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Synthesis and evaluation of a series of homologues of lobelane at the vesicular monoamine transporter-2

Guangrong Zheng, Linda P. Dwoskin, Agripina G. Deaciuc, Peter A. Crooks *

Department of Pharmaceutical Sciences, College of Pharmacy, University of Kentucky, Rose Street, Lexington, KY 40536-0082, USA

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ABSTRACT

A series of lobelane homologues has been synthesized and evaluated for their [^{3}H]DTBZ binding affinity at the vesicular monoamine transporter-2 (VMAT2). The structure–activity relationships (SAR) indicate that for retention of binding affinity at VMAT2, the lengths of the methylene linkers should be no shorter than one methylene unit at C-6 of the piperidine ring, and no shorter than two methylene units at C-2 of the piperidine ring. These results indicate that the intramolecular distances between the piperidine ring and two phenyl rings in lobelane analogues are an important criterion for retention of high affinity at VMAT2.

The brain vesicular monoamine transporter-2 (VMAT2) is believed to play an important role in mediating the neurochemical and behavioral effects of psychostimulants.[1] And this transporter is currently recognized as a valid target for the development of potential treatments for methamphetamine abuse.[2] (−)-Lobeline (**1**, Fig. 1), the major alkaloid of *Lobelia inflata*, has been shown to inhibit dopamine uptake into synaptic vesicles via an interaction with the tetrabenazine (TBZ) binding site on VMAT2.[3] The *des*-oxygen lobeline analogue, lobelane (**2b**, Fig. 1), has increased potency and selectivity for interaction with the TBZ binding site on VMAT2 compared to lobeline.[4] Moreover, lobelane dose dependently decreases methamphetamine self-administration without altering sucrose-maintained responding, which is more specific compared to lobeline, suggesting that it has potential as a novel treatment for methamphetamine abuse.[5]

Extensive structure–activity relationship (SAR) studies on lobelane have been carried out, including structural modifications of both the piperidine ring and two phenyl rings; these have included changing the chirality of piperidinyl C-2 and C-6 stereo centers, and altering the positions of the two phenethyl substituents on the piperidine ring.[4,6] As part of a continuing effort to explore the SAR for lobelane analogues, the goal of the present investigation was to determine the optimal methylene linker lengths between the piperidine ring and each of the two phenyl rings in the lobelane molecule. Thus, we herein investigate a series of lobelane homologues, in which the two ethylene linkers connecting each of the two phenyl rings to the C-6 and C-2 positions of the piperidine ring have been replaced by a range of methylene linkers of different length (0–3 carbons), that is, combinations of 0,0-, 0,1-, 0,2-, 0,3-, 1,1-, 1,2-, 1,3-, 2,3-, and 3,3-carbons at C-6 and C-2 of the piperidine ring, as new ligands for VMAT2 (Fig. 1 and Table 1).

The syntheses of the lobelane homologues are outlined in Schemes 1–3. Compound **3**, which is devoid of methylene units between the central piperidine ring and two phenyl rings, was synthesized by initial *N*-methylation of 2,6-biphenylpyridine (**12**) with methyl *p*-tosylate, and reduction of the resulting quaternary ammonium salt (**13**) with $NaBH_4$, followed by catalytic hydrogenation over Pd-C [Scheme 1(a)]. Analogues in which two phenyl rings are linked to the piperidine ring by one (**4a** and **4b**) or three methylene units (**5a** and **5b**), were synthesized as illustrated in Scheme 1(b). Kumada coupling[7] of 2,6-dibromopyridine (**14**) with the corresponding benzylmagnesium chloride or 3-phenyl-1-propylmagnesium bromide to afford intermediates **15** and **16**, respectively,

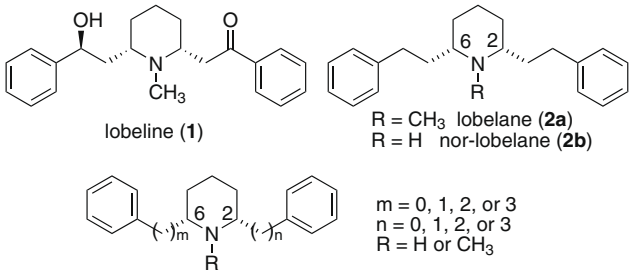

Figure 1. Structures of (−)-lobeline (**1**), nor-lobelane (**2a**), lobelane (**2b**), and lobelane homologues.

* Corresponding author. Tel.: +1 859 257 1718; fax: +1 859 257 7585.
E-mail address: pcrooks@email.uky.edu (P.A. Crooks).

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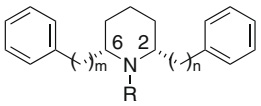
Table 1
Inhibition constants (K_i) for lobelane homologues at the [^{3}H]DTBZ binding site (VMAT2) on rat synaptic vesicle membranes.



Compound	R	m	n	[^{3}H]DTBZ binding K_i, μM, ±SEM[a]
Lobeline (**1**)	—	—	—	2.76 ± 0.64
Nor-lobelane (**2a**)	H	2	2	2.31 ± 0.21
Lobelane (**2b**)	CH_3	2	2	0.97 ± 0.19
3	CH_3	0	0	>100
4a	H	1	1	7.68 ± 1.44
4b	CH_3	1	1	10.5 ± 3.36
5a	H	3	3	4.56 ± 1.03
5b	CH_3	3	3	1.00 ± 0.23
6a	H	0	1	24.1 ± 6.10
6b	CH_3	0	1	13.6 ± 8.14
7a	H	0	2	28.4 ± 7.30
7b	CH_3	0	2	16.1 ± 4.07
8a	H	0	3	28.6 ± 2.44
8b	CH_3	0	3	62.5 ± 16.6
9a	H	1	2	12.2 ± 0.19
9b	CH_3	1	2	2.77 ± 1.77
10a	H	1	3	1.13 ± 0.28
10b	CH_3	1	3	1.62 ± 0.39
11a	H	2	3	4.60 ± 0.56
11b	CH_3	2	3	0.88 ± 0.30

[a] Each K_i value represents data from at least three independent experiments, each performed in duplicate.

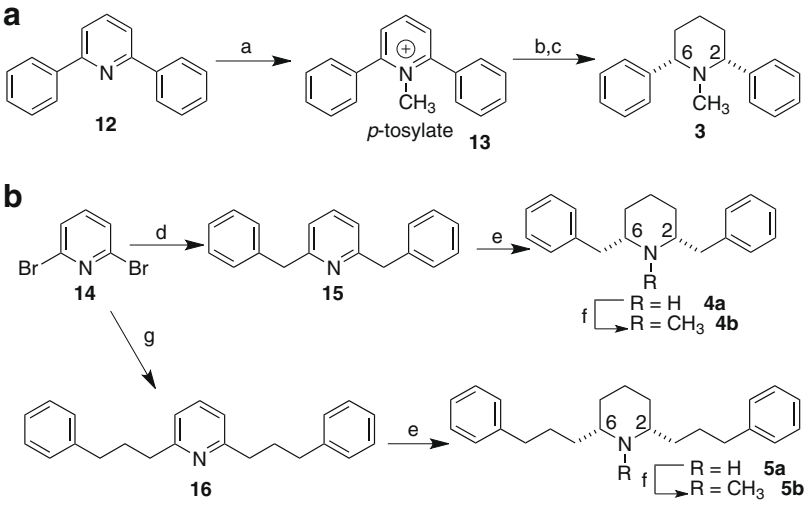
followed by catalytic hydrogenation over Adams catalyst (PtO_2) yielded analogue **4a** or **5a**, respectively. *N*-Methylation of **4a** and **5a** were carried out utilizing $NaBH_3CN/(CH_2O)_n$ to afford analogues **4b** and **5b**, respectively.

Analogue pairs **6a** and **6b, 7a** and **7b, 8a** and **8b**, in which one phenyl ring is attached directly to C-6 of the central piperidine ring, while the other phenyl ring is linked to C-2 by one, two, or three methylene units, were synthesized as illustrated in Scheme 2(a) and 2(b). Kumada coupling of 2-bromo-6-methoxypyridine (**17**) with benzylmagnesium chloride, followed by treatment with $POCl_3$ in DMF afforded 2-chloro-6-benzyl-pyridine (**18**). Suzuki coupling of **18** with phenylboronic acid was achieved using [1,3-bis(2,6-diisopropylphenyl)imidazol-2-ylidene](3-chloropyridyl)-palladium(II) dichloride (PEPPSI-IPr) as a catalyst and KO*t*Bu as a base in *i*-PrOH.[8] The pyridine ring in the resulting coupled product **19** was reduced to the corresponding piperidine ring by catalytic hydrogenation, as described above for the synthesis of compound **4a**, to afford analogue **6a** [Scheme 2(a)]. Compound **21**, the common intermediate for the preparation of analogues **7a** and **8a**, was synthesized by coupling of 2-bromo-6-methylpyridine (**17**) with phenylboronic acid under Suzuki coupling conditions.[9] Condensation of **21** with benzaldehyde in Ac_2O under reflux afforded compound **22**. Compound **21** was also used to synthesize compound **23** via deprotonation of the 2-methyl group followed by an S_N2 reaction with 2-iodoethylbenzene. Catalytic hydrogenation of **22** and **23**, as described above for the synthesis of compound **4a**, afforded compounds **7a** and **8a**, respectively [Scheme 2(b)]. Compounds **6a, 7a**, and **8a**, were converted into **6b, 7b**, and **8b**, respectively, by *N*-methylation, as described in Scheme 1.

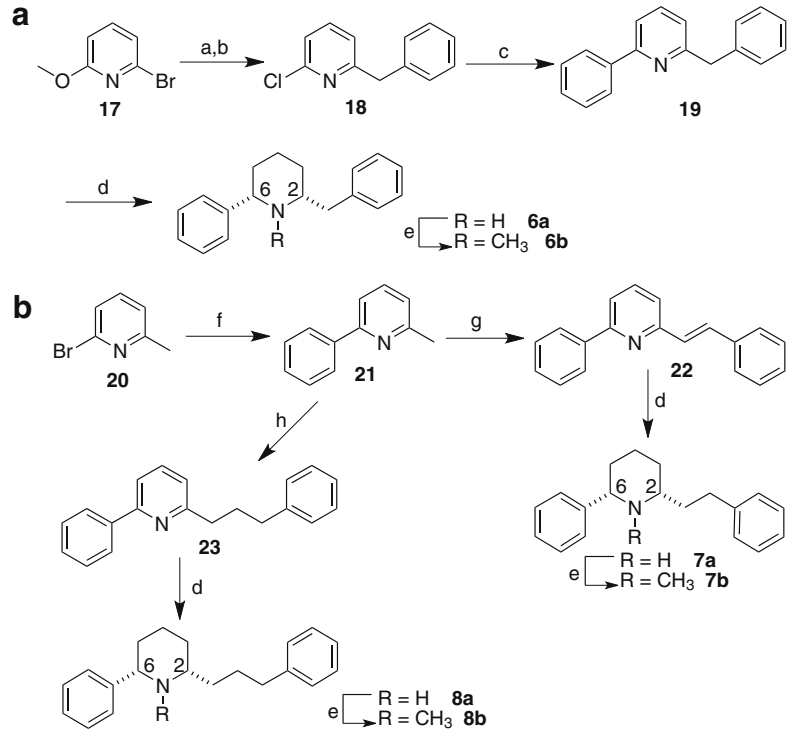
Analogues **9a, 9b, 10a**, and **10b**, which contain a single methylene linker at C-6 and an ethylene or a propylene linker at C-2 of the piperidine ring interconnecting each of the two phenyl rings, were synthesized as illustrated in Scheme 3(a). Compound **18** from Scheme 2(a) was used as a starting material to synthesize these analogues. Negishi coupling of **18** with 2-phenyl-1-ethylzinc iodide or 3-phenyl-1-propylzinc iodide, using PEPPSI-IPr as a catalyst and LiBr as an additive in THF/DMF[10], afforded compound **24** or **25**, respectively. Catalytic hydrogenation of **24** and **25**, as described above for the synthesis of compound **4a**, afforded compounds **9a** and **10a**, respectively, which were converted into **9b** and **10b**, respectively, by *N*-methylation, as described in Scheme 1.

Analogues **11a** and **11b**, in which the two phenyl rings are linked to the central piperidine ring by an ethylene moiety at C-6 and a propylene moiety at C-2, respectively, were synthesized by initial mono-condensation of 2,6-lutidine (**26**) with benzaldehyde, to afford the conjugated product **27**, followed by a similar procedure to that described in Scheme 2(b) for the synthesis of **8a** and **8b** [Scheme 3(b)]. The structures and purities of the analogues were determined by ^{1}H NMR, ^{13}C NMR, GC-MS.[11]

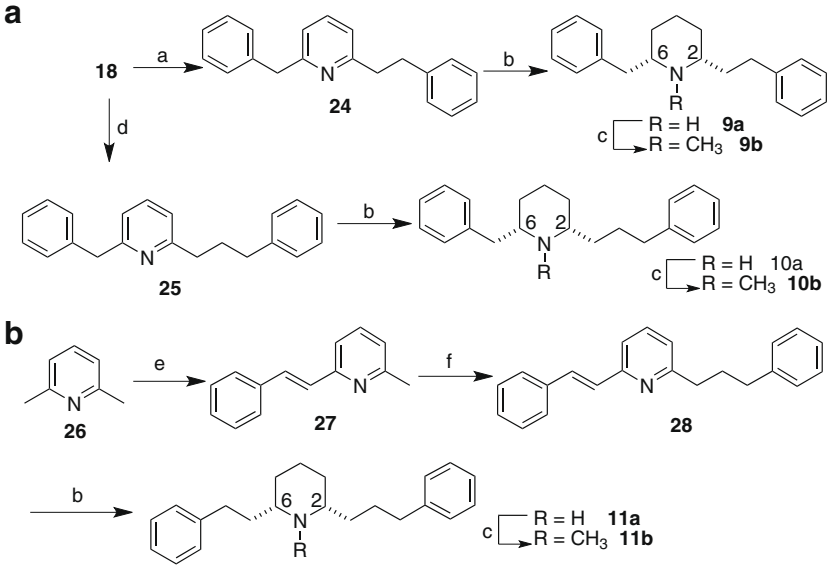
The ability of the above lobelane homologues to interact with VMAT2 was assessed by determining their ability to inhibit [^{3}H]dihydrotetrabenazine ([^{3}H]DTBZ, K_d = 1.67 nM[3b]) binding to rat brain vesicle membranes, utilizing a fast throughput 96-well assay,[12] and the results are summarized in Table 1.



Scheme 1. Reagents and conditions: (a) Methyl *p*-tosylate, 180–200 °C; (b) $NaBH_4$, MeOH, rt; (c) H_2, Pd/C, MeOH, 10 psi, rt; (d) Benzylmagnesium chloride, $NiCl_2$(dppp), THF, rt; (e) H_2, PtO_2, HOAc, 50 psi, rt; (f) $NaBH_3CN$, $(CH_2O)_n$, MeOH, rt; (g) (3-Bromopropyl)benzene, Mg, 1,2-dibromoethane, $NiCl_2$(dppp), THF, rt.



Scheme 2. Reagents and conditions: (a) Benzylmagnesium chloride, $NiCl_2$(dppp), THF, rt; (b) $POCl_3$, DMF, 80 °C; (c) Phenylboronic acid, PEPPSI-IPr, KO-*t*Bu, *i*PrOH; (d) H_2, PtO_2, HOAc, 50 psi, rt; (e) $NaBH_3CN$, $(CH_2O)_n$, MeOH, rt; (f) Phenylboronic acid, $Pd(PPh_3)_4$, K_2CO_3, toluene; (g) Benzaldehyde, Ac_2O, reflux; (h) i—LDA, THF, −78 °C; ii—(2-Iodoethyl)benzene, THF, −78 °C–rt.



Scheme 3. Reagents and conditions: (a) Phenethylmagnesium chloride, Zn, I_2, PEPPSI-IPr, LiBr, THF/DMF; (b) H_2, PtO_2, HOAc, 50 psi, rt; (c) $NaBH_3CN$, $(CH_2O)_n$, MeOH, rt; (d) (3-bromopropyl)benzene, Mg, 1,2-dibromoethane, Zn, I_2, PEPPSI-IPr, LiBr, THF/DMF; (e) Benzaldehyde, Ac_2O, reflux; (f) i—LDA, THF, −78 °C; ii—(2-iodoethyl)benzene, THF, −78 °C–rt.

The *cis*-lobelane/nor-lobelane homologues **3, 4a, 4b, 5a**, and **5b** are structurally symmetrical and isomerically pure *meso*-homologues. The removal of the ethylene linker on both sides of the piperidine ring of lobelane to afford homologue **3** resulted in a complete loss of affinity for the DTBZ binding site on VMAT2. Removal of one methylene unit on each side of the piperidine ring of lobelane, to afford homologue **4b**, also had a detrimental effect on VMAT2 affinity, resulting in a 10-fold loss compared to lobelane. On the other hand, adding one methylene unit to each side of the piperidine ring of lobelane to produce homologue **5b** (K_i = 1.00 μM) resulted in a retention of affinity at VMAT2.

The non-symmetrical racemic homologues **6b, 7b**, and **8b**, in which one phenyl ring is directly attached to the piperidine ring at C-6, while the second phenyl ring is connected to the piperidine ring at C-2 via a one-, two, or three methylene linker unit, respectively, exhibited 14–64 fold less affinity at VMAT2 compared to lobelane. Increasing the length of the C-2 methylene linker in **4b** by one or two additional methylene units to afford racemic homo-

logues **9b** and **10b** (K_i = 2.77 and 1.62 μM, respectively), respectively, resulted in increased affinity at VMAT2 compared to **4b**; these homologues were only 2–3 times less potent than lobelane. Interestingly, the addition of an additional methylene unit to the C-2 ethylene linkers in the lobelane molecule, to produce racemic homologue **11b**, had no significant effect on affinity at VMAT2 compared to lobelane. In fact, **11b** (K_i = 0.88 μM) was slightly more potent than lobelane (K_i = 0.97 μM). The nor analogues **6a–11a** exhibited affinities at VMAT2 which were within an order of magnitude difference compared to their corresponding *N*-methylated analogues **6b–11b**, which is consistent with previous data from the lobelane analogue series.[6a]

The results of this study indicate that for retention of binding affinity at VMAT2, structurally related lobelane homologues should contain a C-6 methylene linker that is no shorter than one methylene unit, and a C-2 methylene linker that is no shorter than two methylene units between the piperidine ring and the phenyl ring. The optimal methylene linker lengths appear to be a combination of a two methylene unit linker at C-6 and a three methylene unit linker at C-2.

In conclusion, a series of lobelane homologues has been synthesized. These results indicate that the intramolecular distances between the piperidine ring and two phenyl rings in lobelane analogues are important for retention of high potency at VMAT2.

Acknowledgment

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Selected spectra data: compound **3**, ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.42–1.86 (m, 6H), 1.79 (s, 3H), 3.05 (dd, J = 10.5, 2.7 Hz, 2H), 7.15–7.50 (m, 10H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 25.43, 37.19, 42.47, 71.05, 126.80, 127.43, 128.45, 146.24 ppm; MS *m/z* 251 (M^+).Compound **4a**, ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.10–1.35 (m, 3H), 1.53–1.82 (m, 4H), 2.50–2.70 (m, 6H), 7.05–7.25 (m, 10H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 24.82, 32.52, 43.78, 58.48, 126.05, 128.27, 129.03, 138.89 ppm; MS *m/z* 264 $(M-1)^+$.Compound **4b**, ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.08 (m, 1H), 1.17–1.43 (m, 4H), 1.58 (m, 1H), 2.50 (s, 3H), 2.35–2.58 (m, 4H), 3.21 (m, 2H), 7.10–7.35 (m, 10H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 24.34, 27.83, 35.06, 41.73, 65.70, 126.04, 128.33, 129.52, 140.27 ppm; MS *m/z* 278 $(M-1)^+$.Compound **5a**, ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.85–1.07 (m, 2H), 1.20–1.46 (m, 1H), 1.50–1.80 (m, 7H), 2.37–2.52 (m, 2H), 2.60 (t, J = 7.5 Hz, 4H), 7.10–7.32 (m, 10H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 25.04, 28.22, 32.83, 36.36, 37.34, 57.20, 125.77, 128.33, 128.47, 142.49 ppm; MS *m/z* 321 $(M)^+$.Compound **5b**, ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.20–1.78 (m, 14H), 2.12 (s, 3H), 2.22–2.38 (m, 2H), 2.50–2.68 (m, 4H), 7.05–7.32 (m, 10H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 24.97, 27.48, 28.48, 32.05, 34.22, 36.41, 63.78, 125.77, 128.35, 128.48, 142.58 ppm; MS *m/z* 335 $(M-1)^+$.Compound **6a**, ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.28 (m, 1H), 1.46 (m, 2H), 1.60–1.95 (m, 4H), 2.66 (ddd, J = 13.2, 8.4, 1.8 Hz, 1H), 2.78 (ddd, J = 13.2, 8.4, 1.8 Hz, 1H), 2.89 (m, 1H), 3.55 (brd, J = 10.2 Hz, 1H), 7.12–7.37 (m, 10H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 25.55, 32.40, 35.23, 44.09, 59.20, 62.45, 126.28, 126.29, 127.00, 128.38, 128.51, 129.30, 129.27, 145.57 ppm; MS *m/z* 250 $(M-1)^+$.Compound **6b**, ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.86 (m, 1H), 1.18–1.40 (m, 2H), 1.45–1.78 (m, 3H), 2.18 (s, 3H), 2.25 (m, 1H), 2.53 (dd, J = 13.2, 9.3 Hz, 1H), 2.97 (dd, J = 11.1, 2.4 Hz, 1H), 3.27 (dd, J = 13.2, 3.6 Hz, 1H), 7.15–7.42 (m, 10H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 24.91, 31.41, 36.59, 41.09, 41.15, 66.49, 71.52, 125.96, 126.83, 127.51, 128.24, 128.48, 129.74, 140.74, 146.16 ppm; MS *m/z* 264 $(M-1)^+$. Compound **7a**, ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.13–1.27 (m, 2H), 1.39–1.58 (m, 2H), 1.64–1.94 (m, 5H), 2.60–2.75 (m, 5H), 3.61 (dd, J = 10.5, 2.4 Hz, 1H), 7.13–7.40 (m, 10H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 25.57, 32.25, 32.54, 34.89, 39.23, 57.47, 62.55, 125.83, 126.83, 127.10, 128.41, 128.44, 142.36, 145.55 ppm; MS *m/z* 265 (M^+).Compound **7b**, ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.35–1.90 (m, 6H), 1.98 (m, 1H), 2.02 (s, 3H), 2.13 (m, 1H), 2.59 (m, 1H), 2.80 (m, 1H), 2.97 (dd, J = 9.9, 4.5 Hz, 1H), 7.12–7.38 (m, 10H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 25.04, 31.16, 31.87, 35.59, 35.85, 40.02, 64.70, 71.46, 125.80, 127.06, 127.57, 128.41, 128.53, 142.68, 145.04 ppm; MS *m/z* 279 (M^+).Compound **8a**, ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.15 (m, 1H), 1.36–1.57 (m, 4H), 1.60–1.94 (m, 6H), 2.55–2.70 (m, 5H), 3.61 (dd, J = 10.8, 2.4 Hz, 1H), 7.10–7.40 (m, 10H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 25.61, 28.24, 32.28, 34.86, 36.38, 37.23, 58.00, 62.74, 125.81, 126.89, 127.13, 128.38, 128.45, 128.50, 142.52, 145.49 ppm; MS *m/z* 278 $(M-1)^+$.Compound **8b**, ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.23–1.87 (m, 9H), 1.93 (s, 3H), 2.02 (m, 1H), 2.51–2.66 (m, 4H), 2.90 (dd, J = 10.5, 2.4 Hz, 1H), 7.10–7.38 (m, 10H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 25.16, 26.98, 31.52, 34.04, 36.56, 36.70, 40.39, 64.41, 71.20, 125.70, 126.66, 127.42, 128.30, 128.35, 128.45, 142.68, 146.19 ppm; MS *m/z* 293 (M^+). Compound **9a**, ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.00–1.38 (m, 4H), 1.50–1.82 (m, 5H), 2.28–2.77 (m, 6H), 6.92–7.35 (m, 10H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 24.88, 32.16, 32.46, 32.81, 38.75, 43.82, 56.21, 58.63, 125.70, 126.31, 128.26, 128.32, 128.48, 129.24, 139.21, 141.83 ppm; MS *m/z* 278 $(M-1)^+$.Compound **9b**, ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.14–1.56 (m, 5H), 1.60–1.78 (m, 2H), 1.92 (m, 1H), 2.32 (s, 3H), 2.30–2.74 (m, 5H), 3.13 (dd, J = 12.0, 2.7 Hz, 1H), 7.15–7.35 (m, 10H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 24.76, 26.93, 27.56, 32.48, 32.87, 36.59, 41.41, 63.05, 65.39, 125.72, 125.95, 128.23, 128.35, 128.47, 129.44, 140.24, 142.64 ppm; MS *m/z* 292 $(M-1)^+$.Compound **10a**, ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.96–1.82 (m, 11H), 2.34–2.76 (m, 6H), 7.03–7.35 (m, 10H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 24.96, 28.01, 32.48, 32.78, 36.18, 37.02, 43.96, 57.08, 58.75, 62.45, 125.75, 126.30, 128.32, 128.42, 128.51, 129.29, 139.25, 142.44 ppm; MS *m/z* 292 $(M-1)^+$.Compound **10b**, ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.13–1.82 (m, 10H), 2.45 (s, 3H), 2.40–2.80 (m, 4H), 2.90 (m, 1H), 3.24 (dd, J = 12.6, 3.0 Hz, 1H), 7.10–7.35 (m, 10H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 23.59, 25.10, 25.99, 28.04, 30.67, 33.02, 35.94, 39.83, 64.43, 66.05, 125.90, 126.65, 128.39, 128.58, 129.44, 137.78, 141.77 ppm; MS *m/z* 306 $(M-1)^+$.Compound **11a**, ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.00–1.20 (m, 2H), 1.28 (m, 1H), 1.40–1.83 (m, 9H), 2.44–2.68 (m, 6H), 7.10–7.30 (m, 10H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 24.84, 28.20, 32.42, 32.63, 36.30, 36.94, 38.76, 57.08, 57.32, 125.81, 125.90, 128.36, 128.41, 128.47, 142.07, 142.45 ppm; MS *m/z* 306 $(M-1)^+$.Compound **11b**, ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.22–1.50 (m, 6H), 1.55–1.78 (m, 5H), 1.87 (m, 1H), 2.13 (s, 3H), 2.35 (m, 1H), 2.54–2.70 (m, 3H), 7.14–7.32 (m, 10H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 25.19, 27.38, 28.38, 31.52, 32.54, 34.38, 36.47, 63.03, 63.49, 125.75, 128.36, 128.39, 128.51, 142.73 ppm; MS *m/z* 321 (M^+).
12. *Preparation of rat brain synaptic vesicles.* Fresh whole brain (excluding cerebellum and brain stem) was homogenized in 20 vol of ice-cold 0.32 M sucrose using a glass homogenizer (7 strokes of a Teflon pestle, clearance = 0.003 in). Homogenates were centrifuged at 1000*g* for 12 min at 4 °C. Resulting supernatants (S1) were centrifuged at 22,000*g* for 10 min. Resulting pellets (P2), containing the synaptosomes, were resuspended in 18 mL ice-cold Milli-Q water for 5 min with 7 strokes of the Teflon pestle homogenizer. Osmolarity was restored by immediate addition of 2 mL of 25 mM HEPES and 100 mM K_2-tartrate buffer (pH 7.5). Samples were centrifuged at 20,000*g* for 20 min. $MgSO_4$ (final concentration, 1 mM) was added to the resulting supernatants (S3). Final centrifugations were performed at 100,000*g* for 45 min. Pellets (P4) were resuspended immediately in ice-cold buffer (see below) providing ~15 μg protein/100 μL. *[^{3}H]DTBZ binding assay.* One hundred microliters of vesicle suspension was incubated in assay buffer (in mM: 25 HEPES, 100 K_2-tartrate, 5 $MgSO_4$, 0.1 EDTA and 0.05 EGTA, pH 7.5, 25 °C) in the presence of 5 nM [^{3}H]DTBZ and 1 nM–1 mM lobelane analogues (final concentrations) for 30 min at room temperature. Nonspecific binding was determined in the presence of 10 μM Ro4-12084. Assays were performed in duplicate using Unifilter−96 96-well GF/B filter plates (presoaked in 0.5% polyethylenimine) and terminated by harvesting using a FilterMate harvester. After washing 5 times with 350 μL of the ice-cold wash buffer (in mM: 25 HEPES, 100 K_2-tartrate, 5 $MgSO_4$ and 10 NaCl, pH 7.5), filter plates were dried, bottom-sealed and each well filled with 40 μL Packard's MicroScint 20 cocktail. Bound [^{3}H]DTBZ was measured using a Packard TopCount NXT scintillation counter and a Packard Windows NT-based operating system.