Vesamicol Receptor Mapping of Brain Cholinergic Neurons with Radioiodine-Labeled Positional Isomers of Benzovesamicol[1]

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Alzheimer's disease is characterized by progressive cerebral cholinergic neuronal degeneration. Radiotracer analogs of benzovesamicol, which bind with high affinity to the vesamicol receptor located on the uptake transporter of acetylcholine storage vesicles, may provide an in vivo marker of cholinergic neuronal integrity. Five positional isomers of racemic iodobenzovesamicol (4′-, 5-, 6-, 7-, and 8-IBVM) were synthesized, exchange-labeled with iodine-125, and evaluated as possible in vivo markers for central cholinergic neurons. Only two isomers, 5-IBVM (**5**) and 6-IBVM (**10**), gave distribution patterns in mouse brain consistent with cholinergic innervation: striatum ≫ hippocampus ≥ cortex > hypothalamus ≫ cerebellum. The 24-h tissue-to-cerebellum concentration ratios for 5-IBVM (**5**) were 3−4-fold higher for striatum, cortex, and hippocampus than the respective ratios for 6-IBVM (**10**). Neither 8-IBVM (**16**) nor 4′-IBVM (**17**) exhibited selective retention in any of the brain regions examined. In the heart, only 5-IBVM (**5**) exhibited an atria-to-ventricles concentration ratio consistent with high peripheral cholinergic neuronal selectivity. The 7-IBVM (**14**) isomer exhibited an anomalous brain distribution pattern, marked by high and prolonged retention in the five brain regions, most notably the cerebellum. This isomer was screened for binding in a series of 26 different biological assays; 7-IBVM (**14**) exhibited affinity only for the σ-receptor with an IC_{50} of ∼30 nM. Drug-blocking studies suggested that brain retention of 7-IBVM (**14**) reflects high-affinity binding to both vesamicol and σ-receptors. Competitive binding studies using rat cortical homogenates gave IC_{50} values for binding to the vesamicol receptor of 2.5 nM for 5-IBVM (**5**), 4.8 nM for 6-IBVM (**10**), and 3.5 nM for 7-IBVM (**14**). Ex vivo autoradiography of rat brain after injection of (−)-5-[^{125}I]IBVM ((−)-[^{125}I]**5**) clearly delineated small cholinergic-rich areas such as basolateral amygdala, interpeduncular nucleus, and facial nuclei. Except for cortex, regional brain levels of (−)-5-[^{123}I]IBVM ((−)-[^{123}I]**5**) at 4 h exhibited a linear correlation (r^2 = 0.99) with endogenous levels of choline acetyltransferase. Conclusion: Vesamicol receptor mapping of cholinergic nerve terminals in murine brain can be achieved with 5-IBVM (**5**) and less robustly with 6-IBVM (**10**), whereas the brain localization of 7-IBVM (**14**) reflects high-affinity binding to both vesamicol and σ-receptors.

Introduction

Benzovesamicols have been shown to bind in vitro with high affinity to a site on the acetylcholine transporter complex[2] in synaptic vesicle preparations isolated from the marine ray Torpedo[3] and rat brain.[4] The binding is highly enantioselective and noncompetitive with acetylcholine, indicating that the vesamicol receptor allosterically modulates the transporter. The acetylcholine transporter from Torpedo electric organ has been recently cloned, expressed in fibroblasts, and shown to harbor vesamicol receptors.[5]

Over the past decade Parsons and co-workers have utilized vesamicol as a pharmacological tool to study cholinergic nerve terminal function.[6] This group has shown that vesamicol also binds to a second site in rat brain called the vesamicol-binding protein.[7] Although vesamicol has a lower affinity for this second site, the higher density of this site, compared to the vesamicol receptor, might complicate efforts to quantitatively map the vesamicol receptor in vivo. However, certain derivatives of vesamicol including 5-substituted benzovesamicols not only bind with higher affinity to the vesamicol receptor than vesamicol itself but show little affinity for the vesamicol-binding protein.[8]

A number of benzovesamicol derivatives have been labeled with positron-emitting isotopes for use in tomographic imaging of cholinergic nerve density in brain.[9] In a previous communication, we reported that (−)-5-[^{125}I]IBVM is a highly specific in vivo marker for cholinergic nerve fields.[10] Selective localization of (−)-5-[^{125}I]IBVM in cholinergic-rich regions of the mouse brain was avid, prolonged, highly stereoselective, and partially blocked by vesamicol. On the basis of these findings, Kuhl and co-workers have used (−)-5-[^{123}I]-IBVM to obtain tomographic measures of cholinergic nerve terminal density in brain of normal volunteers[11] and patients with Alzheimer's disease and Parkinson's disease.[12]

Using an in vitro acetylcholine active-transport assay to determine relative activity, Rogers et al. evaluated over 80 structural variants of vesamicol and found that bulky substituents were tolerated in the 5-position but not the 8-position of benzovesamicol (see Figure 1); 6- and 7-substituted benzovesamicols were not evaluated

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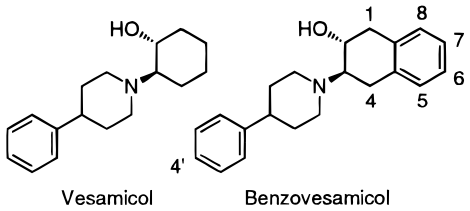


Figure 1. Structures of vesamicol and benzovesamicol showing their *trans*-2*R*,3*R*-configuration. The numbering system is the same as that used previously.[10,13] A different numbering system has been employed by others in a recent report.[8]

in this study.[13] We sought to expand our initial study[10] with 5-IBVM (**5**) by investigating the effect of systematically altering the aromatic ring position of iodine on the in vivo cholinergic nerve-mapping characteristics of IBVM. We report here the synthesis of four new radioiodinated isomers of IBVM and a comparison of their binding and biodistribution behavior with that of 5-IBVM (**5**) in rodents. This study found that vesamicol receptor mapping of cholinergic nerve terminals in murine brain can be achieved with 5-IBVM (**5**) and less robustly with 6-IBVM (**10**). The brain localization of 7-IBVM (**14**), however, reflects high-affinity binding to both vesamicol and σ-receptors.

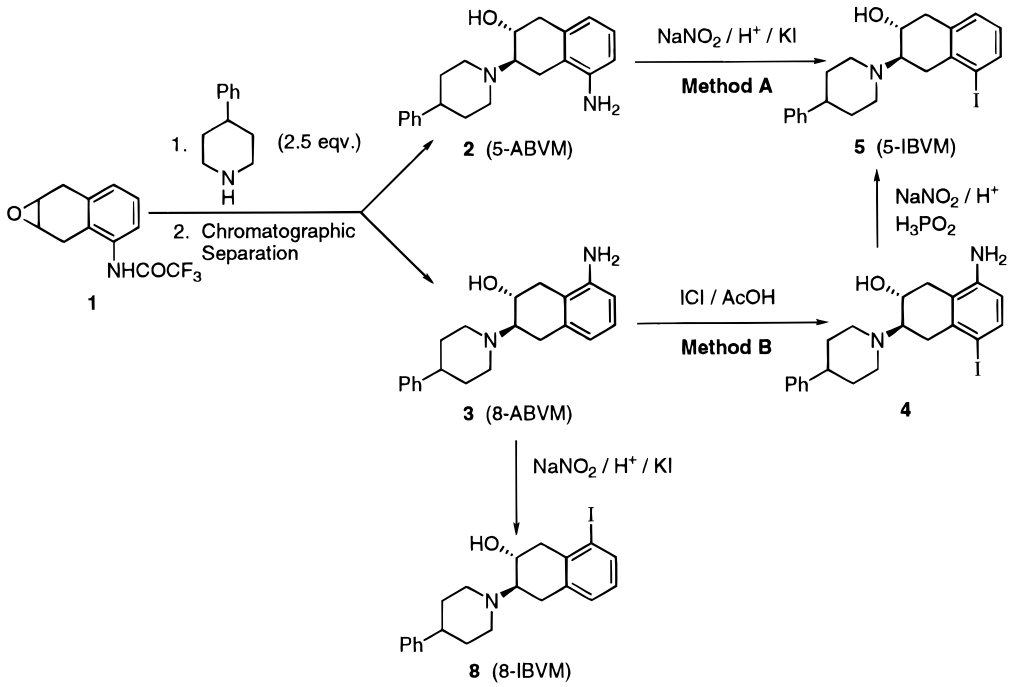
Results

Chemistry. The synthesis of racemic 5-IBVM (**5**)[10] was accomplished by two routes as shown in Scheme 1. Method A is a one-step diazotization of the previously described 5-aminobenzovesamicol (5-ABVM, **2**)[13] in the presence of iodide. Method B is a two-step sequence in which 8-aminobenzovesamicol (8-ABVM, **3**) was regioselectively iodinated in the 5-position using iodine monochloride followed by reductive removal of the 8-amino group with hypophosphorus acid. Method B was developed not because of difficulties with method A but because of the availability of 8-ABVM (**3**) as a major byproduct in the synthesis of 5-ABVM (**5**). The chiral resolution of racemic 5-IBVM (**5**) was achieved by use of (−)-(*S*)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (MTPA) to form the diastereomeric MTPA esters.[14] The (+)- and (−)-diastereomers were readily separated by preparative TLC on silica gel after several developments with hexane/EtOAc (19/1).[10] Base hydrolysis of the MTPA esters provided the enantiomers of 5-IBVM ((+)-**5**, (−)-**5**) with optical purities greater than 98% as determined by chiral HPLC using a Chiracel OD column.[15]

The 8-IBVM (**6**) isomer was prepared from 8-ABVM (**3**) by the diazotization route described in the synthesis of 5-IBVM (**5**). Synthesis of 4′-IBVM (**17**) was achieved by reacting 6,7-epoxy-1-(trifluoroacetamido)-5,8-dihydronaphthalene (**1**) with 4-(4′-iodophenyl)piperidine[16] followed by removal of the amino group of the resulting product through the diazonium salt as was done for 5-IBVM (**5**). Selective functionalization of the 6- and 7-positions of benzovesamicol, however, posed some difficulty. The key step was the regioselective *ortho*-nitration of 5- and 8-acetamidobenzovesamicols using fuming nitric acid in acetic anhydride (Scheme 2). The *ortho*-directing ability of the acetamido group has been demonstrated in other aromatic systems.[17] The desired 6-IBVM (**10**) was prepared from 5-amino-6-nitrobenzovesamicol (**7**) by reductive deamination and subsequent reduction of the nitro group to give 6-ABVM (**9**) followed by diazotization and reaction with potassium iodide. The 7-IBVM (**14**) isomer was synthesized in similar fashion starting from 8-ABVM (**3**).

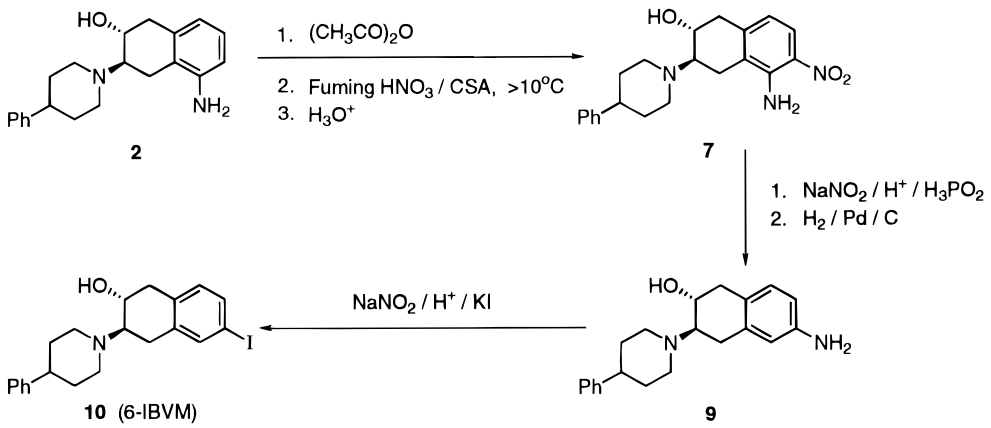
Iodine-125-labeled isomers were synthesized by solid-state exchange labeling of the respective racemic iodo compounds with sodium [^{125}I]iodide. The exchange reaction, which is a refinement of a method first reported by this laboratory in 1982, is conducted under acidic conditions at 140−160 °C and likely involves an electrophilic iodine species.[18] Radiochemical yields ranged from 65% to 89%. Purification was readily

Scheme 1. Synthesis of Racemic 5-IBVM (**5**) and 8-IBVM (**6**)[a]



[a] The alternate synthesis of **5** (method B) was also employed due to availability of 8-aminobenzovesamicol (**3**) as a major byproduct in the synthesis of 5-aminobenzovesamicol (**2**).

Scheme 2. Synthesis of 6-IBVM (**10**)[a]



[a] The 7-IBVM (**14**) isomer was synthesized from 8-ABVM (**3**) using the same strategy.

Table 1. Tissue Distribution of [^{125}I]Iodobenzovesamicols in Mice[a,b]

	5-IBVM (**5**)	6-IBVM (**10**)	7-IBVM (**14**)	8-IBVM (**6**)	4′-IBVM (**17**)
		0.5 h			
cortex	2.29 ± 0.08	2.99 ± 0.32	2.32 ± 0.24	1.53 ± 0.18	3.01 ± 0.29
striatum	3.81 ± 0.73	3.90 ± 0.52	2.34 ± 0.33	1.64 ± 0.22	2.68 ± 0.17
hippocampus	2.09 ± 0.34	2.73 ± 0.34	1.99 ± 0.41		2.91 ± 0.28
hypothal	1.69 ± 0.22	2.51 ± 0.27	2.16 ± 0.33		2.73 ± 0.35
cerebellum	1.28 ± 0.06	2.05 ± 0.24	2.06 ± 0.32	1.22 ± 0.15	2.33 ± 0.26
liver	10.98 ± 1.90	18.07 ± 1.47	19.30 ± 5.72		15.44 ± 1.53
lung	7.57 ± 0.78	3.93 ± 0.23	13.11 ± 5.01		8.86 ± 1.07
atria	1.73 ± 0.41	2.09 ± 0.22	4.37 ± 0.44	1.64 ± 0.73	3.03 ± 0.32
ventricles	1.27 ± 0.44	1.34 ± 0.11	3.29 ± 0.55	1.25 ± 0.10	2.90 ± 0.58
blood	0.82 ± 0.42	1.06 ± 0.07	1.13 ± 0.12	1.00 ± 0.10	1.85 ± 0.15
		4 h			
cortex	1.17 ± 0.20	1.28 ± 0.13	1.62 ± 0.21	0.10 ± 0.02	0.32 ± 0.03
striatum	3.30 ± 0.52	3.07 ± 0.24	1.96 ± 0.36	0.12 ± 0.02	0.33 ± 0.03
hippocampus	1.06 ± 0.19	1.03 ± 0.10	1.27 ± 0.18		0.31 ± 0.03
hypothal	0.61 ± 0.08	0.68 ± 0.06	1.42 ± 0.19		0.28 ± 0.03
cerebellum	0.21 ± 0.04	0.29 ± 0.03	1.23 ± 0.13	0.08 ± 0.02	0.28 ± 0.03
liver	5.92 ± 2.04	6.33 ± 0.50	12.69 ± 1.20		9.12 ± 0.23
lung	1.11 ± 0.90	0.89 ± 0.07	5.86 ± 1.10		2.95 ± 0.59
atria	1.43 ± 0.50	0.97 ± 0.18	2.45 ± 0.66	0.34 ± 0.10	1.35 ± 0.21
ventricles	0.31 ± 0.02	0.53 ± 0.11	1.77 ± 0.32	0.23 ± 0.04	1.88 ± 0.46
blood	0.53 ± 0.08	0.51 ± 0.08	0.47 ± 0.04	0.19 ± 0.03	0.66 ± 0.07
		24 h			
cortex	0.88 ± 0.15	0.22 ± 0.06	1.74 ± 0.14		
striatum	2.20 ± 0.26	0.76 ± 0.15	2.32 ± 0.17		
hippocampus	0.71 ± 0.12	0.16 ± 0.03	1.48 ± 0.12		
hypothal	0.42 ± 0.04	0.10 ± 0.01	1.35 ± 0.18		
cerebellum	0.05 ± 0.01	0.05 ± 0.01	1.06 ± 0.09		
liver	0.89 ± 0.06	0.88 ± 0.08	6.48 ± 0.57		
lung	0.09 ± 0.01	0.10 ± 0.01	1.74 ± 0.20		
atria	0.82 ± 0.38	0.10 ± 0.02	0.77 ± 0.13		
ventricles	0.06 ± 0.01	0.10 ± 0.04	0.27 ± 0.02		
blood	0.04 ± 0.01	0.05 ± 0.004	0.09 ± 0.01		

[a] Tissue radioactivity concentration expressed as % dose/g normalized to a standard 25-g weight (±SD). [b] $N = 5$/time interval for 5-, 6-, and 8-IBVM (**5**, **10**, **6**); $N = 4$ or 5 for 7- and 4′-IBVM (**14**, **17**).

accomplished by silica Sep-Pak chromatography using a hexane/ethyl acetate gradient. We confirmed by HPLC analysis[15] that no ring scrambling of the iodine label occurred during the radioiodide exchange reaction. An alternative radiolabeling approach which provides (−)-5-[^{123}I]IBVM ((−)-[^{123}I]**5**) with specific activities > 20 000 Ci/mmol has been developed for use in clinical studies.[19] However, the solid-state exchange method utilized in this study gave specific activities sufficient (50−200 Ci/mmol) for use in animal studies.[10]

Tissue Distribution Studies. Radioactivity concentrations obtained in mouse brain and peripheral tissues 0.5, 4, and 24 h after intravenous injection of five radioiodinated benzovesamicol isomers are presented in Table 1. At 0.5 h postinjection, all five tracers showed relatively uniform localization in the brain regions evaluated. However, at 4 h postinjection, as further illustrated in Figure 2, three regional brain distribution patterns became evident: The 5- and 6-isomers **5** and **10** were retained in a pattern highly consistent with cholinergic nerve density in mouse brain, striatum ≫ hippocampus ≥ cortex > hypothalamus ≫ cerebellum. In contrast, the 8- and 4′-isomers **6** and **17** effluxed so rapidly and uniformly from the brain that further studies were not performed. A third, unexpected pattern was observed at 4 h with the 7-isomer **14** which was strongly retained in all five brain regions, most notably the cerebellum which is known to have sparse cholinergic innervation.

At 24 h postinjection, the regional brain patterns

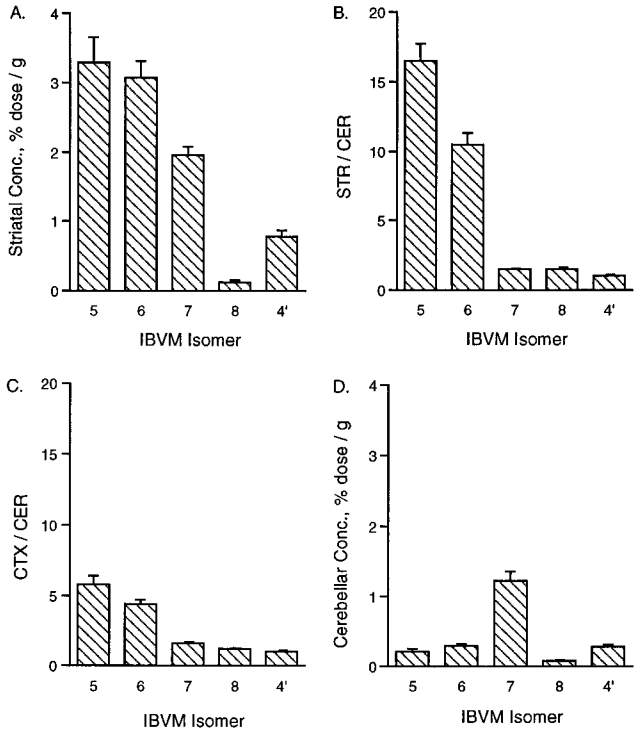


Figure 2. Comparison of key distribution parameters in mouse brain 4 h after iv tracer injection of IBVM isomers. Position of iodine is shown on the *x*-axis. Panel A compares the striatum concentrations; B and C compare the striatum/cerebellum and cortex/cerebellum activity ratios, respectively; D compares the cerebellum concentrations. Tissue concentrations are given in % injected dose/g (±SD) of tissue normalized to a 25-g mouse.

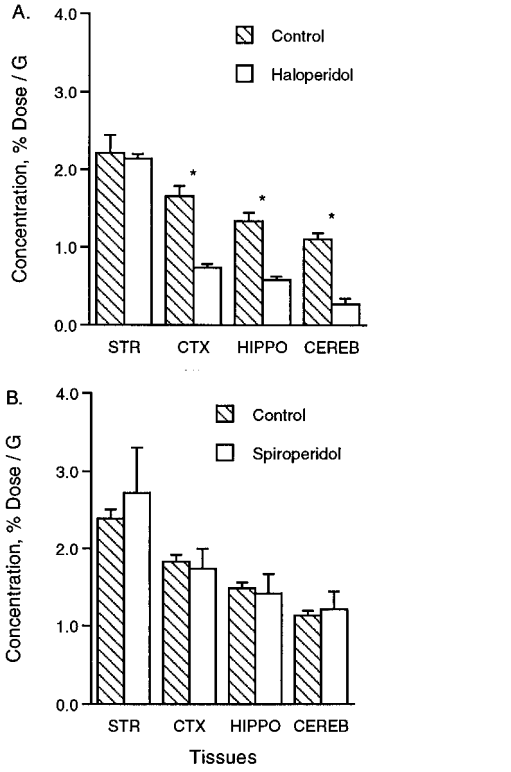


Figure 3. (A) Bar graph showing effect of haloperidol pretreatment (1.0 mg/kg ip) on 24-h regional brain distribution of (±)-7-[^{125}I]IBVM ([^{125}I]**14**) in CD-1 mice. Tissue concentrations are given in % injected dose/g (±SD) of tissue normalized to a 25-g mouse. (B) Effect of spiroperidol pretreatment (0.1 mg/kg ip) using the same protocol. In both studies, those sample pairs for which the means are statistically different ($p < 0.01$) are marked with an asterisk (*).

obtained with 6-IBVM (**10**) and especially with 5-IBVM (**5**) were again highly consistent with cholinergic nerve density. The absolute retention values were higher for the 5-isomer **5** with tissue-to-cerebellum concentration ratios of 44 for striatum, 18 for cortex, and 14 for hippocampus; these ratios were 3−4-fold higher than those obtained with 6-IBVM (**10**). The 24-h distribution of 7-IBVM (**14**), like its 4-h distribution, was anomalous in that all five brain regions retained large amounts of radioactivity. Most notable was the 24-h concentration of 7-IBVM (**14**) in cerebellum, a value 21-fold higher than cerebellar levels of either 5-IBVM (**5**) or 6-IBVM (**10**). The heart atria-to-ventricles concentration ratio, an index of peripheral cholinergic neuronal selectivity,[20,21] was highest for 5-IBVM (**5**), approaching 5 at 4 h postinjection and 14 at 24 h.

Receptor Screening of 7-IBVM (14). To determine if binding to any other known receptor types might be contributing to the anomalous brain retention pattern of 7-IBVM (**14**), the compound was screened for binding in a series of 26 different binding assays. Of the 26 binding assays performed with 7-IBVM (**14**) at a concentration of 1×10^{-5} M, >50% inhibition was observed only in the σ- and α_1-adrenergic receptor assays. Subsequent evaluation of 7-IBVM binding to the σ-receptor at five concentrations of the compound gave an estimated IC_{50} of approximately 30 nM. A similar evaluation of 7-IBVM in a 5-point α_1-adrenergic assay gave an estimated value of $IC_{50} > 500$ nM.

Drug-Blocking Studies. To further determine if σ-receptor binding might be contributing to this anomalous brain retention pattern of 7-IBVM (**14**), the effects of haloperidol and spiroperidol pretreatment on the regional tissue distribution of this radiotracer were evaluated in mice (see Figure 3). Haloperidol, a potent antagonist of both dopamine D_2 and σ-receptors,[22] lowered radioactivity levels 55−58% in cortex, hippocampus, and hypothalamus and 76% in cerebellum ($p < 0.01$); no change was observed in striatal activity. Spiroperidol, a potent D_2 antagonist with only weak σ-receptor blocking activity,[23] did not have a significant effect on radioactivity levels in the five brain areas evaluated.

Vesamicol Receptor Binding. The IC_{50} values for vesamicol receptor binding were determined by means of a competitive binding assay employing rat cortical homogenates and [^{3}H](methylamino)benzovesamicol, [^{3}H]MABV, a ligand that selectively binds to the vesamicol receptor with an apparent $K_d = 0.2$ nM.[24] Placement of the iodine atom in position 5, 6, or 7 gave <5 nM IC_{50} values, with 5-IBVM (**5**) being the most potent at 2.5 nM. A 22-fold decrease in binding affinity occurred when iodine was moved from the 7- to the 8-position. Iodine placement in the 4′-position of the phenylpiperidino group also lowered binding affinity (20.4 nM) but less dramatically. Using this assay, racemic vesamicol had an IC_{50} of 15.2 nM. The estimated IC_{50} values are presented in Figure 4.

Autoradiographic Distribution of (−)-5-[^{125}I]-IBVM. The distribution of (−)-5-[^{125}I]IBVM ((−)-[^{125}I]-**5**) in brain was examined in two rats each at 5 and 120 min after iv injection. Initial tracer uptake delineated gray and white matter structures, with greater levels in the former due most likely to higher blood flow

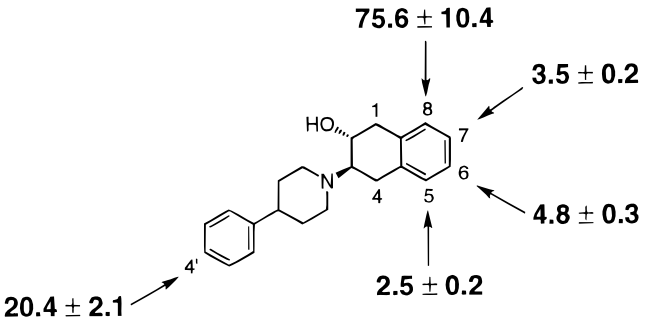


Figure 4. IC_{50} values (nM), concentration of compound required to inhibit 50% of [^{3}H](methylamino)benzovesamicol specific binding to homogenates of rat cerebral cortical synaptosomes; values represent mean of quadruplicate determinations ± SD. The value obtained for racemic vesamicol was 15.2 ± 1.3 nM.

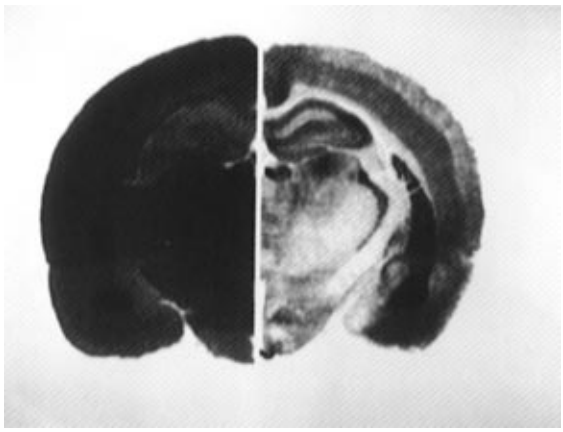


Figure 5. Autoradiographic distribution of 5-[^{125}I]IBVM ([^{125}I]-**5**) in coronal hemisections of rat brain at the level of the hippocampus 5 min (left hemisection) and 120 min (right hemisection) following tracer injection. Areas of highest tracer concentration are represented by increased density in the images, which are scaled independently to demonstrate relative distribution patterns. Note the initial homogeneity of tracer activity in gray matter (left) and the distinct, laminar cortical and hippocampal and selective subcortical retention at longer postinjection times (right).

(Figure 5, left hemisection). However, by 120 min a distinct pattern of tracer retention was observed, resulting in preferential labeling of a subset of gray matter structures (Figure 5, right hemisection). In Figure 6, a color-coded autoradiographic distribution at 120 min posttracer injection indicated densest labeling in the interpeduncular nucleus followed by the striatum (caudate-putamen and olfactory tubercle), anterior and intralaminar thalamic nuclei, cranial nerve motor nuclei, cerebral cortex, and hippocampus. Lowest levels of delayed tracer retention were observed in the cerebellar hemispheres and white matter.

Correlation of Choline Acetyltransferase (ChAT) and (−)-5-[^{123}I]IBVM. Radioactivity concentrations were determined in the atria and ventricles of heart and in four brain regions 4 h after tail-vein injection of (−)-5-[^{123}I]IBVM ((−)-[^{123}I]**5**) in CD-1 mice. After counting, tissue samples were stored at −20 °C for 4 days to allow the iodine-123 to decay before assaying for ChAT. The radioenzymatic method of Fonnum,[25] which utilizes [^{14}C]acetyl-CoA and choline to assay ChAT, was modified when applied to heart tissue to eliminate interference from endogenous carnitine and carnitine-acetyltransferase.[21,26] This modified ChAT assay was also used in the determination of ChAT levels in brain. ChAT activity in atria was approximately 2-fold higher than the activity levels in the ventricles, whereas the iodine-123 activity ratio was 3−4-fold higher in atria. The levels of brain ChAT decreased in the order striatum > hippocampus ≥ cortex > cerebellum; the iodine-123 concentrations decreased in the order striatum > cortex > hippocampus ≫ cerebellum. The linear correlation coefficient for ChAT activities versus iodine-123 concentrations in the four brain regions sampled was only 0.79. If the results from cortex were neglected, however, this relationship became linear ($r^2 = 0.99$). A bar graph, Figure 7, summarizes the results obtained in this correlation study.

Discussion

Radiotracers can often be tested for their potential to map specific neuronal systems by simply determining their gross regional distribution pattern in vivo. This radiotracer screening method is especially applicable to the cholinergic network which is heterogeneously distributed in murine brain.[27,28] The striatum, cortex, and hippocampus are densely innervated, in contrast to the cerebellum which has very sparse cholinergic innervation. Thus, striatum-to-cerebellum and cortex-to-cerebellum concentration ratios may serve as approximate indices of neuronal specificity. Regional brain concentrations at 4 and 24 h strongly suggest that 5-IBVM (**5**) and 6-IBVM (**10**) are specific markers for cholinergic nerves. The 24-h tissue-to-cerebellum concentration ratios for 5-IBVM (**5**) were 3−4-fold higher for striatum, cortex, and hippocampus than the respective ratios for 6-IBVM (**10**). The more pronounced neuronal retention of 5-IBVM (**5**) in brain was also consistent with the observed regional heart distribution of this tracer. The 24-h atria-to-ventricles concentration ratio for 5-IBVM (**5**) was 14-fold higher than the respective ratio for 6-IBVM (**10**). Atria are known to harbor a higher density of cholinergic innervation than ventricles.[20,21,27]

In vivo autoradiography of (−)-5-[^{125}I]IBVM ((−)-[^{125}I]-**5**) in rat brain provided additional support for the cholinergic nerve specificity of 5-IBVM (**5**). The brain distribution of 5-IBVM (**5**) at 5 min postinjection was typical of patterns obtained with cerebral blood flow tracers such as [^{14}C]iodoantipyrene.[29,30] In contrast, the heterogeneous retention pattern of 5-IBVM (**5**) in brain at 120 min postinjection was consistent with the regional density of cholinergic nerve terminals as revealed by in vitro autoradiography of [^{3}H]hemicholinium-3 binding[31−33] and by the regional brain distribution patterns of acetylcholine and its synthesizing and hydrolyzing enzymes ChAT and acetylcholinesterase.[21,28] Small cholinergic-rich areas of the brain such as basolateral amygdala, interpeduncular nucleus, and the facial nuclei could be clearly delineated. On the basis of known brain cholinergic neuroanatomy, the binding of 5-IBVM (**5**) in the striatum likely reflects the presence of cholinergic interneurons, whereas cortical and hippocampal labeling is due predominantly to projection terminals arising in the cholinergic basal forebrain nuclei.[34−37]

The brain distribution pattern of 7-IBVM (**14**) was unique in that high radioactivity levels were observed in all brain regions sampled, including the cerebellum, even 24 h after tracer injection. The evaluation of 7-IBVM by NovaScreen in 26 different biological assays

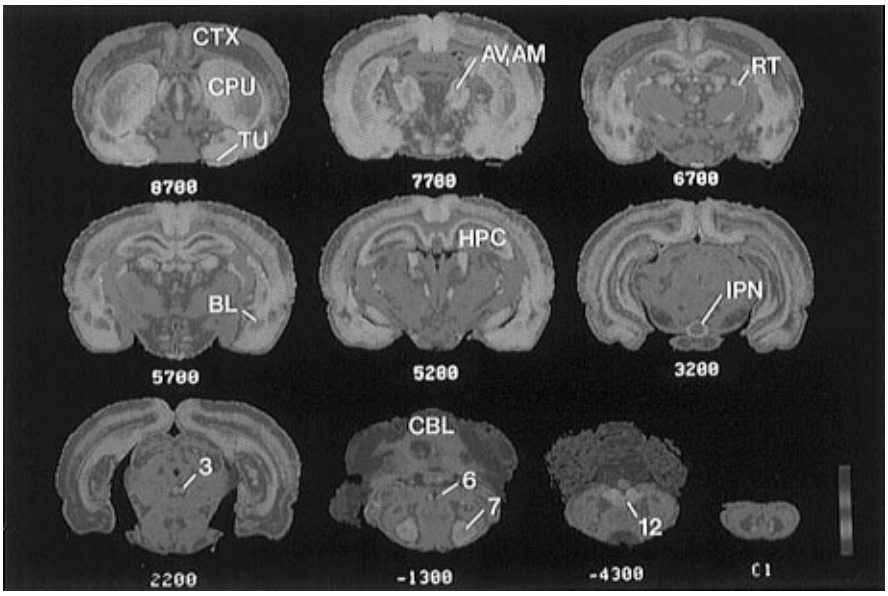


Figure 6. Autoradiographic distribution of 5-[^{125}I]IBVM ([^{125}I]**5**) retention in rat brain 120 min following bolus iv injection. Coronal sections at various rostrocaudal levels are presented from the level of the head of the caudate (upper left) to the cervical spinal cord (lower right). Tracer concentration is indicated by pseudocolor transformation, according to the scale at the lower right, with red and violet representing highest (1920 dpm/μg of protein) and lowest (13 dpm/μg of protein) levels, respectively. Numbers below each section refer to distance from the interaural line in mm, according to the stereotaxic atlas of Paxinos and Watson.[53] Note distinct labeling of cholinergic terminals in the striatum, cerebral neocortex, hippocampus, select diencephalic nuclei, and brainstem and spinal cord motor nuclei. Abbreviations: AM, anteromedial thalamus; AV, anteroventral thalamus; BL, basolateral amygdala; CBL, cerebellum; CPU, caudate-putamen; CTX, cerebral neocortex; HPC, hippocampus; IPN, interpeduncular nucleus; RT, reticular thalamus; TU, olfactory tubercle; 3, occulomotor nucleus; 6, abducens nucleus; 7, facial nucleus; 12, hypoglossal nucleus.

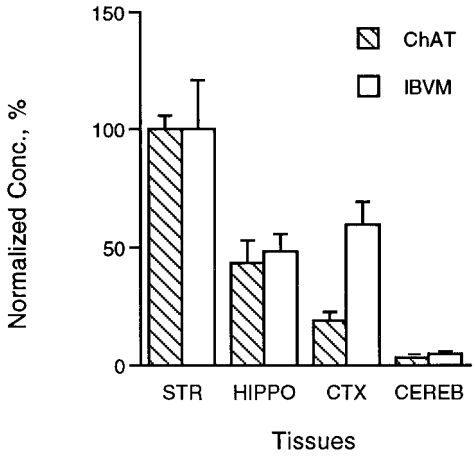


Figure 7. Comparison of choline acetyltransferase (ChAT) activity and (−)-5-[^{123}I]IBVM ((−)-[^{123}I]**5**) concentrations in four regions of CD-1 mouse brain ($N = 5$). The concentrations have been normalized to the levels obtained in striatum and are expressed as a percentage of the striatal level. Mice were sacrificed 4 h after iv injection of radiotracer. Abbreviations: striatum (STR), hippocampus (HIPPO), cortex (CTX), cerebellum (CEREB).

suggested that the σ-receptor might be involved in the cerebellar retention of 7-IBVM. This hypothesis is consistent with the high density of σ-receptors known to be present in the mouse cerebellum.[38] Also, while this work was in progress, Efange et al. reported that certain vesamicol derivatives bind to the σ-receptor with affinities in the 10−25 nM range.[39] Further evidence that σ-receptor binding may be a major determinant of the 24-h brain retention pattern of 7-IBVM (**14**) was obtained from blocking studies with haloperidol, a potent in vivo antagonist of both σ- and dopamine D_2 receptors. Over 75% of 7-IBVM (**14**) cerebellar retention was blocked by a haloperidol dose of 1 mg/kg ip; a dose of 2.5 mg/kg ip (data not shown) decreased cerebellar retention 86%. The lack of effect of spiroperidol pretreatment on 7-IBVM (**14**) brain retention demonstrated that dopamine D_2 receptor binding was not involved. The high cerebellar retention of 7-IBVM (**14**) is also inconsistent with D_2 binding since the density of this receptor is very low in mouse cerebellum.[40,41] The concentrations of radioactivity in liver, lung, and heart 24 h after injection of 7-IBVM (**14**) were also markedly higher than the respective levels obtained with 5- and 6-IBVM (**5**, **10**). Haloperidol (2.5 mg/kg ip) pretreatment lowered radioactivity levels in liver (−74%), lung (−88%), atria (−60%), and ventricles (−38%). This finding is consistent with the high density of σ-receptors in these peripheral tissues.[42,43]

It is notable that, although the mouse striatum has a σ-receptor density only slightly lower than either hippocampus or hypothalamus,[38] haloperidol pretreatment did not block 7-IBVM (**14**) retention in this brain region (Figure 3). It is possible that σ-receptor blockade in the striatum may have been masked by an increase in vesamicol receptor binding. Dopamine D_2 receptors located on striatal cholinergic neurons are generally thought to be involved in the inhibitory control of striatal cholinergic function.[44] Enhanced retention of a ^{18}F-labeled 5-aminobenzovesamicol derivative in monkey striatum in response to pretreatment with haloperidol or the dopamine D_2 antagonist raclopride has been reported by Ingvar et al.[45] They attributed this phenomenon to augmentation of vesamicol receptor binding due to enhanced cholinergic activity occurring in response to dopamine D_2 receptor blockade. However, our observation, that spiroperidol pretreatment at a dose of 0.1 mg/kg ip did not significantly increase striatal retention of racemic 7-IBVM (**14**), is not con-

sistent with this hypothesis. It should be pointed out that the study by Ingvar et al. was performed in monkeys, and striatal radioactivity levels were determined by positron emission tomography up to 60 min after radiotracer injection; the study described here was performed in mice 24 h after tracer injection. A better understanding of the striatal response of 7-IBVM (**14**) to haloperidol pretreatment may be made possible by drug-blocking studies of the enantiomers of 7-IBVM (**14**) which have now become available.[46]

The relative in vitro binding affinities of 5- and 6-IBVM (**5**, **10**) for the vesamicol receptor, as determined in the competitive binding assay using rat cortical homogenates, are consistent with the relative tenacity with which these two isomers map cholinergic innervation in vivo. Although the 5-isomer **5** had only a two-fold lower IC_{50} than the 6-isomer **10**, the former showed a 4−8-fold higher retention in cortical and hippocampal brain regions 24 h after injection.

The ability of 5-IBVM (**5**) to map cholinergic innervation is also supported by its selective retention in the atrial wall of the myocardium. From slightly above unity at 30 min, the atria-to-ventricles radioactivity ratio of 5-IBVM (**5**) rose to almost 5 at 4 h and to over 13 at 24 h. The levels of ChAT in rat heart[21] indicate a density of cholinergic innervation in atria that are approximately 5-fold higher than in ventricles.[22] Our assay of ChAT in CD-1 mouse heart indicated a greater than 2-fold higher level in the atria; the actual ratio, however, may be higher because ChAT activity in ventricle is near the sensitivity level of the assay. Our finding of higher ChAT levels in hippocampus than in cortex of CD-1 mouse brain is consistent with other reports in mice[27] and rats.[28] The low correlation (r^2 = 0.79) obtained between ChAT activities and (−)-5-[^{123}I]-IBVM ((−)-[^{123}I]**5**) levels in the four brain regions of CD-1 mice was due mainly to higher tracer levels in cortex than in hippocampus; a linear correlation (r^2 = 0.99) was obtained if cortical results were omitted. Higher levels of radioactivity in cortex than in hippocampus have also been observed in mouse brain with iodine-125-labeled 5-IBVM (**5**) and 6-IBVM (**10**) (see Table 1) as well as with carbon-11- and fluorine-18-labeled 5-substituted benzovesamicols.[24,47,48] This mismatch between ChAT and radioactivity levels, and thus vesamicol receptor density, was not observed in normal human brain using (−)-5-[^{123}I]IBVM ((−)-[^{123}I]**5**).[11]

The distribution data in Table 1 indicated that 5-IBVM (**5**) and 6-IBVM (**10**) cleared very slowly from nonneuronal areas of the brain. At 4 h after tracer injection, concentrations of radioactivity were still high in cerebellum and blood. The synthesis of new analogs of IBVM which are less lipophilic than 5-IBVM (**5**) may provide tracers that show enhanced brain extraction as well as faster clearance of nonspecifically bound radioactivity. This would potentially enable the determination of brain cholinergic density within a few hours of radiotracer injection as opposed to the 22-h time period presently required to obtain a neuronal map of the human brain.[11,12]

Conclusion

The evaluation of five positional isomers of radioiodinated benzovesamicol as in vivo markers for brain cholinergic nerve density indicates that radioiodine substitution may be optimal at the 5-position. The 6-position is only slightly less favorable. Evidence regarding 7-IBVM (**14**) is not conclusive because of tracer cobinding to the σ-receptor. Final assessment of 7-IBVM (**14**) will require evaluation of its enantiomers.

Experimental Section

^{1}H NMR spectra were obtained in $CDCl_3$ on a Bruker 360 or 300 MHz NMR spectrometer and are reported in parts per million downfield from tetramethylsilane. Mass spectra were obtained on a Finnigan 4021 GC/MS spectrometer in the electron impact (EI) ionization mode at 70 eV. Molecular masses are given in atomic mass units (amu) followed by percent intensity relative to the most abundant ion. Accurate mass spectral determinations were also obtained in the EI mode at 70 eV. Elemental analyses were carried out by Spang Microanalytical Laboratory, Eagle Harbor, MI. Melting points were determined on a Thomas-Hoover capillary melting point apparatus in open capillary tubes and are uncorrected. Flash-chromatography utilized Merck 230−400 mesh silica gel. Thin-layer chromatography (TLC) used Analtech 0.25-mm glass-backed plates with fluorescent background. Visualization was achieved by phosphomolybdic acid (PMA), iodine, or UV illumination. High-pressure liquid chromatography (HPLC) was performed on a Beckman Instrument Model 344 gradient liquid chromatograph with an ABS Model 757 UV/vis detector and a Waters Model 740 data module. Neutral alumina and silica Sep-Paks were purchased from Waters Associates, Milford, MA. Borosilicate glass beads (3 mm) were obtained from Macalaster-Bicknell, Millville, NJ. Reagents and solvents were purchased from commercial sources and used without further purification unless otherwise noted. The 5- and 8-aminobenzovesamicols (**2**, **3**) were prepared by reacting 6,7-epoxy-1-(trifluoroacetamido)-5,8-dihydronaphthalene (**1**) with 4-phenylpiperidine by published procedures.[13] Polysorbate-80, used to dissolve IBVM isomers for in vitro receptor binding studies using rat brain cortices, was purchased from American Research Products Co., Solon, OH.

The iodine-125 was a no-carried-added solution of Na^{125}I (ca. 700−800 mCi/mL) in reductant free 0.1 N NaOH obtained from Nordion International Inc., Kanata, Ontario, Canada. Radioactivity was quantified with a Capintec Model CRC-12 radioisotope calibrator which was calibrated against an NBS standard solution of Na^{125}I. Radio-TLC analyses were performed on 2.5 × 20 cm silica gel-coated glass plates (Whatman K_6F). The plates were analyzed on a Berthold automatic TLC linear analyzer LB2832 radiochromatogram scanner immediately after development and drying. Percent exchange was determined by integration of the chromatogram peaks and calculated as the ratio of activity of exchanged product to the total activity chromatographed.

(±)-*trans*-2-Hydroxy-5-iodo-3-(4-phenylpiperidino)tetralin (5-IBVM) (5). Method A. To a cooled (5 °C) solution of (±)-5-aminobenzovesamicol (**2**) (120 mg, 37.0 μmol) in acetic acid (2 mL) and concentrated HCl (1 mL) was added dropwise a solution of $NaNO_2$ (27 mg, 40.0 μmol) in H_2O (2 mL) while the temperature of the solution was maintained under 10 °C. After the mixture was stirred for 30 min, a solution of KI (74 mg, 44.7 μmol) and I_2 (57 mg, 22.3 μmol) in H_2O (1 mL) was added dropwise. The reaction mixture was stirred at 5 °C for 3 h, allowed to warm to room temperature, and stirred overnight. The reaction mixture was poured into saturated $NaHCO_3$ solution (20 mL) and extracted with ethyl acetate (3 × 20 mL). The combined extracts were washed with 10% $NaHSO_3$ solution, dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The residue was flash-chromatographed on silica gel with 15% ethyl acetate in hexane to afford 109 mg (68%) of (±)-5-iodobenzovesamicol (**5**) as a white solid: mp 138−140 °C (recrystallized from ether/hexane); IR (KBr) 3610−3275 (br), 3059, 3025, 2929, 2907, 1645, 1602 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.68−1.97 (m, 4H), 2.44−3.03 (m, 9H), 3.25 (d, d, J = 16.1, 5.7 Hz, 1H), 3.84 (t, d, J = 10.2, 5.7 Hz, 1H), 4.3 (br s, OH), 6.84 (t, J = 7.7 Hz, 1H), 7.10 (d, J = 7.7 Hz, 1H), 7.19−7.35 (m, 5H), 7.70 (d, J = 7.7 Hz, 1H); MS (EI, 70 eV) m/z (rel intensity) 433 (M^+, 15.12), 306 (1.73), 231 (1.10), 216 (1.13), 202 (6.32), 186 (1.53), 174

(100.00), 160 (22.81), 146 (6.25), 128 (14.46), 115 (26.41), 91 (21.44), 56 (9.80), 42 (6.10); high-resolution MS (EI, 70 eV) calcd for $C_{21}H_{24}INO$ 433.0903, found 433.0910. Anal. ($C_{21}H_{24}INO$) C, H, N.

(±)-*trans*-2-Hydroxy-5-iodo-3-(4-phenylpiperidino)tetralin (5-IBVM) (5). Method B: (±)-*trans*-2-Hydroxy-8-amino-5-iodo-3-(4-phenylpiperidino)tetralin (8-Amino-5-IBVM) (4). To a solution of (±)-8-aminobenzovesamicol (**3**) (4.28 g, 13.27 mmol) in acetic acid (125 mL) was added dropwise a solution of iodine monochloride (2.16 g, 13.27 mmol) in acetic acid (5 mL) at room temperature. The resulting solution was stirred overnight, and the solvent was evaporated under reduced pressure. The residue was dissolved in $CHCl_3$ (30 mL), and the solution was washed with saturated $NaHCO_3$ solution (30 mL) and extracted with $CHCl_3$ (2 × 50 mL). The combined extracts were dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The residue was flash-chromatographed on silica gel with 70% ethyl acetate in hexane to afford 4.97 g (84%) of (±)-8-amino-5-iodobenzovesamicol (**4**) as a white solid: mp 165−169 °C (recrystallized from ether/hexane); ^{1}H NMR ($CDCl_3$) δ 1.68−1.99 (m, 4H), 2.33−2.98 (m, 9H), 3.03 (d, d, J = 16.5, 5.7 Hz, 1H), 3.67 (br s, NH_2), 3.76 (t, d, J = 10.2, 5.7 Hz, 1H), 4.38 (br s, OH), 6.32 (d, J = 8.4 Hz, 1H), 7.15−7.36 (m, 5H), 7.49 (d, J = 8.4 Hz, 1H); MS (EI, 70 eV) m/z (rel intensity) 448 (M^+, 10.76), 321 (6.60), 289 (16.75), 287 (17.38), 269 (4.13), 256 (6.25), 203 (7.61), 174 (33.89), 160 (100.00), 144 (21.87), 143 (23.70), 130 (39.66), 56 (50.77), 42 (36.78); high-resolution MS (EI, 70eV) calcd for $C_{21}H_{25}IN_2O$ 448.1012, found 448.1021. Anal. ($C_{21}H_{25}IN_2O$) C, H, N.

(±)-*trans*-2-Hydroxy-5-iodo-3-(4-phenylpiperidino)tetralin (5-IBVM) (5). A solution of $NaNO_2$ (811 mg, 11.75 mmol) in H_2O (5 mL) was added dropwise to a cooled (5 °C) solution of 8-amino-5-iodobenzovesamicol (**4**) (4.97 g, 11.09 mmol) in 50% hypophosphorous acid (40 mL) and concentrated HCl (10 mL) while maintaining the reaction temperature under 10 °C. The mixture was stirred under 5 °C for 1 h, allowed to warm to room temperature, stirred overnight, and poured into saturated aqueous NaCl solution. The aqueous layer was extracted with CH_2Cl_2 (3 × 40 mL). The combined extracts were washed with saturated $NaHCO_3$ solution, dried over anhydrous sodium sulfate, and concentrated under reduced pressure. The residue was flash-chromatographed on silica gel, eluting with 15% ethyl acetate in hexane to afford 4.18 g (87%) of (±)-5-iodobenzovesamicol (**5**). The ^{1}H NMR and IR spectra of this product were identical with those of **5** prepared from **2** by the diazotization route described above.

Resolution of (±)-*trans*-2-Hydroxy-5-iodo-3-(4-phenylpiperidino)tetralin ((±)-5-IBVM) ((+)-5 and (−)-5). To a solution of (±)-5-iodobenzovesamicol (**5**) (286 mg, 0.66 mmol), 4-(dimethylamino)pyridine (24 mg, 0.20 mmol), and triethylamine (2.76 mL, 1.98 mmol) in dry chloroform (5 mL) was added dropwise (*S*)-(−)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (184 mg, 0.73 mmol) by syringe at room temperature.[10,14] The resulting solution was stirred for 6 h and then poured into ethyl acetate (20 mL). The solution was washed with saturated $NaHCO_3$ solution, and the aqueous layer was extracted with ether (2 × 20 mL). The combined extracts were dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The two diastereomeric MTPA esters were separated by preparative thin layer chromatography on silica gel (20 cm × 20 cm, 1.0 mm) after several developments with 5% ethyl acetate in hexane. The less polar compound (R_f = 0.41, 10% ethyl acetate in hexane) was the (+)-diastereomer ($[\alpha]^{23}_D$ = +36.7°, c = 1.5, EtOH), of which 195 mg (46%) was obtained. The more polar compound (R_f = 0.35, 10% ethyl acetate in hexane) was the (−)-diastereomer ($[\alpha]^{23}_D$ = −56.7°, c = 1.5, EtOH), of which 182 mg (43%) was obtained.

The (−)-diastereomer (163 mg, 0.25 mmol) was dissolved in THF (10 mL) and MeOH (5 mL) to which was added 2 N NaOH solution (10 mL). The mixture was stirred for 10 h at room temperature and then extracted with ethyl acetate (3 × 30 mL). The combined extracts were dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The residue was flash-chromatographed on silica gel, eluting with 15% ethyl acetate in hexane to afford 94 mg (86%) of (−)-5-IBVM ((−)-**5**) (mp 138−140 °C; $[\alpha]^{23}_D$ = −45.3°, c = 1.5, EtOH). The (+)-5-IBVM enantiomer ((+)-**5**) was obtained in 89% yield from the (+)-diastereomer by the above-described method (mp 138−140 °C; $[\alpha]^{23}_D$ = +44.0°, c = 1.5, EtOH). The optical purity of the (−)- and (+)-enantiomers was >98% as determined by chiral HPLC using a Chiracel OD column (4.6 × 250 mm) eluting with 10% 2-propanol in hexane at a flow rate of 1 mL/min with UV detection at 254 nm. Retention times of (−)-**5** and (+)-**5** were 6.8 and 10.0 min, respectively.[15]

(±)-*trans*-2-Hydroxy-8-iodo-3-(4-phenylpiperidino)tetralin (8-IBVM) (6). The 8-iodobenzovesamicol (**6**) was obtained as a white solid in 57% yield from 8-aminobenzovesamicol (**3**) by the same method (method A) described for the synthesis of 5-iodobenzovesamicol (**5**): mp 194−197 °C (recrystallized from ether/hexane); ^{1}H NMR ($CDCl_3$) δ 1.72−2.05 (m, 4H), 2.36 (d, d, J = 11.4, 2.2 Hz, 1H), 2.52−2.99 (m, 8H), 3.41 (d, d, J = 16.8, 6.2 Hz, 1H), 3.87 (t, d, J = 10.1, 6.2 Hz, 1H), 4.34 (br s, OH), 6.83 (t, J = 7.8 Hz, 1H), 7.09 (d, J = 7.8 Hz, 1H), 7.19−7.35 (m, 5H), 7.71 (d, J = 7.8 Hz, 1H); MS (EI, 70 eV) m/z (rel intensity) 433 (17.51), 306 (4.29), 231 (1.04), 216 (1.63), 203 (8.46), 1.86 (1.11), 174 (100.00), 160 (23.03), 145 (9.61), 56 (44.40), 42 (42.08); high-resolution MS (EI, 70 eV) calcd for $C_{21}H_{24}INO$ 433.0903, found 433.0894. Anal. ($C_{21}H_{24}INO$) C, H, N.

(±)-*trans*-5-Amino-2-hydroxy-6-nitro-3-(4-phenylpiperidino)tetralin (5-Amino-6-nitro-BVM) (7). (±)-5-Aminobenzovesamicol (**2**) (3.04 g, 9.42 mmol) was added to acetic anhydride (50 mL). After the resulting solution was stirred for 30 min, *p*-toluenesulfonic acid monohydrate (2.15 g, 11.30 mmol) was added. The solution was cooled at 5 °C in an ice bath, and 90% fuming nitric acid (1.12 mL, 22.60 mmol) was added dropwise at a rate which maintained the reaction temperature under 7 °C. After stirring at 5 °C for 3.5 h, the solution was poured with stirring into ice water, and the aqueous layer was extracted with CH_2Cl_2 (3 × 30 mL). The combined organic layers were washed with saturated $NaHCO_3$ solution and evaporated to dryness under reduced pressure. The residue was refluxed in 6 N HCl (40 mL) solution with stirring for 2 h to hydrolyze the acetamide. After adjusting to pH 10 by the addition of 6 N NaOH, the solution was extracted with CH_2Cl_2 (3 × 30 mL), and the combined extracts were dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The residue was flash-chromatographed on silica gel, eluting with 40% ethyl acetate in hexane to afford 2.95 g (85%) of (±)-5-amino-6-nitrobenzovesamicol (**7**) as a yellow solid: mp 176−180 °C; ^{1}H NMR ($CDCl_3$) δ 1.70−1.99 (m, 4H), 2.42−3.02 (m, 9H), 3.26 (d, d, J = 17.0, 5.5 Hz, 1H), 3.86 (t, d, J = 10.4, 5.5 Hz, 1H), 4.21 (br s, OH), 6.28 (br s, NH_2), 6.47 (d, J = 8.8 Hz, 1H), 7.19−7.35 (m, 5H), 7.97 (d, J = 8.8 Hz, 1H); MS (EI, 70 eV) m/z (rel intensity) 367 (M^+, 10.88), 350 (1.81), 322 (2.10), 206 (2.69), 203 (5.30), 190 (16.62), 174 (27.82), 160 (51.22), 144 (11.38), 130 (16.65), 43 (100.00); high-resolution MS (EI, 70eV) calcd for $C_{21}H_{25}N_3O_3$ 367.1896, found 367.1896.

(±)-*trans*-2-Hydroxy-6-nitro-3-(4-phenylpiperidino)tetralin (6-Nitro-BVM) (8). To a cooled (5 °C) solution of 5-amino-6-nitrobenzovesamicol (**7**) (2.93 g, 7.97 mmol) in acetic acid (20 mL) was added dropwise a solution of $NaNO_2$ (605 mg, 8.77 mmol) in H_2O (5 mL) while maintaining the reaction temperature under 10 °C. The mixture was stirred at 5 °C for 1 h, and then hypophosphorous acid (30 mL) was added. The mixture was allowed to warm to room temperature, stirred overnight, and then poured into saturated saline solution. The aqueous layer was extracted with CH_2Cl_2 (3 × 40 mL). The combined extracts were washed with a saturated $NaHCO_3$ solution, dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The residue was flash-chromatographed on silica gel, eluting with 40% ethyl acetate in hexane to afford 2.61 g (93%) of (±)-6-nitrobenzovesamicol (**8**) as a yellow solid: mp 174−177 °C; ^{1}H NMR ($CDCl_3$) δ 1.68−1.98 (m, 4H), 2.40 (t, d, J = 11.5, 2.2 Hz, 1H), 2.58 (m, 1H), 2.77−3.09 (m, 7H), 3.42 (d, d, J = 17.0, 5.9 Hz, 1H), 3.91 (t, d, J = 10.1, 5.9 Hz, 1H), 4.30 (br s, OH), 7.19−7.36 (m, 6H), 7.97 (d, J = 9.3 Hz, 1H), 7.99 (s, 1H); MS (EI, 70 EV) m/z (rel intensity) 352 (M^+, 29.26), 335 (3.38), 321 (1.46), 203 (7.86), 191 (2.04), 174 (100.00), 160 (20.10), 144 (11.54), 128 (19.13),

115 (45.31); high-resolution MS (EI, 70 eV) calcd for $C_{21}H_{24}N_2O_3$ 352.1787, found 352.1791.

(±)-*trans*-2-Hydroxy-6-amino-3-(4-phenylpiperidino)-tetralin (6-Amino-BVM) (9). The 6-nitrobenzovesamicol (**8**) (1.24 g, 3.51 mmol) was dissolved in ethyl acetate (20 mL) and ethanol (30 mL), and 10% palladium on activated carbon (400 mg) was added. The mixture was hydrogenated at 40 psi overnight. After filtration, the solution was concentrated under reduced pressure, and the residue was flash-chromatographed on silica gel, eluting with 50% ethyl acetate in hexane to afford 1.05 g (93%) of (±)-6-aminobenzovesamicol (**9**) as a white solid: mp 172−173 °C; ^{1}H NMR ($CDCl_3$) δ 1.68−1.94 (m, 4H), 2.36 (t, d, J = 11.4, 2.1 Hz, 1H), 2.55 (m, 1H), 2.65−2.86 (m, 6H), 2.96 (d, J = 11.2 Hz, 1H), 3.21 (d, d, J = 15.5, 5.8 Hz, 1H), 3.54 (br s, NH_2) 3.83 (t, d, J = 9.7, 5.8 Hz, 1H), 4.38 (br s, OH), 6.44 (d, J = 2.4 Hz, 1H), 6.51 (d, d, J = 8.1, 2.4 Hz, 1H), 6.90 (d, J = 8.1 Hz, 1H), 7.19−7.34 (m, 5H); MS (EI, 70 eV) m/z (rel intensity) 322 (M^+, 5.63), 304 (20.46), 291 (4.36), 279 (1.99), 215 (2.67), 202 (4.48), 174 (42.65), 161 (9.34), 160 (9.67), 144 (100.00), 131 (23.37), 57 (39.74), 43 (85.29); high-resolution MS (EI, 70 eV) calcd for $C_{21}H_{26}N_2O$ 322.2045, found 322.2036. Anal. ($C_{21}H_{26}N_2O$) C, H, N.

(±)-*trans*-2-Hydroxy-6-iodo-3-(4-phenylpiperidino)tetralin (6-IBVM) (10). 6-Iodobenzovesamicol (**10**) was obtained as a white solid in 66% yield from 6-aminobenzovesamicol (**9**) by using method A described for the synthesis of 5-iodobenzovesamicol (**5**): mp 142−143 °C (recrystallized from ether/hexane); ^{1}H NMR ($CDCl_3$) δ 1.71−1.95 (m, 4H), 2.36 (t, d, J = 11.4, 2.2 Hz, 1H), 2.52−2.57 (m, 1H), 2.69-2.97 (m, 7H), 3.26 (d, d, J = 16.3, 5.8 Hz, 1H), 3.84 (t, d, J = 10.0, 5.8 Hz, 1H), 4.35 (br s, OH), 6.85 (d, J = 8.1 Hz, 1H), 7.19−7.35 (m, 5H), 7.44 (d, J = 8.1 Hz, 1H), 7.46 (s, 1H); MS (EI, 70 eV) m/z (rel intensity) 433 (M^+, 19.27), 415 (2.15), 314 (1.10), 307 (4.17), 245 (2.01), 231 (2.26), 203 (8.37), 202 (10.41), 174 (100.00), 160 (8.58), 146 (7.62), 128 (24.39), 115 (41.54), 56 (49.43), 42 (56.26); high-resolution MS (EI, 70 eV) calcd for $C_{21}H_{24}INO$ 433.0903, found 433.0889. Anal. ($C_{21}H_{24}INO$) C, H, N.

(±)-*trans*-8-Amino-2-hydroxy-7-nitro-3-(4-phenylpiperidino)tetralin (8-Amino-7-nitro-BVM) (11). 8-Amino-7-nitrobenzovesamicol (**11**) was obtained in 78% yield from 8-aminobenzovesamicol (**3**) by using the method described for the synthesis of 5-amino-6-nitrobenzovesamicol (**7**): mp 153−154 °C (recrystallized from ether/hexane); ^{1}H NMR ($CDCl_3$) δ 1.69−1.98 (m, 4H), 2.36−2.48 (m, 2H), 2.53−2.62 (m, 1H), 2.80−3.03 (m, 6H), 3.12 (d, d, J = 15.7, 6.3 Hz, 1H), 3.94 (t, d, J = 9.4, 6.3 Hz, 1H), 6.30 (br s, NH_2), 6.48 (d, J = 8.9 Hz, 1H), 7.19−7.36 (m, 5H), 7.98 (d, J = 8.9 Hz, 1H); MS (EI, 70 eV) m/z (rel intensity) 367 (M^+, 9.39), 350 (2.12), 349 (3.14), 332 (1.87), 229 (2.03), 216 (3.15), 206 (2.63), 204 (2.38), 203 (11.49), 190 (6.79), 189 (15.46), 174 (100.00), 172 (18.37), 162 (52.40), 161 (22.50), 160 (50.92), 144 (11.54), 143 (17.76), 130 (30.96), 117 (28.74), 115 (29.11), 103 (30.36), 91 (48.06); high-resolution MS (EI, 70 eV) calcd for $C_{21}H_{25}IN_3O_3$ 367.1896, found 367.1887.

(±)-*trans*-2-Hydroxy-7-nitro-3-(4-phenylpiperidino)tetralin (7-Nitro-BVM) (12). 7-Nitrobenzovesamicol (**12**) was obtained in 86% yield from 8-amino-7-nitrobenzovesamicol (**11**) by using the method described for the synthesis of 6-nitrobenzovesamicol (**8**): mp 166−170 °C; ^{1}H NMR ($CDCl_3$) δ 1.73−1.96 (m, 4H), 2.23−3.09 (m, 9H), 3.42 (d, d, J = 16.3, 5.8 Hz, 1H), 3.90 (t, d, J = 10.0, 5.8 Hz, 1H), 4.32 (br s, OH), 7.20−7.36 (m, 6H), 7.97 (d, J = 8.7 Hz, 1H), 7.99 (s, 1H); MS (EI, 70 eV) m/z (rel intensity) 352 (M^+, 100.00), 335 (26.3), 322 (11.6), 307 (11.1), 219 (8.0), 203 (20.2), 174 (91.2), 160 (20.8), 145 (14.5), 115 (26.0), 91 (23.7); high-resolution MS (EI, 70 eV) calcd for $C_{21}H_{24}N_2O_3$ 352.1787, found 352.1791.

(±)-*trans*-2-Hydroxy-7-amino-3-(4-phenylpiperidino)-tetralin (7-Amino-BVM) (13). 7-Aminobenzovesamicol (**13**) was obtained in 73% yield from 7-nitrobenzovesamicol (**12**) by using the method described for the synthesis of 6-amino-BVM (**9**): mp 178−180 °C; ^{1}H NMR ($CDCl_3$) δ 1.69−1.94 (m, 4H), 2.36 (t, d, J = 11.4, 2.2 Hz, 1H), 2.54 (m, 1H), 2.70−2.98 (m, 7H), 3.21 (d, d, J = 16.0, 5.8 Hz, 1H), 3.54 (br s, NH_2), 3.84 (m, 1H), 4.38 (br s, OH), 6.46 (d, J = 2.3 Hz, 1H), 6.50 (d, d, J = 8.1, 2.3 Hz, 1H), 6.89 (d, J = 8.1 Hz, 1H), 7.19−7.34 (m, 5H); MS (EI, 70 eV) m/z (rel intensity) 322 (M^+, 100.00), 291 (3.1), 203 (14.2), 189 (6.9), 174 (36.5), 161 (10.2), 144 (16.6), 132 (13.8), 120 (16.3), 91 (12.4); high-resolution MS (EI, 70 eV) calcd for $C_{21}H_{26}N_2O$ 322.2045, found 322.2028. Anal. ($C_{21}H_{26}N_2O$) C, H, N.

(±)-*trans*-2-Hydroxy-7-iodo-3-(4-phenylpiperidino)tetralin (7-IBVM) (14). 7-Iodobenzovesamicol (**14**) was obtained as a white solid in 45% yield from 7-aminobenzovesamicol (**13**) by using method A described for the synthesis of 5-iodobenzovesamicol (**5**): mp 185−187 °C (recrystallized from ether/hexane); ^{1}H NMR ($CDCl_3$) δ 1.65−1.95 (m, 4H), 2.37 (t, d, J = 11.4, 2.2 Hz, 1H), 2.51−2.58 (m, 1H), 2.70-2.99 (m, 7H), 3.26 (d, d, J = 16.3, 5.8 Hz, 1H), 3.84 (t, d, J = 10.0, 5.8 Hz, 1H), 4.37 (br s, OH), 6.84 (d, J = 8.1 Hz, 1H), 7.19−7.35 (m, 5H), 7.43 (d, J = 8.1 Hz, 1H), 7.47 (s, 1H); MS (EI, 70 eV) m/z (rel intensity) 433 (M^+, 25.00), 203 (11.70), 174 (100.00), 160 (14.38), 146 (7.30), 128 (20.58), 115 (37.65), 91 (31.77), 56 (47.96), 42 (46.73); high-resolution MS (EI, 70 eV) calcd for $C_{21}H_{24}INO$ 433.0903, found 433.0881. Anal. ($C_{21}H_{24}INO$) C, H, N.

(±)-*trans*-5-Amino-2-hydroxy-3-[4-(4′-iodophenyl)piperidino]tetralin (5-Amino-4′-iodo-BVM) (15) and (±)-*trans*-8-Amino-2-hydroxy-3-[4-(4′-iodophenyl)piperidino]-tetralin (8-Amino-4′-iodo-BVM) (16). To a solution of 6,7-epoxy-1-(trifluoroacetamido)-5,8-dihydronaphthalene (**1**) (420 mg, 1.63 mmol) in EtOH (20 mL) was added 4-(4′-iodophenyl)-piperidine[16] (920 mg, 3.32 mmol). The resulting solution was refluxed for 32 h and concentrated under reduced pressure. The residue was flash-chromatographed on silica gel, eluting with 50% ethyl acetate in hexane to afford 5-amino-4′-iodobenzovesamicol (**15**) (301 mg, 41%) and 8-amino-4′-iodobenzovesamicol (**16**) (289 mg, 39%). **15** (R_f 0.27, 50% ethyl acetate in hexane): mp 198−201 °C; ^{1}H NMR ($CDCl_3$) δ 1.68−1.94 (m, 4H), 2.41−3.01 (m, 9H), 3.25 (d, d, J = 16.0, 5.5 Hz, 1H), 3.58 (br s, NH_2), 3.86 (t, d, J = 10.4, 5.5 Hz, 1H), 6.55 (d, J = 7.7 Hz, 1H), 6.59 (d, J = 7.7 Hz, 1H), 6.99 (t, J = 7.7 Hz, 1H), 7.00 (d, J = 8.4 Hz, 2H), 7.63 (d, J = 8.4 Hz, 2H); MS (EI, 70 eV) m/z (rel intensity) 448 (M^+, 8.03), 300 (20.77), 286 (21.32), 229 (2.23), 217 (2.43), 174 (4.62), 163 (59.72), 162 (34.30), 160 (17.54), 144 (100.00), 130 (43.38), 115 (23.86), 56 (43.60); high-resolution MS (EI, 70 eV) calcd for $C_{21}H_{25}IN_2O$ 448.1012, found 448.1006. Anal. ($C_{21}H_{25}IN_2O$) C, H, N.

16 (R_f 0.20, 50% ethyl acetate in hexane): ^{1}H NMR ($CDCl_3$) δ 1.62−1.92 (m, 4H), 2.30−3.01 (m, 9H), 3.13 (d, d, J = 15.7, 6.3 Hz, 1H), 3.62 (br s, NH_2), 3.92 (m, 1H), 6.56 (d, J = 7.7 Hz, 2H), 6.98 (t, J = 7.7 Hz, 1H), 7.00 (d, J = 8.4 Hz, 1H), 7.63 (d, J = 8.4 Hz, 2H).

(±)-*trans*-2-Hydroxy-3-[4-(4′-iodophenyl)piperidino]tetralin (4′-IBVM) (17). 4′-Iodobenzovesamicol (**17**) was obtained in 81% yield from 8-amino-4′-iodobenzovesamicol (**16**) by using the second step of method B (Scheme 1) described for the synthesis of 5-iodobenzovesamicol (**5**) from 8-amino-5-iodobenzovesamicol (**4**): mp 148−149 °C (recrystallized from ether/hexane); ^{1}H NMR ($CDCl_3$) δ 1.66−1.92 (m, 4H), 2.39 (t, d, J = 11.3, 1.9 Hz, 1H), 2.51 (t, t, J = 11.9, 3.9 Hz, 1H), 2.78−3.01 (m, 7H), 3.32 (d, d, J = 16.1, 5.8 Hz, 1H), 3.89 (t, d, J = 10.1, 5.8 Hz, 1H), 7.00 (d, J = 8.4 Hz, 2H), 7.10−7.19 (m, 4H), 7.63 (d, J = 8.4 Hz, 2H); MS (EI, 70 eV) m/z (rel intensity) 443 (M^+, 38.49), 328 (6.04), 300 (100.00), 288 (12.61), 229 (5.14), 217 (6.15), 174 (18.29), 156 (9.06), 144 (13.10), 129 (58.68), 115 (66.64), 56 (94.65), 42 (98.26); high-resolution MS (EI, 70 eV) calcd for $C_{21}H_{24}INO$ 433.0903, found 433.0904. Anal. ($C_{21}H_{24}INO$) C, H, N.

Radiochemical Syntheses. In a general procedure, solid-phase radioiodide exchange reactions were performed in a septum-closed 3-mL multidose vial by modification of our previously described method.[18,19,49] Heating was accomplished with an oil bath, and the temperature was that of the equilibrated oil bath.

A solution of $(NH_4)_2SO_4$ (5.0 mg in 15 μL of deionized H_2O), (±)-5-iodobenzovesamicol (**5**) (20 μg in 20 μL of ethanol), and three layers of 3-mm glass beads were successively placed in a 3-mL multidose vial; 8 mCi of $Na^{125}I$ was added via syringe. The syringe was rinsed with acetone (2 × 50 μL), which was added to the reaction vial, and the sides of the reaction vial were washed down with EtOH (2 × 50 μL). The reaction vial was crimped and fitted with a distillate condenser, and the reaction mixture was heated to dryness at 145 °C in an oil

bath. Air (20 mL) was then slowly introduced via syringe through the septum of the reaction vial for 1 min to completely dry the reaction mixture. The reaction mixture was maintained at 155 °C for an additional 30 min, cooled to room temperature, dissolved in acetone (1 mL), and subjected to radio-TLC analysis (silica gel, $CHCl_3$/EtOH, 97/3). The radiochemical yield was 89%.

Following removal of the acetone by a gentle stream of argon, the dry reaction mixture was dissolved in CH_2Cl_2 (3 × 1 mL) and transferred to a silica Sep-Pak cartridge. Elution with hexane (6 mL) followed by hexane/ethyl acetate (9/1, 14 mL) removed the less polar impurities. Further elution with hexane/ethyl acetate (1/1, 8 mL) afforded 6.36 mCi of (±)-5-[^{125}I]iodobenzovesamicol ([^{125}I]**5**) in >96% radiochemical purity as determined by radio-TLC. The specific activity of the exchanged product was then estimated based on starting quantities of substrate and final radiochemical yield. Prior to biological evaluation, the hexane/ethyl acetate solution was evaporated to dryness under argon flow. The radioligand was formulated in ethyl alcohol/0.005 M acetate-buffered saline, pH 4.5 (5/95).

Tissue Distribution Studies in Mice. These studies were performed in 6−8 week old female CD-1 mice (18−32 g) obtained from Charles River, Inc. For each time interval evaluated, 4−5 mice under light ether anesthesia received bolus tail-vein injections of 7.0−13.3 μCi of racemic ^{125}I-labeled compound in 0.05−0.10 mL of isotonic acetate buffer, pH 4.5. The mice were sacrificed by decapitation at designated time intervals. The whole brain was rapidly separated from the skull, and the entire cerebral cortex, cerebellum, striatum, hippocampus, and approximately 75% of the hypothalamus were excised, weighed, and counted on a Packard 5260 Autogamma counter. The remainder of the brain was also weighed and counted, as were samples of four peripheral tissues and blood (0.10 mL) obtained by cardiac puncture. Tissue concentrations are expressed as % injected dose/g of tissue (% dose/g ± SD) normalized to a standard 25-g mouse weight.

Receptor Screening Assays. Receptor screening assays were performed by NovaScreen, a commercial research and development support service, located in Hanover, MD. The study was funded by the National Institute of Mental Health as part of their Psychoactive Drug Discovery Program.

1. Binding Assays. The hydrochloride salt of 7-IBVM (**14**) dissolved in 4% aqueous dimethyl sulfoxide was screened in a panel of 26 assays ($N = 2$) at a concentration of 1×10^{-5} M. Binding of 7-IBVM (**14**) was determined in 14 neurotransmitter receptor assays (adenosine, α_1, α_2, β, dopamine 1, dopamine 2, $GABA_A$, $GABA_B$, serotonin 1, serotonin 2, NMDA, kainate, quisqualate, and strychnine-sensitive glycine), five regulatory site assays (central benzodiazine, strychnine-insensitive glycine, PCP, MK-801, and σ), four ion channel binding assays (calcium type N, calcium types T and L, chloride, and low-conductance potassium), and three second messenger site assays (forskolin, phorbol ester, and inositol triphosphate).

2. σ-Receptor Binding Assay. Solutions of [^{3}H]di(2-tolyl)-guanidine (DTG)[50] were added to preparations of guinea pig brain membranes containing varying concentrations of 7-IBVM (**14**) in 50 mM Tris-HCl buffer, pH 7.4. The final concentration of DTG was 2.0 nM. The preparations were incubated at 25 °C for 60 min. The reaction was terminated by rapid vacuum filtration onto glass fiber filters. The amount of radioactivity retained on the filters was determined by liquid scintillation counting. The degree of nonspecific binding was determined in the presence of 10 μM haloperidol. Reference compounds using this assay had the following K_i values: haloperidol (2.8 nM), DTG (31.0 nM), (+)-3-PPP (124.6 nM), (+)SKF-10047 (1263.3 nM).

Drug-Blocking Studies. Haloperidol lactate (im haldol brand) was purchased from McNeil Pharmaceutical, Spring House, PA, and spiroperidol hydrochloride from RBI, Natick, MA. Female CD-1 mice (20−27 g), prepared as described above, received ip injections of either haloperidol (1.0 mg/kg in 10−15 μL of vehicle) or spiroperidol (0.1 mg/kg in 150−200 μL of physiological saline); control animals received equal volumes of vehicle; $N = 5$/group. One hour later, all mice received bolus tail-vein injections of 9.5−10.5 μCi of racemic 7-[^{125}I]IBVM ([^{125}I]**14**) in 150−200 μL of isotonic acetate buffer. The mice were sacrificed 24 h after radiotracer injection; regional brain and peripheral tissue samples were processed as described above. The same experiment was repeated at higher drug dose levels: haloperidol (2.5 mg/kg), spiroperidol (0.5 mg/kg); $N = 5$/drug group and $N = 2$ for the control group.

IC_{50} Determinations. The competitive binding assay employed here is a modification of that reported by Ruberg[51] which was derived from the in vitro autoradiographic method of Marien and co-workers.[52] In place of [^{3}H]vesamicol, we utilized a tracer developed recently in our laboratory, [^{3}H]-MABV, to avoid possible complications that might arise from the reported binding of [^{3}H]vesamicol to a second site called the vesamicol-binding protein.[7] [^{3}H]MABV (82 Ci/mmol) was labeled using a procedure analogous to that described for the preparation of [^{11}C]MABV[24] except that [^{3}H]methyl iodide was used in place of [^{11}C]methyl iodide. There was no significant difference in results obtained from incubations conducted at 25 or 37 °C. Therefore, the latter temperature was utilized.

1. Tissue Preparation. Five female Sprague−Dawley rats weighing 200−250 g were decapitated while under ether anesthesia, and whole cerebral cortices were quickly removed, pooled, and homogenized in 9 vol of pH 7.2 Tris buffer (120 mM NaCl, 50 mM Tris, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, HCl to adjust pH) with a polytron at a setting of 7 for 10−15 s. The crude homogenate was centrifuged at 50000*g* for 1 h, the supernatant discarded, and the pellet resuspended in 5 vol of buffer and recentrifuged at 50000*g* for 1 h. The resulting pellet was reserved and reconstituted in buffer to 10 times the original volume of tissue to furnish the final homogenate preparation. Protein concentration, measured by the Bradford assay, was 3−4 μg/μL. Preparations were stored at −70 °C until used.

2. Binding Assay Conditions for IC_{50} Determinations. Incubations were performed at 37 °C for 1 h in a total volume of 500 μL of Tris-salt buffer, containing test drug dissolved in 90/10 Tris buffer/ethanol:Polysorbate-80 (4:1), 0.6 nM [^{3}H]-MABV (~400 fmol of MABV/sample), and 150 μg of cortex homogenate protein (~30−40 fmol of specific binding sites). Nonspecific binding of [^{3}H]MABV was determined in the presence of either 4 μM unlabeled (±)-MABV or 10 μM (±)-vesamicol. Incubations were terminated by filtration through 0.1% poly(ethylenimine)-treated Whatman GF/B filters in a Brandel cell harvester followed by washing of the filters with fill-suction cycles with room temperature buffer (~10 mL total volume) and a final rinse with deionized water. Filters were removed and counted by liquid scintillation using 10 mL of scintillation cocktail. The mean ± SD of each data set was calculated and corrected versus negative controls (no homogenate). The percent of inhibition of control [^{3}H]MABV binding versus the log of the test drug concentration was plotted to determine the IC_{50} value for each drug. Inhibition data were fit to a single binding site model using a nonlinear least-squares algorithm. The equation used to relate specific binding relative to control specific binding (% of control binding) to the concentration of inhibitor added (I) is

$$\%\ \text{of control binding} = (100.0 - \text{BB})\frac{IC_{50}}{IC_{50} + I}$$

where BB is a constant background binding term that represents the nondisplaceable binding seen at the highest concentrations of added inhibitor. This nondisplaceable binding may represent binding of [^{3}H]MABV to a second, low-affinity binding site.

In Vivo Autoradiography. Female Sprague−Dawley rats underwent cannulation of a femoral vein under light ether anesthesia. Following recovery for a minimum of 1 h, 0.75−1.0 mCi of (−)-5-[^{125}I]IBVM ((−)-[^{125}I]**5**) was injected as an intravenous bolus followed immediately by flushing of the catheter with fresh saline solution. Animals were killed by injection of pentobarbital and KCl for cardioplegia followed by decapitation. Brains were dissected, cut coronally, caudal

to the inferior colliculus, and frozen in crushed dry ice. Brains were mounted on microtome chucks and coated with frozen section embedding medium (Lipshaw, Inc., Detroit, MI) to prevent desiccation. Triplicate, 20 μm-thick, coronal sections were cut throughout the brain at intervals of 0.5 mm on a cryostat microtome at −18 °C. Frozen sections were mounted on chilled glass microscope slides and rapidly desiccated on a hotplate at 60−70 °C. Autoradiograms of radioactivity distribution were obtained by apposition of the tissue to tritium-sensitive X-ray film (Hyperfilm-^{3}H; Amersham) for 3−7 days. Films were scanned and analyzed with the use of a microcomputer-assisted video densitometry system (MCID system, Imaging Research, St. Catherines, Ontario).

Correlation Between ChAT and (−)-5-[^{123}I]IBVM ((−)-[^{123}I]5) in Mouse Brain. 1. Tissue Distribution Analysis of (−)-[^{123}I]5. The tracer (−)-[^{123}I]**5** was synthesized by the method of Van Dort and co-workers.[19] Five female CD-1 mice under light ether anesthesia were injected via the femoral vein with 35−46 μCi of (−)-[^{123}I]**5**. Four hours later, the animals were decapitated and selected tissues were quickly excised, weighed, and counted in an autogamma counter. The tissues were stored in their counting vials at −70 °C for 6 or more days (>10 iodine-123 half-lives) and thawed as needed for subsequent ChAT assay.

2. ChAT Assay. Brain samples from five of the mice were homogenized in a Kontes glass tissue homogenizer; heart tissue samples were homogenized with a polytron; 50 μL of a buffered choline solution,[25] prepared from 1.49 g (4.0 mmol) of EDTA (disodium salt), 6.5 mg (0.02 mmol) of eserine (physostigmine), and 368 mg (2.0 mmol) of choline hydrobromide dissolved in 100 mL of 0.05 M Tris-HCl buffer, was mixed with 10 μL of [^{14}C]acetyl-*S*-coenzyme A (DuPont NEN; CAT assay grade, 0.01 μCi/μL, 2.5 × 10^{-3} M). The above mixture was combined with 40 μL of brain homogenate supernatant and incubated at ambient temperature for 15 min. The final concentrations of the reagents in the incubation mixture were 2.5 × 10^{-4} M acetyl-*S*-coenzyme A (2.2 × 10^3 dpm/μL), 8.0 × 10^{-3} M choline hydrobromide, 2 × 10^{-2} M EDTA, and 1 × 10^{-4} M eserine.

The incubation mixture was carefully transferred by micropipet to a preequilibrated neutral alumina Sep-Pak and eluted with acetone−water (9:1, v/v). Fifteen fractions of approximately 0.5 mL each were collected directly into scintillation vials, mixed with 10 mL of biodegradable scintillation fluid, and counted. Control studies were conducted demonstrating that under typical assay conditions, elution of [^{14}C]acetylcholine was complete after 15 fractions (>80% recovery), while [^{14}C]acetyl-*S*-coenzyme A was completely retained.

Statistical Analysis. Data are expressed as the mean ± SD. To compare means, the two-tailed *t*-test for unpaired samples was used. Linear regression analysis was performed with the linear curve-fit option in CA-Cricket Graph III, version 1.5.2, for the Macintosh computer, Computer Associates International, Inc., Islandia, NY.

Humane Treatment of Animals. In all experiments with living animals, studies were performed in accordance with the United States Department of Agriculture Animal Welfare Act.

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