 (CH, C-20), 23.0 (CH_2, C-19′), 22.5 (CH_2, C-14, C-14′),11.0 (CH_3, C-18′); MALDIMS (matrix, α-cyano-4-hydroxycinnamic acid in MeOH−MeCN−H_2O, 2:1:1) *m/z* 842.45 $[M - 2Br + 2H]^+$ (100%); *anal.* C 59.62, H 5.86, N 7.65%, calcd for $C_{52}H_{56}Br_2N_6O_5{\cdot}2H_2O$ C 60.00, H 5.81, N 8.07%.

4,4′-Dimethyl-19,20,19′,20′-tetrahydro-18,18′-dideoxybisnortoxiferinium I Diiodide (3a). Compound **3a** (55 mg, 91%) was obtained from **3** (40 mg, 72 mmol) and MeI (0.2 mL) as a white solid: ^{1}H NMR (DMSO-d_6, 400 MHz) δ 7.47 (2H, d, *J* = 7.3 Hz, H-9, H-9′), 7.22 (2H, m, H-11, H-11′), 6.95 (2H, m, H-10, H-10′), 6.66 (2H, d, *J* = 7.5 Hz, H-12, H-12′), 6.20 (2H, s, H-17, H-17′), 5.22 (2H, s, H-2, H-2′), 4.20 (2H, br s, H-3, H-3′), 3.91−3.79 (4H, m, CH_2-5, CH_2-5′), 3.50 (2H, m, H-21a, H-21a′), 3.40 (6H, s, N^+-CH_3, N'^+-CH_3), 3.32−3.26 (2H, m, H-21b, H-21b′), 2.52−2.39 (4H, m, H-15, H-15′, H-6a, H-6a′), 2.34−2.23 (4H, m, H-6b, H-6b′, H-14a, H-14a′), 2.10 (2H, m, H-20, H-20′), 1.60 (2H, m, H-14b, H-14b′), 1.31 (2H, m, H-19a, H-19a′), 1.21 (2H, m, H-19b, H-19b′), 0.89 (6H, t, *J* = 7.3 Hz, H-18, H-18′); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 147.0 (C, C-13, C-13′), 132.8 (CH, C-17, C-17′), 132.6 (C, C-8, C-8′), 129.6 (CH, C-11, C-11′), 122.9 (CH, C-9, C-9′), 121.1 (CH, C-10, C-10′), 112.8 (C, C-16, C-16′), 110.3 (CH, C-12, C-12′), 72.0 (CH, C-3, C-3′), 69.9 (CH, C-2, C-2′), 64.7 (CH_2, C-5, C-5′), 59.2 (CH_2, C-21, C-21′), 53.0 (C, C-7, C-7′), 47.7 (CH_3, N^+-CH_3, N'^+-CH_3), 39.3 (CH_2, C-6, C-6′), 37.4 (CH, C-20, C-20′), 33.6 (CH, C-15, C-15′), 23.0 (CH_2, C-19, C-19′), 22.5 (CH_2, C-14, C-14′), 10.9 (CH_3, C-18, C-18′); MALDIMS (matrix, 2,5-dihydroxybenzoic acid in MeOH−H_2O, 1:3) *m/z* 739.5 $[M - I + Na + 3H]^+$, 713.4 $[M - I]^+$ (100%); *anal.* C 54.60, H 5.90, N 6.11%, calcd for $C_{40}H_{50}N_4I_2{\cdot}2H_2O$ C 54.80, H 6.21, N 6.39%.

4,4′-Diallyl-19,20,19′,20′-tetrahydro-18,18′-dideoxybisnortoxiferinium I Dibromide (3b). Compound **3b** (81 mg, 80%) was obtained from **3** (70 mg, 0.126 mmol) and allyl bromide (0.2 mL) as a white solid; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 7.47 (2H, d, *J* = 7.5 Hz, H-9, H-9′), 7.22 (2H, m, H-11, H-11′), 6.95 (2H, m, H-10, H-10′), 6.66 (2H, d, *J* = 7.9 Hz, H-12, H-12′), 6.31−6.16 (2H, m, 2 x $N^+-CH_2-C\underline{H}=CH_2$), 6.21 (2H, s, H-17, H-17′), 5.81−5.86 (4H, m, 2 × $N^+-CH_2-CH=C\underline{H}_2$), 5.50 (2H, s, H-2, H-2′), 4.54 (2H, dd, *J* = 12.6, 7.9 Hz, 2 × $N^+-C\underline{H}H-CH=CH_2$), 4.22 (2H, dd, *J* = 12.6, 5.5 Hz, 2 × N^+-$CH\underline{H}$-$CH=CH_2$), 4.18 (2H, br s, H-3, H-3′), 3.93 (2H, dd, *J* = 19.0, 10.1 Hz, H-5a, H-5a′), 3.67−3.59 (2H, m, *J* = 9.2 Hz, H-5b, H-5b′), 3.47 (2H, t, *J* = 13.6 Hz, H-21a, H-21a′), 3.32−3.26 (2H, m, H-21b, H-21b′), 2.62−2.50 (4H, m, H-15, H-15′, H-6a, H-6a′), 2.34−2.25 (4H, m, H-6b, H-6b′, H-14a, H-14a′), 2.16−2.07 (2H, m, H-20, H-20′), 1.60 (2H, d, *J* = 12.3 Hz, H-14b, H-14b′), 1.40−1.20 (4H, m, H-19a, H-19a′, H-19b, H-19b′), 0.87 (6H, t, *J* = 7.3 Hz, H-18, H-18′); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 147.1 (C, C-13, C-13′), 132.8 (CH, C-17, C-17′), 132.4 (C, C-8, C-8′), 129.6 (CH, C-11, C-11′), 127.5 (CH_2, 2 × $N^+-CH_2-CH=\underline{C}H_2$), 126.8 (CH, 2 × $N^+-CH_2-\underline{C}H=CH_2$), 122.8 (CH, C-9, C-9′), 121.0 (CH, C-10, C-10′), 112.6 (C, C-16, C-16′), 110.1 (CH, C-12, C-12′), 71.8 (CH, C-3, C-3′), 69.3 (CH, C-2, C-2′), 61.5 (CH_2, C-5, C-5′), 60.6 (CH_2, 2 × $N^+-\underline{C}H_2-CH=CH_2$), 55.4 ($CH_2$, C-21, C-21′), 52.7 (C, C-7, C-7′), 39.8 (CH_2, C-6, C-6′), 36.9 (CH, C-20, C-20′), 33.7 (CH, C-15, C-15′), 22.9 (CH_2, C-19, C-19′), 22.5 (CH_2, C-14, C-14′), 10.9 (CH_3, C-18, C-18′); MALDIMS (matrix, 2,5-dihydroxybenzoic acid in MeOH−H_2O, 1:3) *m/z* 791.56 $[M - 5H]^+$ 719.46 $[M - Br + 2H]^+$ (100%); *anal.* C 61.37, H 6.64, N6.21%, calcd for $C_{44}H_{54}N_4Br_2{\cdot}3H_2O$ C 61.97, H 7.09, N 6.57%.

4,4′-Di(4-nitrobenzyl)-19,20,19′,20′-tetrahydro-18,18′-dideoxybisnortoxiferinium I Dibromide (3c). Compound **3c** (150 mg, 84%) was obtained from **2** (100 mg, 0.180 mmol) and 4-nitrobenzyl bromide (200 mg, 0.92 mmol) as a yellow solid: ^{1}H NMR (DMSO-d_6, 400 MHz) δ 8.42 (4H, d, *J* = 8.5 Hz, 4 × $O_2N-C-CH$), 7.92 (4H, d, *J* = 8.5 Hz, 4 × $O_2N-C-CH=C\underline{H}$), 7.51 (2H, d, *J* = 7.1 Hz, H-9, H-9′), 7.24 (2H, m, H-11, H-11′), 6.95 (2H, m, H-10, H-10′), 6.63 (2H, d, *J* = 7.5 Hz, H-12, H-12′), 6.26 (2H, s, H-17, H-17′), 5.70 (2H, s, H-2, H-2′), 5.26 (2H, d, *J* = 12.1 Hz, 2 × $N^+-C\underline{H}H-C_6H_4-NO_2$), 4.95 (2H, d, *J* = 12.1 Hz, 2 × $N^+-CH\underline{H}-C_6H_4-NO_2$), 4.31 (2H, dd, *J* = 18.8, 9.5 Hz, H-5a, H-5a′), 4.25 (2H, br s, H-3, H-3′), 3.45 (2H, t, *J* = 12.5 Hz, H-21a, H-21a′), 3.37−3.27 (2H, m, *J* = 9.2 Hz, H-5b, H-5b′), 2.88 (2H, d, *J* = 12.5 Hz, H-21b, H-21b′), 2.70−2.63 (4H, m, H-15, H-15′, H-6a, H-6a′), 2.54−2.31 (6H, m, H-14a, H-14a′, H-6b, H-6b′, H-20, H-20′), 1.70 (2H, d, *J* = 11.5 Hz, H-14b, H-14b′), 1.49−1.38 (2H, m, H-19a, H-19a′), 1.34−1.24 (2H, m, H-19b, H-19b′), 0.91 (6H, t, *J* = 7.3 Hz, H-18, H-18′); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 148.8 (C, 2 × C-NO_2), 146.8 (C, C-13, C-13′), 135.8 (C, 2 × C1 benzyl), 133.9 (CH, 2 × C2 benzyl), 133.2 (CH, C-17, C-17′), 132.2 (C, C-8, C-8′), 129.7 (CH, C-11, C-11′), 124.2 (CH, 2 × C3 benzyl), 123.0 (CH, C-9, C-9′), 120.7 (CH, C-10, C-10′), 112.1 (C, C-16, C-16′), 109.6 (CH, C-12, C-12′), 73.8 (CH, C-3, C-3′), 68.6 (CH, C-2, C-2′), 61.6 (CH_2, C-5, C-5′), 59.6 (CH_2, 2 × CH_2 benzyl), 54.0 (CH_2, C-21, C-21′), 52.4 (C, C-7, C-7′), 38.3 (CH_2, C-6, C-6′), 36.7 (CH, C-20, C-20′), 33.3 (CH, C-15, C-15′), 23.0 (CH_2, C-19, C-19′), 22.5 (CH_2, C-14, C-14′), 11.0 (CH_3, C-18, C-18′); MALDIMS (matrix, 2,5-dihydroxybenzoic acid in MeOH−H_2O, 1:3) *m/z* 909.60 $[M - Br + 2H]^+$, 719.46 $[M - Br + 2H]^+$, 692.58 $[M - Br - CH_3C_6H_4NO_2 - Br + H]^+$ (100%); *anal.* C 59.58, H 5.85, N 7.72, calcd for $C_{52}H_{56}N_6O_4Br_2 \times 3H_2O$ C 59.89, H 5.99, N 8.06%.

Ca^{2+}/Fluo-4 Assay. The α7-GH_3 cell line[32] was cultured in Dulbecco's modified Eagle medium supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, 10% fetal bovine serum, and 0.1 mg/mL G-418. The Ca^{2+}/Fluo-4 assay was performed essentially as previously described.[33] The cells were split into poly-D-lysine-coated, black, 96-well plates with a clear bottom (BD Biosciences, Bedford, MA, USA), and the assay was performed 64−72 h later. The culture medium was aspirated, and the cells were incubated in 50 μL of loading buffer [Hank's buffered saline solution (HBSS) containing 20 mM HEPES, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 2.5 mM probenecid, pH 7.4], supplemented with 6 mM Fluo-4/AM (Molecular Probes,

dx.doi.org/10.1021/np500259j | J. Nat. Prod. 2014, 77, 2006−2013

Eugene, OR, USA) at 37 °C for 1 h. The loading buffer was removed, the cells were washed once with 100 μL of assay buffer [HBSS containing 20 mM HEPES, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 2.5 mM probenecid, pH 7.4], and then 100 μL of assay buffer containing 100 μM genistein and various concentrations of the test compounds was added to the wells. Following a 30 min incubation at 37 °C in a humidified 5% CO_2 incubator, the 96-well plate was assayed in a NOVOstar microplate reader (BMG Labtechnologies, Offenburg, Germany), measuring emission (in fluorescence units) at 520 nm caused by excitation at 485 nm before and up to 60 s after addition of 33 μL of agonist solution (the agonists were dissolved in assay buffer). The compounds were characterized in duplicate at least three times using EC_{80}–EC_{90} concentrations of ACh as agonist.

Radioligand Binding Studies at the Muscle-Type nAChRs (Competition Assays). (±)-[^{3}H]Epibatidine (56.3 Ci/mmol) was obtained from PerkinElmer. All other chemicals were obtained from Sigma-Aldrich. Frozen samples of the *T. californica* electric organ were purchased from Dr. Charles Winkler, San Pedro, CA, USA. Membrane preparation and radioligand binding assays were performed according to a previously published procedure.[15]

Membrane Preparation. Briefly, frozen samples of the *T. californica* electric organ were placed on ice and allowed to thaw slowly before the membrane preparation. The tissue was homogenized (Polytron) in ice-cold Hepes solid solution (HSS) and centrifuged (15000*g*, 10 min, 4 °C). The pellets were collected, washed four times with HSS buffer through rehomogenization and centrifugation at the same settings, resuspended in HSS buffer, and stored as aliquots at −80 °C.

Competition Assay. Each assay sample, with a total volume of 500 μL, contained 200 μL of the test compound, 100 μL of (±)-[^{3}H]-epibatidine, 100 μL of *T. californica* electroplax (60–70 μg), and 100 μL of HSS buffer. Nonspecific binding was determined in the presence of (−)-nicotine. The samples were incubated for 90 min at 22 °C. The incubation was terminated by vacuum filtration (Brandel harvester) through glass fiber filters (GF/B) presoaked in 1% PEI solution. The filters were rinsed three times with TRIS buffer. Radioactivity was measured using a liquid scintillation counter (PerkinElmer TriCarb 2910 TR).

Competition binding data were analyzed using nonlinear regression methods. K_i values were calculated by the Cheng–Prusoff equation ($K_i = IC_{50}/(1 + L/K_D)$, where L is the used radioligand concentration) based on the measured IC_{50} values and K_D = 2 nM for binding of (±)-[^{3}H]epibatidine. The K_D values were obtained from five independent experiments performed on the same membrane preparations that were used for the competition assays.

M_2 Receptor Binding Assays. Preparation and storage of cardiac porcine membranes was carried out as described elsewhere.[27] Protein content was determined by the Lowry method and amounted to 3.3 mg/mL. The [^{3}H]NMS filtration binding assay was carried out as described earlier.[27] The buffer contained 4 mM Na_2HPO_4 and 1 mM KH_2PO_4, pH 7.4 at 23 °C. [^{3}H]NMS equilibrium binding assays applied 0.2 nM [^{3}H]NMS. Nonspecific [^{3}H]NMS binding was assessed in the presence of 1 μM atropine and did not exceed 5% of total binding. Homologous competition equilibrium binding experiments were carried out for 2 h in a 1.5 mL volume to determine the M_2 equilibrium binding characteristics of the radioligand [^{3}H]NMS. Specific binding of [^{3}H]NMS under control conditions was characterized by the negative log equilibrium dissociation constant, $pK_D = 10.41 \pm 0.32$, $n = 3$.

In dissociation experiments, membranes were incubated with the respective radioligand for 30 min at 23 °C. Thereafter, aliquots of the mixture were added to excess unlabeled ligand in buffer over a total period of 120 min followed by filtration of the samples. To determine the effect of the test compounds on the dissociation of [^{3}H]NMS ($t_{1/2,control}$: 5.27 ± 0.03, n = 70), dissociation was measured by addition of 1 μM atropine in combination with the respective test compounds. Three-point kinetic experiments were performed in analogy to two-point kinetic experiments with measurements of specific [^{3}H]NMS binding at t = 0, t = 10 min, and t = 30 min, respectively.[34] Receptor-bound radioactivity was separated by filtration and measured as described earlier.[35] The binding data from individual experiments were analyzed by computer-aided, nonlinear regression analysis using Prism 5.03 (GraphPad Software, San Diego, CA, USA).

[^{3}H]NMS dissociation data were analyzed assuming a mono-exponential decay as described previously.[26] The slowing actions of the allosteric agents on [^{3}H]NMS dissociation were analyzed as described elsewhere.[36] Homologous competition data obtained with [^{3}H]NMS were analyzed using a four-parameter logistic function to yield estimates of the bottom and top plateaux, the inflection point (IC_{50}), and the slope factor, n, of the curve. If the observed slope factors did not differ significantly from unity (F-test, $p > 0.05$), the IC_{50} values were estimated with n constrained to −1. The pK_D value of [^{3}H]NMS equilibrium binding to the M_2 receptor was calculated according to ref 37.

[^{3}H]NMS (specific activity 82 Ci/mmol) was purchased from PerkinElmer Life and Analytical Sciences (Homburg, Germany). Atropine sulfate and all laboratory reagents were >99% pure and purchased from Sigma Chemicals (Taufkirchen, Germany).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

^{1}H and ^{13}C NMR spectra of compounds **2**, **2a–c**, **3**, **3a–c**. NOESY spectrum of compound **3**. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: darius.zlotos@guc.edu.eg. Tel: +20 2 2758 1041. Fax: +20 2 2758 1041.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank M. Kepe, Institute of Pharmacy, University of Bonn, for her skillful technical assistance as well as Dr. M. Grüne and E. Ruckdeschel, Institute of Organic Chemistry, University of Würzburg, for recording 600 MHz NMR spectra. Dr. D. Feuerbach (Novartis Institutes for Biomedical Research, Basel, Switzerland) is thanked for the generous gift of the α7-GH3 cell line. A.A.J. thanks the Novo Nordisk Foundation for financial support. This work was financially supported in part by the National Institutes of Health P20RR016467 (D.G.)

■ REFERENCES

(1) Jensen, A. A.; Frølund, B.; Liljefors, T.; Krogsgaard-Larsen, P. *J. Med. Chem.* **2005**, *48*, 4705–4745.
(2) Levin, E. D. *Nicotinic Receptors in the Nervous System*; CRC Press: Boca Raton, FL, 2002.
(3) Cassels, B. K.; Bermudez, I.; Dajas, F.; Abin-Carriquiry, J. A.; Wonnacott, S. *Drug Discovery Today* **2006**, *10*, 1657–1665.
(4) Papke, R. L. *Biochem. Pharmacol.* **2014**, *89*, 1–11.
(5) Romanelli, M. N.; Gualtieri, F. *Med. Res. Rev.* **2003**, *23*, 393–426.
(6) Bunnelle, W. H.; Dart, M. J.; Schrimpf, M. R. *Curr. Top. Med. Chem.* **2004**, *4*, 299–334.
(7) Posados, I.; López-Hernández, B.; Cena, V. *Curr. Neuropharmacol.* **2013**, *11*, 298–314.
(8) Prochaska, J. J.; Das, S.; Benowitz, N. L. *Br. Med. J.* **2013**, *347*, f5198.
(9) Young, J. M.; Shytle, R. D.; Sanberg, P. R.; George, T. P. *Clin. Ther.* **2001**, *23*, 532–565.
(10) Shytle, R. D.; Silver, A. A.; Lukas, R. J.; Newman, M. B.; Sheehan, D. V.; Sanberg, P. R. *Mol. Psychiatry.* **2002**, *7*, 525–535.
(11) Papke, R. L.; Bencherif, M.; Lippiello, P. *Neurosci. Lett.* **1996**, *213*, 201–204.
(12) Horenstein, N. A.; Leonik, F. M.; Papke, R. L. *Mol. Pharmacol.* **2008**, *74*, 1496–1511.

(13) Meyer, E.; Kuryatov, A.; Gerzanich, V.; Lindstrom, J.; Papke, R. L. *J. Pharmacol. Exp. Ther.* **1998**, *287*, 918−925.
(14) Jensen, A. A.; Zlotos, D. P.; Liljefors, T. *J. Med. Chem.* **2007**, *50*, 4616−4629.
(15) Zlotos, D. P.; Gündisch, D.; Ferraro, S.; Tilotta, M. C.; Stiefl, N.; Baumann, K. *Bioorg. Med. Chem.* **2004**, *12*, 6277−6285.
(16) Zlotos, D. P.; Buller, S.; Tränkle, C.; Mohr, K. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 2529−2532.
(17) Zlotos, D. P.; Buller, S.; Stiefl, N.; Baumann, K.; Mohr, K. *J. Med. Chem.* **2004**, *47*, 3561−3571.
(18) Da Silva, B. A.; de Araujo Filho, A. P.; Mukherjee, R.; Chiappeta, De A. *Phytother. Res.* **1993**, *7*, 419−424.
(19) Massiot, G.; Thepenier, P.; Jacquier, M. J.; Le-Men Olivier, L.; Delaude, C. *Phytochemistry* **1992**, *31*, 2873−2876.
(20) Bernauer, K.; Berlage, D.; von Philipsborn, W.; Schmid, H.; Karrer, P. *Helv. Chim. Acta* **1958**, *41*, 2293−2308.
(21) Boekelheide, V.; Ceder, O.; Crabb, T.; Kawazoe, Y.; Knowles, R. N. *Tetrahedron Lett.* **1960**, *26*, 1−4.
(22) Zlotos, D. P. *J. Nat. Prod.* **2000**, *63*, 864−865.
(23) Zlotos, D. P. *J. Nat. Prod.* **2003**, *66*, 119−120.
(24) Zlotos, D. P. *Eur. J. Org. Chem.* **2004**, 2375−2380.
(25) *Tridant V.1.1.2*; Wavefunction, Inc.: Irvine, CA, USA, 2006.
(26) Tränkle, C.; Kostenis, E.; Burgmer, U.; Mohr, K. *J. Pharmacol. Exp. Ther.* **1996**, *279*, 926−933.
(27) Tränkle, C.; Dittmann, A.; Schulz, U.; Weyand, O.; Buller, S.; Jöhren, K.; Heller, E.; Birdsall, N. J.; Holzgrabe, U.; Ellis, J.; Höltje, H. D.; Mohr, K. *Mol. Pharmacol.* **2005**, *68*, 1597−1610.
(28) Tränkle, C.; Mies-Klomfass, E.; Botero, C. H. M.; Holzgrabe, U.; Mohr, K. *Mol. Pharmacol.* **1998**, *54*, 139−145.
(29) Maass, A.; Mohr, K. *Eur. J. Pharmacol.* **1996**, *305*, 231−234.
(30) Bock, A.; Merten, N.; Schrage, R.; Dallanoce, C.; Bätz, J.; Klöckner, J.; Schmitz, J.; Matera, C.; Simon, K.; Kebig, A.; Peters, L.; Müller, A.; Schrobang-Ley, J.; Tränkle, C.; Hoffmann, C.; De Amici, M.; Holzgrabe, U.; Kostenis, E.; Mohr, K. *Nat. Commun.* **2012**, *3*, 1044.
(31) Boller, A.; Els, H.; Furst, A. U.S. Patent 3080373, 1961.
(32) Feuerbach, D.; Lingenhöhl, K.; Dobbins, P.; Mosbacher, J.; Corbett, N.; Nozulak, J.; Hoyer, D. *Neuropharmacology* **2005**, *48*, 215−227.
(33) Armishaw, C.; Jensen, A. A.; Balle, T.; Clark, R. J.; Harpsøe, K.; Skonberg, C.; Liljefors, T.; Strømgaard, K. *J. Biol. Chem.* **2009**, *284*, 9498−9512.
(34) Kostenis, E.; Mohr, K. *Trends Pharmacol. Sci.* **1996**, *17*, 280−283.
(35) Tränkle, C.; Andresen, I.; Lambrecht, G.; Mohr, K. *Mol. Pharmacol.* **1998**, *53*, 304−312.
(36) Fang, L.; Jumpertz, S.; Zhang, Y.; Appenroth, D.; Fleck, C.; Mohr, K.; Tränkle, C.; Decker, M. *J. Med. Chem.* **2010**, *53*, 2094−20103.
(37) DeBlasi, A.; O'Reilly, K.; Motulsky, H. J. *Trends Pharmacol. Sci.* **1989**, *10*, 227−229.

■ NOTE ADDED AFTER ASAP PUBLICATION

This paper was published ASAP on September 5, 2014, with errors to Table 1 and the Results and Discussion Section. The corrected version reposted on September 9, 2014.