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Synthesis, nicotinic acetylcholine receptor binding, antinociceptive and seizure properties of methyllycaconitine analogs

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Abstract—A series of methyllycaconitine (**1a**, MLA) analogs was synthesized where the (*S*)-2-methylsuccinimidobenzoyl group in MLA was replaced with a (*R*)-2-methyl, 2,2-dimethyl-, 2,3-dimethyl, 2-phenyl-, and 2-cyclohexylsuccinimidobenzoyl (**1b–f**) group. The analogs **1b–f** were evaluated for their inhibition of [^{125}I]iodo-MLA binding at rat brain α7 nicotinic acetylcholine receptors (nAChR). In order to determine selectivity, MLA and the analogs **1b–f** were evaluated for inhibition of binding to rat brain α,β nAChR using [^{3}H]epibatidine. At the α7 nAChR, MLA showed a K_i value of 0.87 nM, analogs **1b–e** possessed K_i values of 1.67–2.16 nM, and **1f** showed a K_i value of 26.8 nM. Surprisingly, the analog **1e** containing the large phenyl substituent (K_i = 1.67 nM) possessed the highest affinity. None of the compounds possessed appreciable affinity for α,β nAChRs. MLA antagonized nicotine-induced seizures with an AD_{50} = 2 mg/kg. None of the MLA analogs were as potent as MLA in this assay. MLA and all of the MLA analogs, with the exception of **1b**, antagonized nicotine's antinociceptive effects in the tail-flick assay. Compound **1c** (K_i = 1.78 nM at α7 nAChR) with an AD_{50} value of 1.8 mg/kg was 6.7 times more potent than MLA (AD_{50} = 12 mg/kg) in antagonizing nicotine's antinociceptive effects but was 5-fold less potent than MLA in blocking nicotine-induced seizures. Since MLA has been reported to show neuroprotection against β-amyloid$_{1–42}$, these new analogs which have high α7 nAChR affinity and good selectivity relative to α,β nAChRs will be useful biological tools for studying the effects of α7 nAChR antagonist and neuroprotection.

1. Introduction

Nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels formed by the association of five subunits, leading to heteromeric and homomeric structures. The α7 receptor is the only homomeric nAChR widely distributed in the mammalian central nervous system. Binding sites in the brain that show high affinity for [^{125}I]α-bungarotoxin ([^{125}I]α-BTX) have been correlated with the α7 nAChR.[1–4] Methyllycaconitine (**1a**, MLA), an alkaloid isolated from *Delphinium brownii*,[5,6] is reported to be the most potent non-protein competitive α7 nAChR antagonist presently available.[7] Recent studies have suggested that α7 nAChRs may play an important role in cognitive dysfunction, neurodegenerative diseases, vestibular function, epilepsy, and possibly smoking cessation.[8] It is particularly interesting to note that MLA (**1a**) protects against the toxicity produced by the Alzheimer's disease-related peptide β-amyloid$_{1–42}$(Aβ$_{42}$).[9] The availability of potent and selective α7 nAChR antagonist will enhance our ability to study these processes. Although MLA binds potently to the α7 subtype, recent evidence suggests that MLA can also interact with α4β2 nAChR and presynaptic α3/α6 nAChR.[10] Clearly, there is a need for additional and selective α7 nAChR antagonists to further characterize the α7 nAChR pharmacophore and to further study its biological and pharmacological roles.

In this study, we report the syntheses and inhibition of [^{125}I]iodo-MLA and [^{3}H]epibatidine binding at the α7 and α,β nAChRs for MLA (**1a**) and the MLA analogs **1b–f**. Unfortunately, there is a lack of selective behavioral measure for α7-mediated response. Therefore, we evaluated the ability of MLA analogs to antagonize nicotine-induced seizures and antinociception in mice using both the tail-flick and hot-plate assays because of MLA's unique actions in these three assays. These assays were chosen because seizures are mediated at least in part through α7 receptors (sensitive to MLA),

Keywords: Methyllycaconitine; α7 nAChR; Antagonist; MLA analogs.

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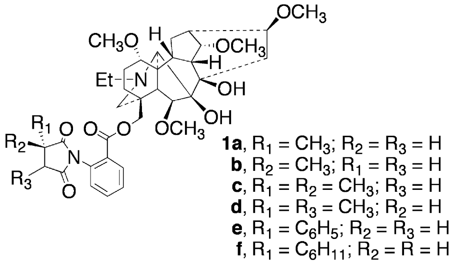
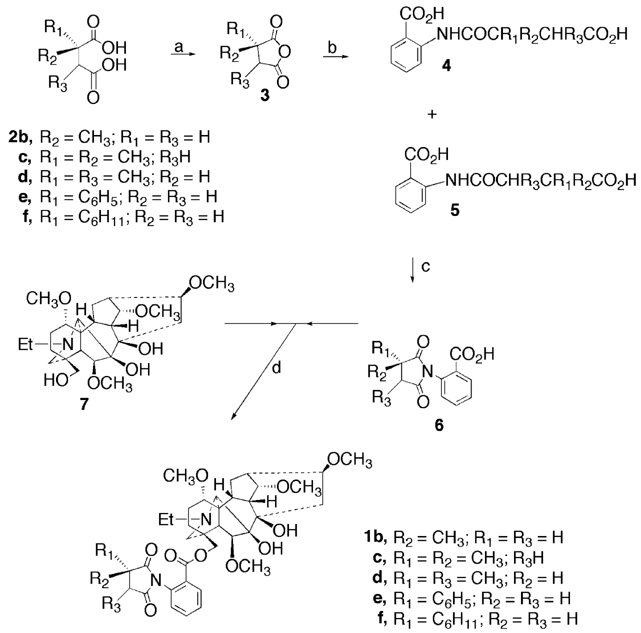
the hot-plate assay is α4β2-receptor-mediated (insensitive to MLA), and the tail-flick assay is mediated through multiple nAChRs (sensitive to MLA).





Scheme 1. Reagents and conditions: (a) acetic anhydride, reflux at 12 h; (b) anthranilic acid; (c) toluene, $(C_2H_5)_3N$ reflux at 12 h; (d) $CH_3C_6H_4SO_2Cl$, pyridine.

1.1. Chemistry

The MLA analogs **1b–f** were synthesized as outlined in Scheme 1. The succinic acids **2b–f** were converted to the corresponding succinic anhydrides **3** by refluxing the appropriate acid with acetic anhydride for 12 h. Treatment of each anhydride with anthranilic acid in chloroform provided the desired substituted methyllycoctonic acids, which are most likely a mixture of **4b–f** and **5b–f** except for the symmetrical analog (**4d** or **5d**).[11] The mixture of acids was refluxed under a Dean Stark tube in toluene containing triethylamine for 12 h to yield the appropriate 2-substituent or 2,3-disubstituted succinimidobenzoic acids **6b–f**. The acids **6b–f** were coupled to the primary hydroxyl group of lycoctonine (**7**) in the presence of *p*-toluenesulfonyl chloride and pyridine to give the desired MLA analogs **1b–f**.[12]

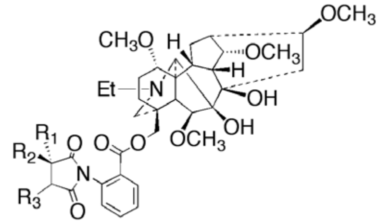
1.2. Biology

The K_i values for the inhibition of [^{125}I]iodo-MLA and [^{3}H]epibatidine binding for MLA (**1a**) and the MLA analogs **1b–f** were determined using previously reported procedures and are listed in Table 1.[13,14] MLA (**1a**) and the MLA analogs **1b–f** were evaluated for their ability to antagonize the effects of nicotine in the tail-flick, hot-plate, and seizure test using previously reported procedures and the AD_{50} values are listed in Table 2.[15]

2. Results and discussion

The α7 nAChR subtype is the second most prevalent in the brain and has been implicated as playing a key role in conditions such as nicotine addiction, schizophrenia, Alzheimer's disease, and epilepsy. Despite recent progress in the synthesis of competitive agonists selective for the α7 nAChR subtype, very few antagonists are known that bind with high affinity and selectivity at this receptor. These include the peptide toxins α-bungarotoxin and the norditerpenoid alkaloid methyllycaconitine (MLA, **1a**).

Table 1. Radioligand binding data for MLA (**1a**) and the MLA analogs **1b–f**[a]



Compound	RTI-7527-	R_1	R_2	R_3	α7 nAChR[^{125}I]iodo-MLA K_i, nM (−*n*H)	α,β nAChR[^{3}H]epibatidine K_i, nM (−*n*H)
1a	MLA	CH_3	H	H	0.87 ± 0.13 (1.09 ± 0.15)	739 ± 43 (0.68 ± 0.02)
1b	58	H	CH_3	H	2.12 ± 0.47 (1.2 ± 0.34)	352 ± 47 (0.89 ± 0.08)
1c	60	CH_3	CH_3	H	1.78 ± 0.15 (0.92 ± 0.05)	602 ± 127 (0.93 ± 0.09)
1d	62	CH_3	H	CH_3	2.46 ± 0.87 (0.98 ± 0.18)	321 ± 33 (0.88 ± 0.08)
1e	59	C_6H_5	H	H	1.67 ± 0.75 (1.44 ± 0.42)	219 ± 23 (0.75 ± 0.04)
1f	61	C_6H_{11}	H	H	26.8 ± 3.62 (0.88 ± 0.21)	1103 ± 400 (1.21 ± 0.02)

−*n*H is the Hill coefficient determined from a four-parameter logistic fit to the data.

[a] Data represent means ± SE from at least three independent experiments

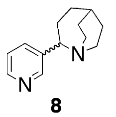
Table 2. Pharmacological evaluation of MLA (**1a**) and MLA analogs **1b–f** as nicotinic antagonists[a]

Compound	Seizures (AD_{50}, mg/kg)	Tail-flick (AD_{50}, mg/kg)	Hot-plate (AD_{50} mg/kg)
1a	2 (0.5–4)	12 (2–59)	15% at 20
1b	40% at 50	10% at 30	0% at 30
1c	10 (9–11)	1.8 (0.4–9)	13% at 20
1d	0% at 50	7.0 (3–15)	25% at 20
1e	0% at 50	18.3 (10–31)	7% at 20
1f	33 (10–113)	7.0 (2.5–18)	0% at 20

Results were expressed as AD_{50} (mg/kg) ± confidence limits (CL) or % effect at the highest dose tested.

[a] Dose–response curves were determined using a minimum of four different doses of test compound and at least eight mice were used per dose group.

β-Amyloid$_{1–42}$ binds to α7 nAChRs with high affinity.[16] Functional studies have shown both α7 nAChR activation[17,18] and inhibition[19] with this peptide. Many studies suggest that α7 nAChRs agonist, such as TC-1698 (**8**),[20] shows neuroprotection against β-amyloid$_{1–42}$ effects on α7 nAChR function. It is well-known that α7 nAChRs undergo extensive desensitization [21–24] so long-term agonist treatment may not be desirable. Interestingly, Martin et al. reported that α7 nAChR antagonist MLA (**1a**) has neuroprotective actions against Aβ$_{42}$-induced neurotoxicity and suggested that α7 nAChR antagonist might be useful pharmacotherapies in treating neurodegenerative disorders such as Alzheimer's disease.[9] Unfortunately, lack of highly selective agonists and antagonists has complicated the elucidation of the physiological function of α7 nAChRs.



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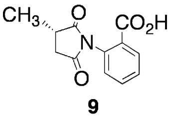

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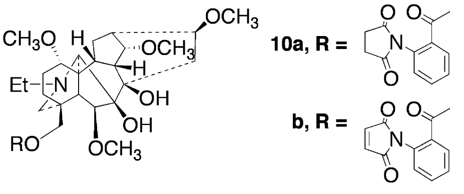

MLA (**1a**) is an ester composed of the alcohol lycoctonine (**7**) and the acid, (*S*)-2-methylsuccinimidobenzoic acid. A number of truncated analogs of the parent MLA (**1a**) have been synthesized.[25] None of the compounds showed appreciable activity.[25,26] Reports from the literature have shown that neither the alcohol **7** nor the acid **9** possesses appreciable affinity for the α7 nAChR.[12,27] Thus, it appears that the (*S*)-2-methylsuccinimidobenzoyl group connected to the C18 oxygen of lycoctonine is essential for potent α7 nAChR binding. In addition, esters formed from the acid **9** and alcohols possessing parts of the lycoctonine structure as well as esters formed from lycoctonine with other acids were reported to possess low affinity for the α7 nAChR.[27,28] In addition, the lycoctonine ester (**10a**), a nonditerpenoid alkaloid lacking the 2-(*S*)-methyl group on the succinimidobenzoyl group of MLA, and the dehydro analog (**10b**) are reported to show 5- and 10-fold loss in affinity relative to MLA at the α7 nAChR, which suggests that the angular-2-methyl-group on the succinimidobenzoyl group is essential for potent α7 nAChR binding.[12,29]

In the present study, we found that replacement of (*S*)-2-methylsuccinimidobenzoyl group in MLA (**1a**, $K_i = 0.87$ nM) with the enantiomeric (*R*)-2-methylsuccinimidobenzoyl group to give **1b**, $K_i = 2.12$ nM, resulted in a 2.4-fold loss in affinity for the α7 nAChR. Compounds **1c**, $K_i = 1.78$ nM, and **1d**, $K_i = 2.62$ nM, which possess an additional methyl group at the 2- or 3-position, showed 2.1- and 3-fold loss in affinity, respectively. Surprisingly, replacement of the 2-methyl group on the 2-methylsuccinimido group of MLA with a phenyl ring to give **1e** resulted in a K_i value of 1.67 nM. Thus, the MLA pharmacophore will accommodate large flat aromatic groups in this part of the structure. In contrast, replacement of the 2-methyl group with a cyclohexyl group to give **1f** resulted in a K_i value of 26.8 nM and thus a 31-fold loss of affinity. This alteration suggests that even though a phenyl ring is allowed in the 2-position of the 2-methylsuccinimido group of MLA, larger more bulky groups like cyclohexyl are not allowed. The weak ability of MLA and all the analogs **1b–f** to inhibit [^{3}H]epibatidine binding indicates that these compounds have low affinity for α,β nAChRs.

MLA analogs **1b–e** showed equipotent binding affinities toward the α7 nAChR. However, their in vivo profile in the seizures and tail-flick assays was different from each other. MLA analogs **1d–f** were weak antagonists in the tail-flick assay ($AD_{50} = 7–18.3$ mg/kg). Analog **1b**, which differs from MLA in only the stereochemistry of the methyl group [(*R*)-methyl] on the succinimidobenzoyl group, was inactive in this assay. The most potent compound, **1c**, with an AD_{50} value of 1.8 mg/kg, was 6.7 times more potent than MLA, which has an AD_{50} of 12 mg/kg. The nAChR subtype or subtypes that mediate the tail-flick response are not known, but the lack of a high correlation between α7 receptor affinity and antagonistic potency in the tail-flick assay, particularly the discrepancy with enantioselectivity found in the 2-methylbenzoyl group, does not support a role for α7 receptors in mediating antinociception in this assay. It should be noted that earlier reports failed to observe a significant blockade of nicotine's effects in the tail-flick test by MLA after sc injection in mice (5 mg/kg)[30] and icv administration in rats (10 μg).[31] However, the doses of MLA used in the present study are much higher than the reported ones. This difference in the effect observed could mean MLA lacks nAChR selectivity at higher doses or it is operating by some other mechanism. MLA and all the analogs had poor affinity for αβ nAChRs as determined in the [^{3}H]epibatidine binding assay and they were inactive at antagonizing the nicotinic effects in the hot-plate test. This is consistent with earlier observations that α4β2 receptors are required for this effect.[32]

MLA was also effective in blocking nicotine-induced seizures in an enantioselective manner. The seizures are mediated by α7 nAChRs and these results are consistent with an α7 mechanism. However, as with the tail-flick assay, there was a poor correlation between α7 receptor affinity and antagonistic potency. The results from the seizure study also support the view that nicotine-induced seizures and antinociception may be mediated by non-α4β2 and non-α7 receptor subtypes. Indeed, pharmacological and genetic approaches initially suggested the involvement of α7 nicotinic subtypes in nicotine-induced seizures. However, additional studies on different mouse inbred strains and transgenic mice have also implicated α4, α5, α3, and β4.[33] The in vivo results show also that analogs **1b** and **1e** are much less potent than MLA in blocking nicotinic effects despite having good affinity for the α7 nAChR. This suggests that these two analogs have a different selectivity profile than MLA. The difference seen between in vitro binding affinity and in vivo potency in the various behavioral tests suggests that these MLA analogs have lower selectivity for the α7 receptor subtype. Ultimately, in vitro functional selectivity on various nAChR subtypes in expressed cells would confirm this in vivo selectivity. While MLA and its derivatives antagonize both nicotine-induced antinociception and seizures, they apparently do so by acting at multiple sites or different mechanisms, since there is little correlation between antagonistic potencies in these two measures. The fact that MLA does not readily cross the blood–brain barrier raises the question of whether pharmacokinetic differences may explain some of these findings. However, it seems unlikely that the pharmacokinetics would be so different among these analogs to explain their pharmacological dissimilarities.

3. Conclusions

Replacement of the (*S*)-2-methylsuccinimidobenzoyl group in MLA (**1a**) by an (*R*)-2-methylsuccinimido- benzoyl to give **1b** caused only a small reduction in binding at the α7 nAChR, however, **1b** showed no ability to antagonize seizures and antinociception induced by nicotine. Surprisingly, compound **1c** which has methyl groups present in both the (2*S*) and (2*R*)-position of the succinimido group is 6.7 times more potent than MLA in antagonizing the antinociception induced by nicotine in the tail-flick test. However, **1c** has essentially the same affinity for the α7 nAChR as **1b**. These analogs have a pharmacological profile distinct from that of MLA despite relatively modest changes in structure. It would seem that they are acting at sites other than α7 receptors. Regardless of their mechanism of action, the (*S*)-2-substituted succinimidobenzoyl group is critical. Further development of MLA analogs may lead to even better probes for characterizing these receptor sites and to biological tools for studying the effects of α7 nAChR antagonist in several neurological disorders.

4. Experimental

Melting points were determined on a Thomas–Hoover capillary tube apparatus and are not corrected. Elemental analyses were obtained by Atlantic Microlabs Inc., and are within ±0.4% of the calculated values. All optical rotations were determined at the sodium D-line using a Rudolph Research Autopol III polarimeter (1-dm cell). ^{1}H NMR were determined on a Bruker Avance DPX-300 or Bruker AMX-500 spectrometer using tetramethylsilane as an internal standard. Silica gel 60 (230–400 mesh) was used for all column chromatography. All reactions were followed by thin-layer chromatography using Whatman silica gel 60 TLC plates and were visualized by UV or by charring using 5% phosphomolybdic acid in ethanol. All solvents were reagent grade.

MLA was isolated from *Delphenium elatum* (pacific giant) seeds (purchased from Flowers of Tomorrow Inc., Parma, Idaho 83660) and hydrolyzed to lycoctonine (**7**).[34,35] [^{3}H]epibatidine (s.a. = 66.6 ci/mmol) was obtained from DuPont New England Nuclear (Boston, MA).

4.1. (*R*)-Methylsuccinamic acids (4b and 5b)

A suspension of (*R*)-methylsuccinic acid (**2b**) (0.89 g, 6.75 mmol) in acetic anhydride (10 mL) was heated to reflux overnight. After removal of the excess of acetic anhydride by distillation, the residue was triturated and dried under reduced pressure to give the anhydride **3b** that was dissolved in 10 mL $CHCl_3$ and added to a suspension of anthranilic acid (0.925 g, 6.75 mmol) in 10 mL $CHCl_3$. The reaction mixture was heated on a steam bath for 30 min, and the $CHCl_3$ removed under reduced pressure. The product was purified on silica gel using hexane/ethyl acetate/methanol/acetic acid (125:45:5:4) as eluent to give 1.56 g (92%) of succinamic acids (**4b** and **5b**): mp 165–167 °C; ^{1}H NMR (CD_3OD) δ 1.25–1.36 (2d, 3H), 2.50–2.60 (m, 1H), 2.76–2.99 (m, 2H), 7.12–7.18 (t, 1H, aromatic), 7.53–7.56 (t, 1H, aromatic), 8.08–8.11 (d, 1H, aromatic), 8.54–8.57 (d, 1H, aromatic).

4.2. (*R*)-Methyllycactonic acid (6b)

A solution of the above (*R*)-methylsuccinamic acids (**4b** and **5b**) (1.5 g, 6 mmol) and triethylamine (3.05 g, 30.1 mmol) in 200 mL of toluene was heated to reflux overnight. The toluene was removed by simple distillation, and the residue was purified on silica gel using hexane/ethyl acetate/methanol/acetic acid (125:45:5:4) as eluent to obtain 0.9 g (64%) of pure (*R*)-methyllycactonic acid (**6b**): mp 117–119 °C; ^{1}H NMR (CD_3OD) δ 1.38–1.40 (d, 3H), 2.41–2.61 (m, 1H), 3.04–3.15 (m, 2H), 7.28–7.31 (d, 1H, aromatic), 7.56–7.58 (t, 1H, aromatic), 7.65–7.68 (t, 1H, aromatic), 8.11–8.14 (d, 1H, aromatic). Anal. ($C_{12}H_{11}NO_4 \cdot 0.5H_2O$) C, H, N.

4.3. (*R*)-Methyllycaconitine (1b) citrate

To a stirred solution of imido acid **6b** (49.9 mg, 0.214 mmol) in 1 mL of dry pyridine was added 76.4 mg (0.428 mmol) of *p*-toluenesulfonyl chloride. The mixture was cooled to 0 °C, 100 mg (0.214 mmol) of lycoctonine was added, and the solution was stirred for additional 1 h at 0 °C and then placed in the refrigerator for 24 h. The yellow solution was dissolved in 10 mL NH_4OH (aq, pH 9) and extracted with 3× 10 mL $CHCl_3$. Organic phases were combined and washed with 10 mL NH_4OH (aq, pH 9) and dried over anhydrous Na_2SO_4. After removal of the solvent, the residue was purified on silica gel using cyclohexane:$CHCl_3$/$(Et)_3N$ (6:4:1) as eluent to give 66 mg (45%) of pure (*R*)-methyllycaconitine (**1b**). An analytical sample was prepared as the citrate salt: mp 99 °C (dec); $[\alpha]_D^{20} + 48.3°$ (*c*, 0.64, EtOH) (free base). Anal. ($C_{43}H_{58}N_2O_{17}{\cdot}1.5H_2O$) C, H, N.

4.4. 2,2-Dimethylsuccinamic acids (4c and 5c)

2,2-Dimethylsuccinic anhydride (**3c**) (2.0 g, 15.6 mmol) was dissolved in 15 mL $CHCl_3$ and added to a suspension of anthranilic acid (2.14 g, 15.6 mmol) in 15 mL $CHCl_3$. The reaction mixture was heated on a steam bath for 30 min and $CHCl_3$ removed under reduced pressure. The product was purified on silica gel using hexane/ethyl acetate/methanol/acetic acid (125:45:5:4) as eluent to obtain 3.5 g (85%) of 2,2-dimethylsuccinamic acids (**4c** and **5c**): mp 138–140 °C; ^1H NMR ($CDCl_3$) δ 1.41 (s, 6H, $2CH_3$), 2.78 (s, 2H, -CH_2), 7.07–7.20 (t, 1H, aromatic), 7.56–7.61 (t, 1H, aromatic), 8.05–8.08 (d, 1H, aromatic), 8.77–8.80 (d, 1H, aromatic). Anal. ($C_{13}H_{15}NO_5{\cdot}0.5H_2O$) C, H, N.

4.5. 2,2-Dimethyllycactonic acid (6c)

A solution of the above 2,2-dimethylsuccinamic acids (**4c** and **5c**) (3.5 g, 13.2 mmol) and triethylamine (6.6 g, 66 mmol) in 300 mL of toluene was heated to reflux overnight. The toluene was removed by simple distillation, and the residue was purified on silica gel using hexane/ethyl acetate/methanol/acetic acid (125:45:5:4) as eluent to obtain 1.3 g (40%) of pure 2,2-dimethyllycactonic acid (**6c**): mp 134–136 °C; ^1H NMR ($CDCl_3$) δ 1.39 (s, 6H, $2CH_3$), 2.70 (s, 2H, -CH_2), 7.23–7.26 (d, 1H, aromatic), 7.47–7.52 (t, 1H, aromatic), 7.63–7.68 (t, 1H, aromatic), 8.11–8.14 (d, 1H, aromatic). Anal. ($C_{13}H_{13}NO_4{\cdot}0.25H_2O$) C, H, N.

4.6. 2,2-Dimethyllycaconitine (1c) citrate

To a stirred solution of imido acid **6c** (105.8 mg, 0.428 mmol) in 2 mL of dry pyridine was added 152.8 mg (0.856 mmol) of *p*-toluenesulfonyl chloride. The mixture was cooled to 0 °C, 100 mg (0.214 mmol) of lycactonine was added, and the solution was stirred for an additional 1 h at 0 °C and then placed in the refrigerator for 15 h. The deep-red solution was dissolved in 15 mL NH_4OH (aq, pH 9) and extracted with 3× 10 mL $CHCl_3$. Organic phases were combined and washed with 10 mL NH_4OH (aq, pH 9) and dried over anhydrous Na_2SO_4. After the solvent was removed, the residue was purified on silica gel using cyclohexane/$CHCl_3$/$(Et)_3N$ (6:4:1) as eluent to obtain 117.5 mg (79%) of the pure 2,2-dimethyllycaconitine (**1c**). An analytical sample was prepared as the citrate salt: mp 104 °C (dec). Anal. ($C_{44}H_{60}N_2O_{17}{\cdot}H_2O$) C, H, N.

4.7. 2,3-Dimethylsuccinamic acids (4d and 5d)

A suspension of 2,3-dimethylsuccinic acid (**2d**) (2.0 g, 13.7 mmol) in acetic anhydride (15 mL) was heated to reflux overnight. After removal of the excess of acetic anhydride by distillation, the residue was triturated and dried under reduced pressure to give the anhydride **3d** which was dissolved in 15 mL $CHCl_3$ and added to a suspension of anthranilic acid (1.88 g, 13.7 mmol) in 15 mL $CHCl_3$. The reaction mixture was heated on a steam bath for 30 min and the $CHCl_3$ removed under reduced pressure. The product was purified on silica gel using hexane/ethyl acetate/methanol/acetic acid (125:45:5:4) as eluent to obtain 3 g (83%) of 2,3-dimethylsuccinamic acids **4d** and **5d**: ^1H NMR ($CDCl_3$) δ 130–138 (m, 6H), 2.75–2.85 (m, 2H), 7.07–7.12 (t, 1H, aromatic), 7.54–7.59 (t, 1H, aromatic), 8.01–8.04 (d, 1H, aromatic), 8.64–8.67 (d, 1H, aromatic).

4.8. 2,3-Dimethyllycactonic acid (6d)

A solution of the above 2,3-dimethylsuccinamic acids **4d** and **5d** (3 g, 11.3 mmol) and triethylamine (5.65 g, 56.5 mmol) in 300 mL of toluene was heated to reflux overnight. The toluene was removed by simple distillation, and the residue was purified on silica gel using hexane/ethyl acetate/methanol/acetic acid (125:45:5:4) as eluent to obtain 0.8 g (29%) of pure 2,3-dimethyllycactonic acid (**6d**): mp 163–165 °C; ^1H NMR ($CDCl_3$) δ 1.32–1.44 (2 d, 6H), 2.62–3.16 (2 m, 2H), 7.26–7.29 (d, 1H, aromatic), 7.51–7.56 (t, 1H, aromatic), 7.67–7.70 (t, 1H, aromatic), 8.17–8.20 (d, 1H, aromatic). Anal. ($C_{13}H_{13}NO_4{\cdot}25H_2O$) C, H, N.

4.9. 2,3-Dimethyllycaconitine (1d) citrate

To a stirred solution of imido acid **6d** (105.8 mg, 0.428 mmol) in 2 mL of dry pyridine was added 152.8 mg (0.856 mmol) of *p*-toluenesulfonyl chloride. The mixture was cooled to 0 °C and 100 mg (0.214 mmol) of lycactonine was added, and the solution was stirred for additional 1 h at 0 °C and was refrigerated for 18 h. The deep-red solution was taken up in 15 mL NH_4OH (aq, pH 9) and extracted with 3× 10 mL $CHCl_3$. Organic phases were combined and washed with 10 mL NH_4OH (aq, pH 9) and dried over anhydrous Na_2SO_4. After removal of the solvent, the residue was purified on silica gel using cyclohexane/$CHCl_3$/$(Et)_3N$ (6:4:1) as eluent to obtain 74.6 mg (50%) of pure 2,3-dimethyllycaconitine (**1d**). An analytical sample was prepared as citrate salt: mp 84 °C (dec). Anal. ($C_{44}H_{60}N_2O_{17}{\cdot}0.5H_2O$) C, H, N.

4.10. Phenylsuccinamic acids (4e and 5e)

Phenylsuccinic anhydride (**3e**) (2.0 g, 11.36 mmol) was dissolved in 15 mL $CHCl_3$ and was added to a suspension of anthranilic acid (1.55 g, 11.36 mmol) in 15 mL $CHCl_3$. The reaction mixture was heated on a steam bath for 30 min, and then the $CHCl_3$ solvent was evaporated to dryness under reduced pressure. The product was purified on silica gel, eluting with hexane/ethyl acetate/methanol/acetic acid (125:45:5:4) to obtain 3 g (84%), phenylsuccinamic acids (**4e** and **5e**): mp 169–171 °C; ^1H NMR (DMSO) δ 2.75–2.82 (dd, 1H), 3.11–3.20 (dd, 1H), 4.02–4.10 (dd, 1H), 7.12–7.17 (m, 1H, aromatic), 7.26–7.38 (m, 5H, –C_6H_5), 7.55–7.60 (m, 1H, aromatic), 7.93–7.99 (m, 1H, aromatic), 8.44–8.50 (m, 1H, aromatic).

4.11. Phenyllycactonic acid (6e)

A solution of the above phenylsuccinamic acids (**4e** and **5e**) (3 g, 9.58 mmol) and triethylamine (4.79 g, 47.9 mmol) in 250 mL of toluene was heated to reflux overnight. The toluene solvent was removed by simple distillation, and the residue was purified on silica gel, eluting with hexane/ethyl acetate/methanol/acetic acid (125:45:5:4) to obtain 1.1 g (39%) of pure phenyllycactonic acid (**6e**): mp 80–82 °C; ^{1}H NMR ($CDCl_3$) δ 2.98–3.11 (dd, 1H), 3.36–3.45 (dd, 1H), 4.21–4.26 (dd, 1H), 7.33–7.40 (m, 6H, aromatic), 7.56–7.61 (m, 1H, aromatic), 7.72–7.76 (m, 1H, aromatic), 8.22–8.25 (m, 1H, aromatic). Anal. ($C_{17}H_{13}NO_4{\cdot}0.6H_2O$) C, H, N.

4.12. Phenyllycaconitine (1e) citrate

To a stirred solution of imido acid **6e** (126.3 mg, 0.428 mmol) in 2 mL of dry pyridine was added 152.8 mg (0.856 mmol) of *p*-toluenesulfonyl chloride. The mixture was cooled to 0 °C, 100 mg (0.214 mmol) of lycactonine was added, and the solution was stirred for additional 1 h at 0 °C and was then placed in the refrigerator for 18 h. The deep-red solution was taken up in 15 mL NH_4OH aqueous solution (pH ~9) and extracted with 3× 10 mL $CHCl_3$. Organic phases were combined and washed with 10 mL NH_4OH aqueous solution (pH ~9) and dried over anhydrous Na_2SO_4. After the solvent was removed, the residue was purified on silica gel, eluted with cyclohexane/$CHCl_3$/$(Et)_3N$ (6:4:1) to obtain 100 mg (63%) of the pure phenyllycaconitine (**1e**). An analytical sample was prepared as the citrate salt: mp 105 °C (dec). Anal. ($C_{48}H_{60}N_2O_{17}{\cdot}0.5H_2O$) C, H, N.

4.13. Cyclohexylsuccinamic acids (4f and 5f)

A suspension of cyclohexylsuccinic acid (**2f**) (2.0 g, 10 mmol) in acetic anhydride (15 mL) was heated to reflux overnight. The excess of acetic anhydride was removed by sample distillation, the residue (**3f**) was triturated and dried under reduced pressure. The anhydride (**3f**) (1.78 g, 9.78 mmol) was dissolved in 15 mL $CHCl_3$ and was added to a suspension of anthranilic acid (1.34 g, 9.78 mmol) in 15 mL $CHCl_3$. The reaction mixture was heated on a steam bath for 30 min, and the $CHCl_3$ solvent was evaporated to dryness under reduced pressure. The product was purified on silica gel, eluting with hexane/ethyl acetate/methanol/acetic acid (125:45:5:4) to obtain 2.9 g (92%) of cyclohexylsuccinamic acids (**4f** and **5f**): mp 171–173 °C, ^{1}H NMR ($CDCl_3$) δ 1.05–2.04 (m, 11H, -C_6H_{11}), 2.61–2.68 (m, 1H), 2.76–2.88 (m, 2H), 7.08–7.13 (t, 1H, aromatic), 7.56–7.61 (t, 1H, aromatic), 8.06–8.08 (d, 1H, aromatic), 8.70–8.73 (d, 1H, aromatic).

4.14. Cyclohexyllycactonic acid (6f)

A solution of the above cyclohexylsuccinamic acids (**4f** and **5f**) (2.77 g, 8.68 mmol) and triethylamine (4.34 g, 43.4 mmol) in 300 mL of toluene was heated to reflux overnight. The toluene solvent was removed by simple distillation. The residue was purified on silica gel, eluting with hexane/ethyl acetate/methanol/acetic acid (125:45:5:4) to obtain 1.1 g (42%) of pure cyclohexyllycactonic acid (**6f**): mp 68–70 °C; ^{1}H NMR ($CDCl_3$) δ 1.12–1.35 (m, 5H), 1.69–1.81 (m, 5H), 2.06–2.11 (m, 1H), 2.71–3.10 (m, 3H), 7.22–7.26 (d, 1H, aromatic), 7.51–7.56 (t, 1H, aromatic), 7.67–7.72 (t, 1H, aromatic), 8.16–8.19 (d, 1H, aromatic). Anal. ($C_{17}H_{19}NO_4{\cdot}0.33H_2O$) C, H, N.

4.15. Cyclohexyllycaconitine (1f) citrate

To a stirred solution of the imido acid (**6f**) (128.98 mg, 0.428 mmol) in 2 mL of dry pyridine was added 152.8 mg (0.856 mmol) of *p*-toluenesulfonyl chloride. The mixture was cooled to 0 °C, and 100 mg (0.214 mmol) of lycactonine was added. The solution was stirred for additional 1 h at 0 °C and was then placed in the refrigerator for 18 h. The deep-red solution was taken up in 15 mL NH_4OH aqueous solution (pH ~9) and extracted with 3× 10 mL $CHCl_3$. Organic phases were combined and washed with 10 mL NH_4OH aqueous solution (pH ~9) and dried over anhydrous Na_2SO_4. After the solvent was removed, the residue was purified on silica gel, eluting with cyclohexane/$CHCl_3$/$(Et)_3N$ (6:4:1) to obtain 105.7 mg (66%) of the pure cyclohexyllycaconitine (**1f**). An analytical sample was prepared as citrate salt: mp 72 °C (dec). Anal. ($C_{48}H_{66}N_2O_{17}{\cdot}1.5H_2O$) C, H, N.

4.16. [^{125}I]Iodo-MLA binding assay

Frozen male rat cerebral cortex (includes hippocampus; Pel-Freez Biologicals, Rogers, AK) was homogenized (polytron) in 39 volumes of ice-cold 50 mM Tris buffer (assay buffer; pH 7.4 at 4 °C) containing 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, and 1 mM $MgCl_2$. The homogenate was centrifuged at 35,000*g* for 10 min at 4 °C and the supernatant discarded. The pellet was washed twice more with the original volume of buffer. After the last centrifugation, the pellet was resuspended in 1/10 the original volume of buffer and stored at −80 °C until needed. The competition binding experiments were carried out in 1.4 mL polypropylene tubes (Matrix Technologies Corporation, Hudson, NH) in a 96-well array. In a final volume of 0.5 mL triplicate samples contained approximately 3 mg of tissue (wet weight; added last), 40–50 pM [^{125}I]iodo-MLA, and 10–12 different concentrations of the test compounds. The dilutions of the test compounds were made using assay buffer containing 10% DMSO (1% final concentration). The total binding and nonspecific binding (300 μM nicotine) samples also contained a final concentration of 1% DMSO. The test compounds were pipetted using a MultiProbe II_{EX} (Packard Instruments, Meriden, CT) robotic liquid handling system. The samples were incubated on ice for 2 h. A MultiMate harvester (Packard) was used to separate bound radioligand from free by rapid vacuum filtration onto GF/B filters presoaked for at least 30 min in assay buffer containing 0.15% bovine serum albumin. The filters were washed with approximately 4 mL of ice cold

10 mM Tris buffer (pH 7.4 at 4 °C) without salts and dried prior to the addition of 35 μL of Microscint 20 scintillant (Packard). The amount of radioligand remaining on each filter was determined using a Packard TopCount microplate scintillation counter (70% efficiency).

4.17. [^{3}H]Epibatidine binding assay

Adult male rat cerebral cortices (Pelfreeze Biological, Rogers, AK) were homogenized in 39 volumes of ice-cold 50 mM Tris buffer (pH 7.4 at 4 °C) containing 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, and 1 mM $MgCl_2$, and centrifuged at 37,000*g* for 10 min at 4 °C. The supernatant was discarded, the pellet resuspended in the original volume of buffer, and the wash procedure repeated twice more. After the last centrifugation, the pellet was resuspended in 1/10 its original homogenization volume and stored at −80 °C until needed. In a final volume of 0.5 mL, each assay tube contained 3 mg wet weight of male rat cerebral cortex homogenate (added last), 0.5 nM [^{3}H]epibatidine (NEN Life Science Products, Wilmington, DE), and one of 10–12 different concentrations of test compound dissolved in buffer (pH 7.4 at room temperature) containing 10% DMSO resulting in a final DMSO concentration of 1%. Total and nonspecific bindings were determined in the presence of vehicle and 300 μM (−)-nicotine, respectively. After 4-h incubation at room temperature, the samples were vacuum-filtered over GF/B filter papers presoaked in 0.03% polyethylenimine using a Brandel 48-well harvester and washed with 6 mL of ice-cold buffer. The amount of radioactivity trapped on the filter was determined by standard liquid scintillation techniques in a TriCarb 2200 scintillation counter (Packard Instruments, Meriden, CT) at approximately 50% efficiency.

4.18. Data handling

The specific binding data were fit using the nonlinear regression equations in Prism (GraphPad Prism v. 3.0; GraphPad Software, San Diego, CA). The Cheng–Prusoff equation[36] [$K_i = IC_{50}/(1 + ([L]/K_d)$] was used to calculate the K_i from the IC_{50}. The data are reported as means ± SEM from at least three independent experiments. K_d values for [^{125}I]iodo-MLA and [^{3}H]epibatidine were 1.8 and 0.02 nM, respectively. These K_d values were determined under conditions identical to their respective assays.

4.19. Tail-flick Test

Antinociception was assessed by the tail-flick method of D'Amour and Smith.[37] A control response (2–4 s) was determined for each mouse before treatment, and a test latency was determined after drug administration. In order to minimize tissue damage, a maximum latency of 10 s was imposed. Antinociceptive response was calculated as percent maximum possible effect (% MPE), where $\%MPE = [(test - control)/(10 - control)] \times 100$. Groups of 8–12 animals were used for each dose and for each treatment. The mice were tested 5 min after it injections of epibatidine analogs for the dose–response evaluation. Eight to 12 mice were treated per dose, and a minimum of four doses were performed for dose–response curve determination. Antagonism studies were carried out by pretreating the mice sc with either saline MLA (**1a**) or MLA analogs **1b–f** at different times before nicotine. The animals were tested 5 min after administration of nicotine.

4.20. Hot-plate test

Mice were placed into a 10-cm wide glass cylinder on a hot plate (Thermojust Apparatus) maintained at 55.0 °C. Two control latencies at least 10 min apart were determined for each mouse. The normal latency (reaction time) was 8–12 s. Antinociceptive response was calculated as percent maximum possible effect (% MPE), where $\%MPE = [(test - control)/40 - control) \times 100]$. The reaction time was scored when the animal jumped or licked its paws. Eight mice per dose were injected sc with epibatidine analogs and tested 5 min thereafter in order to establish a dose–response curve.

4.21. Nicotine-induced seizures

Male ICR (20–25 g) obtained from Harlan Laboratories (Indianapolis, IN) were used throughout the study. Following injection of nicotine, each animal was placed in a $30 \times 30\ cm^2$ Plexiglas cage and observed for 3 min. Whether a clonic seizure occurred within a 3-min time period was noted for each animal after sc administration of nicotine. This time was chosen because seizures occur very quickly after nicotine administration. Antagonism studies were carried out by pretreating the mice sc with either saline or different MLA analogs 10 min before nicotine. The percentage of animals exhibiting a seizure was calculated and dose–response curves were constructed and AD_{50} value determined for each of the MLA analogs.

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