Synthesis and Characterization of Fluorescent Ligands for the Norepinephrine Transporter: Potential Neuroblastoma Imaging Agents

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Radiolabeled *m*-iodobenzylguanidine (MIBG) is a tumor-seeking radioactive drug used in the diagnosis and treatment of pheochromocytomas and neuroblastomas. It is transported into the tumor cells by the neuronal norepinephrine (NE) transporter (NET) which is expressed in almost all neuroblastoma cells. Here, we describe the synthesis and some pharmacological properties of a series of fluorescent compounds structurally related to the NET substrate, MIBG, or to the NET inhibitors, (−)-(*2R,3S*)-cocaine and nisoxetine. Three of 10 synthesized fluorescent compounds, 1-(1-naphthylmethyl)guanidinium sulfate (**1**), 1-[2-(dibenz[*b,f*]azepin-5-yl)ethyl]-guanidinium sulfate (**2**), and (*2R,3S*)-2β-ethoxycarbonyl-3β-tropanyl 5-(dimethylamino)naphthalene-1-sulfonate (**6**), exhibited high affinity (IC_{50} about 50 nM) for the NET. The nisoxetine derivatives **8** (*rac*-*N*-[(3-methylamino-1-phenyl)propyl]-5-(dimethylamino)-1-naphthalenesulfonamide) and **9** (*rac*-4-[(3-methylamino-1-phenyl)propyl]amino-7-nitro-2,1,3-benzoxadiazole) and especially the guanidine derivative **4** (1-[4-(4-phenyl-1,3-butadienyl)benzyl]guanidinium sulfate) which are characterized by intermediate affinity for the NET (IC_{50} 370−850 nM) caused significant and nisoxetine-sensitive cell fluorescence. At least the guanidine derivative **4** might represent a potentially useful agent for imaging of neuroblastoma cells.

Introduction

Neuroblastomas and pheochromocytomas are neural crest-derived tumors of the sympathetic nervous system. Neuroblastomas belong to the most common extracranial solid tumors in young children. About 10−20% of neuroblastomas show spontaneous regression, and few mature to benign ganglioneuroma, whereas most patients with metastatic disease have an unfavorable prognosis for long-term survival.[1,2] Although the tumor responds initially to chemotherapy, it regrows from residual resistant cells resulting in a fatal outcome for the patients. Since bone marrow is the predominant (80% of cases) metastatic site in neuroblastoma, serial bone marrow aspirations are performed during the course of the disease to evaluate response to therapy. In this respect the detection of minimal residual disease is important. A specific technique to image those cells would be therefore a powerful tool. *m*-Iodobenzylguanidine (MIBG) is considered a selective agent which is accumulated by the neuroblastoma cells;[3] however, its accumulation is microscopically not visible. Thus, a fluorescent substance with a neuroblastoma specificity similar to that of MIBG would be helpful.

As a tumor of the sympathetic nervous system, neuroblastomas have many properties in common with this system, such as synthesis, storage, release, and reuptake of norepinephrine (NE). Thus, NE and its metabolites are renally excreted in larger than normal amounts in patients with neuroblastomas, and elevated urinary levels are used in the diagnosis of this tumor.[4]

Tumors of neuroectodermal origins such as neuroblastomas have preserved the ability of neuronal cells to take up NE through the neuronal NE transporter (NET), a specific transport system (also termed "uptake-1") which is located in the neuronal plasma membrane (see below). MIBG is a metabolically stable NE analogue derived from the adrenergic neuron-blocking agents bretylium and guanethidine. MIBG accumulates in neuroendocrine tissues and thereby competes with NE for uptake and storage.[4] Due to these features, MIBG labeled with either ^{131}I or ^{123}I has been used for about 15 years in the scintigraphic visualization (diagnosis) and therapy of pheochromocytomas and neuroblastomas.[5−7]

Uptake of MIBG has been studied in human neuroblastoma cell lines such as SK-N-SH-SY5Y cells.[8−10] The sodium- and chloride-dependent NET is inhibited by (−)-(*2R,3S*)-cocaine, nisoxetine, or desipramine.[11−12]

Fluorescent ligands are useful tools to study receptors.[13] Fluorescent ligands with affinity for the NET, which is often overexpressed in neural crest tumors such as neuroblastomas, could represent potentially useful agents for the diagnosis of these tumors. In the present study, it was our goal to synthesize a series of fluorescent compounds structurally related to MIGB (**1**−**5**), (−)-(*2R,3S*)-cocaine (**6**, **7**), and nisoxetine (**8**−**10**) as potential ligands for the NET. Using the human neuroblastoma cell line SK-N-SH-SY5Y, we also investigated their affinities for the NET. As fluorescent markers, we selected condensed aromatic groups (**1**), aryl olefins such as stilbene derivatives (**2**, **3**), 1,4-diphenyl-1,3-butadiene

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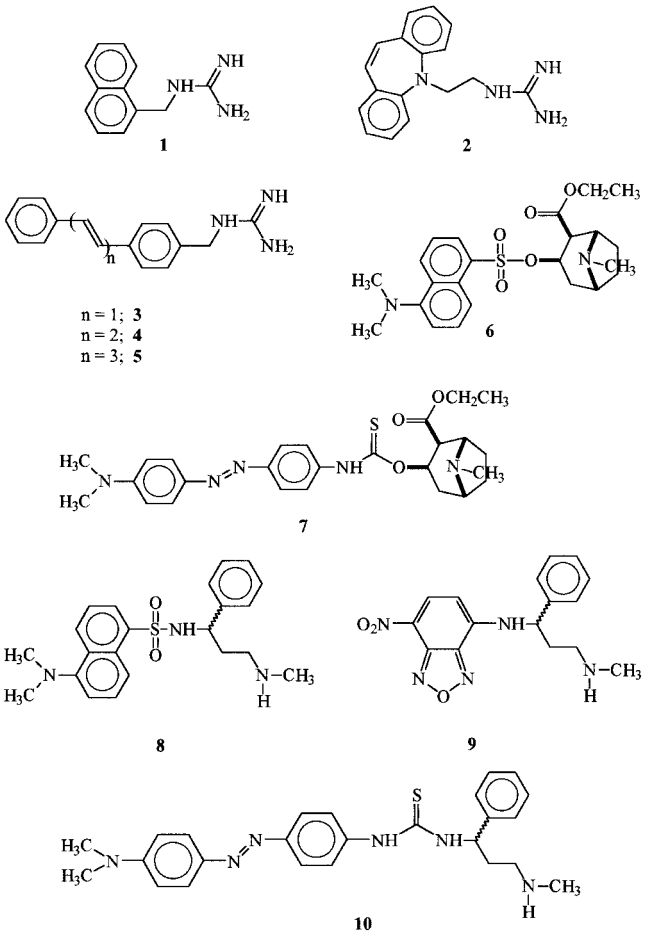


Figure 1. Chemical structures and code numbers of fluorescent norepinephrine transporter ligands (for chemical names, see Table 1).

(**4**), and 1,6-diphenyl-1,3,5-hexatriene (**5**), the dansyl group (**6**, **8**), azobenzene functions (**7**, **10**), and the 7-nitro-2,1,3-benzoxadiazole (NBD) group (**9**).

Chemistry

Figure 1 shows the chemical structures and the code numbers of the synthesized fluorescent derivatives of guanidine (**1**−**5**), (−)-(*2R,3S*)-cocaine (**6**, **7**), and nisoxetine (**8**−**10**). In Table 1 their chemical names together with wavelengths for optimal excitation and maximal fluorescence emission in aqueous solution are given. All 10 substances are new, i.e., they have not yet been described before. However, from 1-(1-naphthylmethyl)-guanidinium sulfate (**1**), a corresponding iodide has already been synthesized earlier.[14]

All five guanidines were synthesized from their corresponding amines by treatment with *S*-methylthiouronium sulfate. These compounds are not iodinated in the meta-position such as in MIBG, but they contain a successively enlarged π-system, which is responsible for their fluorescent properties as described for diphenylbutadiene compounds.[15,16] The conjugated C=C double bonds in 1-[4-(4-phenyl-1,3-butadienyl)benzyl]guanidinium sulfate (**4**) and 1-[4-(6-phenyl-1,3,5-hexatrienyl)-benzyl]guanidinium sulfate (**5**) were obtained through a Wittig reaction using cinnamaldehyde as the main building block (Scheme 1). The main difficulties in the synthesis of the fluorescent guanidine derivatives **3**−**5** were due to their low solubility in all solvents except DMSO; this also complicated their purification and analysis.

The fluorescent (−)-(*2R,3S*)-cocaine derivatives (*2R,-3S*)-2β-ethoxycarbonyl-3β-tropanyl 5-(dimethylamino)-naphthalene-1-sulfonate (**6**) and (*2R,3S*)-*O*-(2β-ethoxycarbonyl-3β-tropanyl)-4-(dimethylamino)azobenzole-4′-thiocarbamide (**7**) were synthesized from ethylecgonine by condensation with dansyl chloride (for **6**) or 4-(dimethylamino)-4'-isocyanoazobenzene (for **7**). Ethylecgonine was obtained through hydrolysis of (−)-(*2R,3S*)-cocaine in ethanolic HCl (Scheme 2).

The fluorescent nisoxetine derivatives *rac*-*N*-[(3-methylamino-1-phenyl)propyl]-5-(dimethylamino)-1-naphthalenesulfonamide (**8**), *rac*-4-[(3-methylamino-1-phenyl)propyl]amino-7-nitro-2,1,3-benzoxadiazole (**9**), and *rac*-*N*-[4-(4-(dimethylamino)phenylazo)phenyl]-*N′*-[(3-methylamino-1-phenyl)propyl]thiourea (**10**) were synthesized in seven steps from 3-chloropropiophenone. The hydroxy function, which was obtained by reduction of the carbonyl group, was replaced by an amino function using the Mitsunobu reaction with potassium phthalimide followed by hydrazinolysis. In the following, the fluorescent groups were introduced by reaction of the amine function with dansyl chloride (for **8**), NBD chloride (for **9**), or 4-(dimethylamino)-4'-isocyanoazobenzene (for **10**) (Scheme 3).

Biological Studies

To obtain a measure of affinity (potency) of the various ligands for the NET, IC_{50} values of the compounds for inhibition of uptake of [^{3}H]NE in human SK-N-SH-SY neuroblastoma cells were determined. All compounds inhibited [^{3}H]NE uptake with Hill coefficients of the inhibition curves not significantly different from unity. As shown in Table 2, the rank order of potency for inhibition of NE uptake was **2** > **1** = **6** > **3** > **8** = **4** > **5** = **9** > **10** > **7**. The affinities of the guanidine derivatives were all in the submicromolar range and thus similar to that of MIBG.[8,9] This indicates that the replacement of the benzyl group of MIBG by a naphthylmethyl residue (**1**) or a dibenzoazepine (*cis*-stilbene structure) residue (**2**) or the addition of a styryl group in the para-position of benzylguanidine (**3**) caused no dramatic affinity change in these MIBG derivatives (Table 2). It remains to be shown whether these fluorescent MIBG derivatives are transported by the NET. Due to their relatively high affinity for the NET, they might represent potential candidates for fluorescent labeling of cells expressing the NET such as neuroblastoma cells.

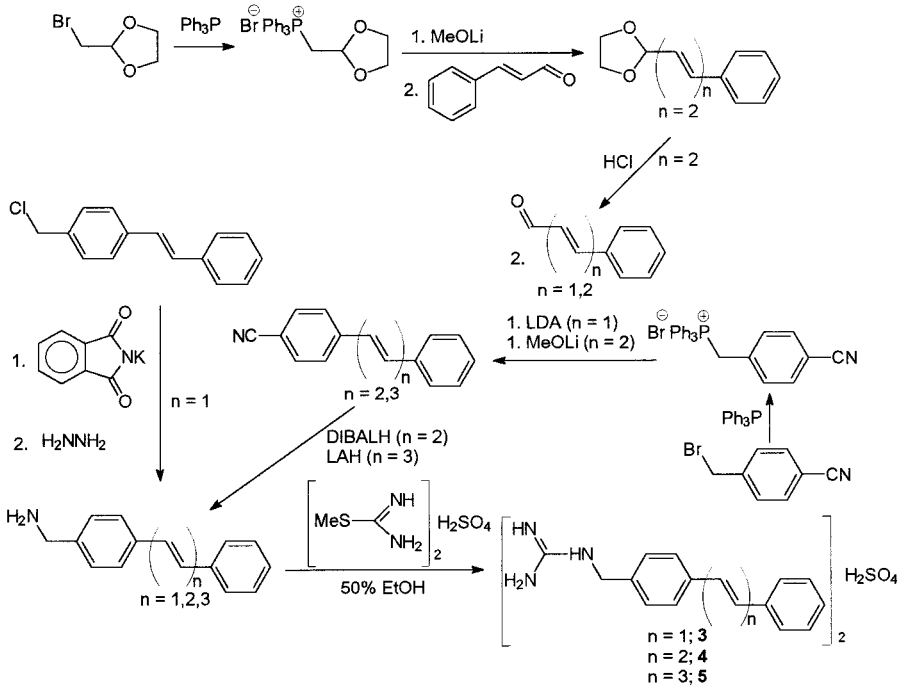
The (−)-(*2R,3S*)-cocaine IC_{50} values for inhibition of NE uptake by means of the NET are at about 1 μM.[11,17] The introduction of a fluorescent 4-(dimethylamino)-azobenzene group into the (−)-(*2R,3S*)-cocaine molecule (**7**) caused only a slight decrease in the affinity for the NET. However, the (−)-(*2R,3S*)-cocaine derivative with a fluorescent dansyl group (**6**) exhibited even increased affinity for the NET (Table 2) compared to (−)-(*2R,3S*)-cocaine. The high affinity to the NET of this fluorescent compound indicates that it might be used for fluorescent imaging of NET expressing tumors.

Nisoxetine is known to bind with high affinity (K_D about 5 nM) to the NET.[18,19] However, and in contrast

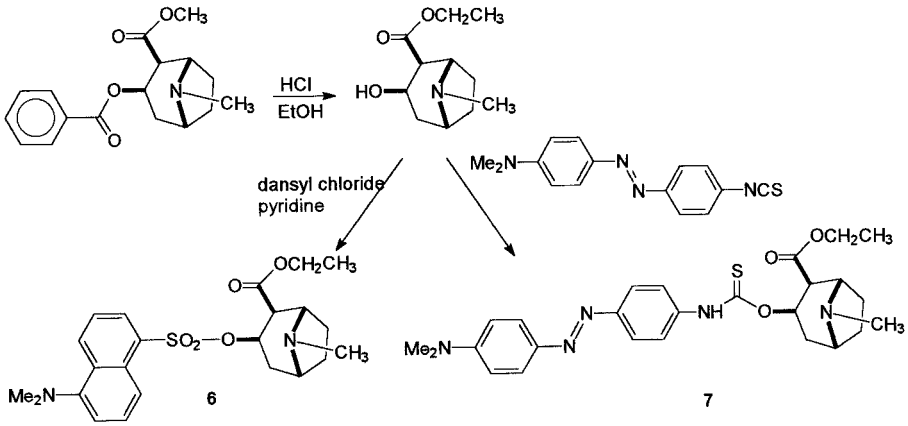
Table 1. Fluorescent Derivatives of Guanidine, (−)-(*2R,3S*)-Cocaine, and Nisoxetine and Their Optimal Excitation and Maximal Fluorescence Emission Wavelengths

derivatives of	code no.	chemical name	excit/emiss (nm)
guanidine	**1**	1-(1-naphthylmethyl)guanidinium sulfate	313/334, 672
	2	1-[2-(dibenz[*b,f*]azepin-5-yl)ethyl]guanidinium sulfate	384/440
	3	1-(*trans*-stilben-4-ylmethyl)guanidinium sulfate	342/458
	4	1-[4-(4-phenyl-1,3-butadienyl)benzyl]guanidinium sulfate	340/384
	5	1-[4-(6-phenyl-1,3,5-hexatrienyl)benzyl]guanidinium sulfate	352/448
(−)-(*2R,3S*)-cocaine	**6**	(*2R,3S*)-2β-ethoxycarbonyl-3β-tropanyl 5-(dimethylamino)naphthalene-1-sulfonate	356/518
	7	(*2R,3S*)-*O*-(2β-ethoxycarbonyl-3β-tropanyl)-4-(dimethylamino)azobenzole-4′-thiocarbamide	340/681
nisoxetine	**8**	*rac*-*N*-[(3-methylamino-1-phenyl)propyl]-5-(dimethylamino)-1-naphthalenesulfonamide	402/504
	9	*rac*-4-[(3-methylamino-1-phenyl)propyl]amino-7-nitro-2,1,3-benzoxadiazole	481/521
	10	*rac*-*N*-[4-(4-(dimethylamino)phenylazo)phenyl]-*N*′-[(3-methylamino-1-phenyl)propyl]thiourea	339/684

Scheme 1



Scheme 2



to the corresponding (−)-(*2R,3S*)-cocaine derivatives, the fluorescent dansyl (**8**) or 4-(dimethylamino)azobenzene (**10**) derivatives of nisoxetine exhibited a relatively low affinity for the NET (Table 2) compared to nisoxetine. This also holds true for the niosoxetine derivative with a 7-nitro-2,1,3-benzoxadiazole group (**9**). A prerequisite for a useful fluorescent ligand for labeling NET-expressing cells is either to be transported and intracellularly accumulated or to bind to the NET with high affinity and thus dissociate slowly from the target protein. Since the fluorescent nisoxetine derivatives are presumably not transported and exhibit relatively low affinity for the NET, they are probably less useful imaging ligands.

For microscopic observation of fluorescence, the emitted fluorescence of the substance should be strong and in the visible range (380−800 nm). Determination of the emission wavelengths of the various compounds (Table 1) showed that compounds **1**−**3** caused in the visible range only relatively weak fluorescence intensities. The

Scheme 3

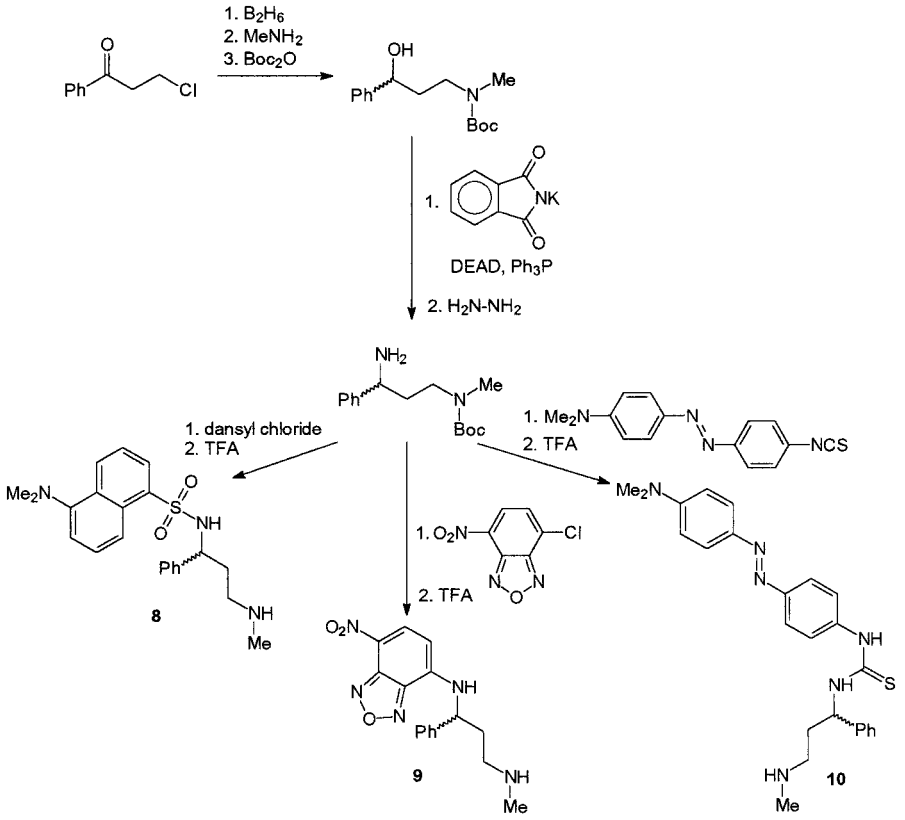


Table 2. IC_{50} Values of the Various Ligands for Inhibition of [^{3}H]NE Uptake into Human SK-N-SH-SY5Y Neuroblastoma Cells[a]

compd	log IC_{50} (−log M)	IC_{50} (μM)
2	7.52 ± 0.35	0.03
1	7.20 ± 0.34	0.06
6	7.18 ± 0.45	0.06
3	6.92 ± 0.33	0.12
8	6.43 ± 0.07	0.37
4	6.40 ± 0.19	0.40
5	6.07 ± 0.54	0.85
9	6.07 ± 0.43	0.85
10	5.99 ± 0.28	1.02
7	5.88 ± 0.12	1.32

[a] SK-N-SH-SY5Y cells were incubated with 10 nM [^{3}H]NE in the presence of the indicated ligands in 5 different concentrations and in the absence or presence of 10 μM nisoxetine (to determine nonspecific uptake). Shown are means ± SEM from 3-4 determinations of IC_{50} values for inhibition of specific [^{3}H]NE uptake.

other synthesized compounds showed clearly stronger fluorescence peaks.

In a preliminary series of experiments, the fluorescent compounds listed in Table 1 were studied for their ability to label human SK-N-SH-SY neuroblastoma cells due to transport by or binding to the NET and for their specific fluorescence qualities. After 30 min of incubation of the cells with one of the fluorescent compounds (at a concentration of 10 μM and in the absence or presence of nisoxetine), nisoxetine-sensitive fluorescence of the cells was examined qualitatively by fluorescence microcopy and quantitatively by measuring spectrofluorimetrically the cell extract. Inspection of the cells under the fluorescence microscope showed that only five of the compounds (**4**−**6**, **8,** and **9**) caused a clearly higher intensity of substance-induced blue or yellow cellular fluorescence in cells incubated in the absence of the NET inhibitor nisoxetine than in its presence. In addition, only in cells incubated with one of these five compounds the extracts of cells incubated in the absence of nisoxetine (compared to those in its presence) showed a higher content of the fluorescent compound; this is illustrated in Figure 2 for the guanidine derivative **4**.

For cells incubated with compounds **5**, **6**, **8**, **9**, and **4**, the substance-specific fluorescence peak was by a factor of 1.12, 1.29, 2.22, 2.56, and 2.70, respectively, higher in the extracts from cells incubated in the absence of nisoxetine than in its presence. Thus, the guanidine derivative **4** showed the highest nisoxetine-sensitive cellular retention which presumably was due to NET-mediated uptake of this compound. However, also the nisoxetine derivatives **8** and **9** which are not expected to be transported but to bind to the NET exhibited a more than 2-fold difference in the substance-specific fluorescence peaks. Since net rates of uptake of transported substrates decrease with time and initial rates of specific uptake relative to "uptake" by diffusion are highest at nonsaturating substrate concentrations, a reduction in the incubation time (to measure initial uptake rates) and a reduction of the substrate concentration (below IC_{50} or K_m) should result in an increase in the specific uptake of a substrate compared to nonspecific diffusion. Whether this holds true for the guanidine derivative **4** remains to be shown.

Conclusions

Methods have been developed for the preparation of 10 fluorescent derivatives of the NET ligands MIBG, (−)-(*2R,3S*)-cocaine, and nisoxetine. All 10 compounds inhibited uptake of [^{3}H]NE in human neuroblastoma

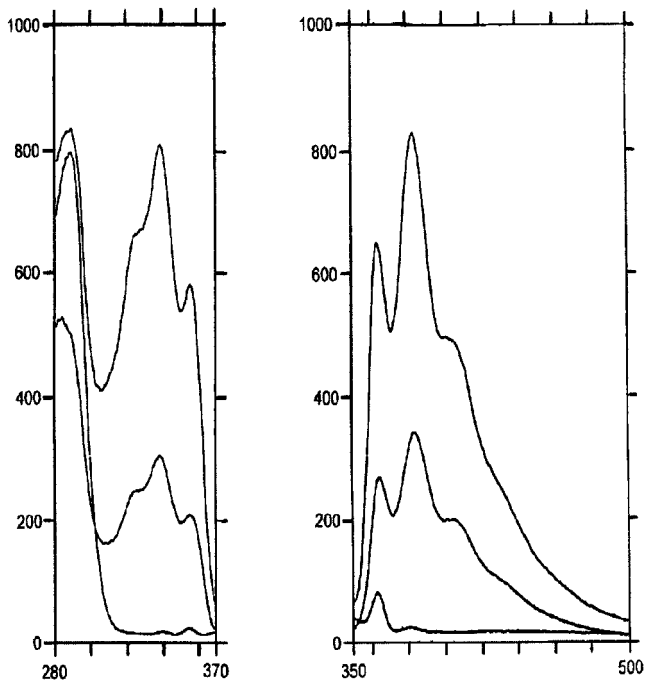


Figure 2. Excitation spectra (left) and emission spectra (right) of extracts of human SK-N-SH-SY neuroblastoma cells incubated with the fluorescent guanidine derivative **4** in the absence (uppermost curves) or presence (middle curves) of nisoxetine; spectra of cells incubated in the absence of the fluorescent compound (lowest curves).

cells with IC_{50} values ranging from 30 to 1320 nM. Two of the five synthesized fluorescent MIBG derivatives, 1-(1-naphthylmethyl)guanidinium sulfate (**1**) and 1-[2-(dibenz[*b,f*]azepin-5-yl)ethyl]guanidinium sulfate (**2**), as well as one of the two fluorescent (−)-(*2R,3S*)-cocaine analogues, (*2R,3S*)-2β-ethoxycarbonyl-3β-tropanyl 5-(dimethylamino)naphthalene-1-sulfonate (**6**), inhibited [^{3}H]-NE uptake with high potency (IC_{50} = 30 and 60 nM, respectively). However, only the nisoxetine derivatives *rac-N*-[(3-methylamino-1-phenyl)propyl]-5-(dimethylamino)-1-naphthalenesulfonamide (**8**) and *rac*-4-[(3-methylamino-1-phenyl)propyl]amino-7-nitro-2,1,3-benzoxadiazole (**9**) and especially the guanidine derivative 1-[4-(4-phenyl-1,3-butadienyl)benzyl]guanidinium sulfate (**4**) caused more than 2-fold (nisoxetine-sensitive) cellular retention and, when inspected under the fluorescence microscope, nisoxetine-sensitive cell fluorescence. After improvement of some experimental conditions, at least the guanidine derivative **4** might represent a potentially useful diagnostic agent for labeling of neuroblastoma tumor cells.

Experimental Section

A. General Methods. Melting points were determined with a Reichert Kofler-Heiztisch microscope and are uncorrected. Thin-layer chromatography was performed on 0.2-mm silica gel 60 F_{254} sheets (Merck, Darmstadt). ^{1}H and ^{13}C NMR spectra of all compounds were consistent with the assigned structure. NMR spectra were recorded on a Bruker AC 200 instrument, and chemical shifts are reported in ppm (δ) relative to the residual signal on the deuterated solvent ($CHCl_3$, δ 7.26; CHD_2-OD, δ 3.30; $(CD_3)_2SO$, δ 2.49). Purity and structures of precursors were checked by gas chromatography combined with mass spectrometry (GC-5890 and MSD-5970 from Hewlett-Packard). Excitation and emission spectra were recorded on a Shimadzu spectrophotometer RF-5000. For observation of the fluorescence, a Leitz Diavert microscope was applied with an Ploemopak illuminator and with Leitz filters A (450−490 nm) or G (350−460 nm). Flash chromatography was performed with silica gel (0.04−0.063 mm) from Merck Darmstadt. All chemicals for syntheses were obtained from Aldrich Chemical Co., except NBD-chloride which was obtained from Sigma and (−)-(*2R,3S*)-cocaine HCl which was obtained from Merck Darmstadt. Dansyl chloride had to be separated from the acid by flash chromatography, eluting with dichloromethane. If not stated otherwise, all substances used for biological tests were obtained from Sigma.

B. Syntheses. 1-(1-Naphthylmethyl)guanidinium Sulfate (1). A solution of 1-(aminomethyl)naphthalene (0.79 g) and *S*-methylthiouronium sulfate (0.7 g) was heated under reflux for 24 h in 15 mL of boiling 50% ethanol. The solvent was removed in vacuo followed by addition of another portion of the 50% ethanol which was subsequently again removed in vacuo. This procedure was repeated three times. The precipitated product was recrystallized from water to give 0.71 g (57%) of **1** as a white solid. Mp: 245−247 °C. ^{1}H NMR (200 MHz, DMSO-d_6): 4.68 (2 H, s, CH_2), 7.3−8.1 (7 H, m, arom), 7.9 (4 H, s, $(H_2N)_2C$), 8.96 (1 H, s, HNC_2). ^{13}C NMR (50.3 MHz, DMSO-d_6): 42.28 (1 C, CH_2), 123.27 (1 C, C^8), 123.98 (1 C, C^3), 125.50 (1 C, C^6), 125.83 (1 C, C^7), 126.21 (1 C, C^4), 127.54 (1 C, C^2), 128,. 5 (1 C, C^5), 130.45 (1 C, C^{8a}), 132.95 (1 C, C^{4a}), 133.21 (1 C, C^1), 157.54 (1 C, CN_3).

1-[2-(Dibenz[*b,f*]azepin-5-yl)ethyl]guanidinium Sulfate (2). 1.44 mL of bromoacetonitrile was added to a mixture of 500 mg of 5*H*-dibenz[*b,f*]azepine and 2 g of sodium bicarbonate in 20 mL of dichloromethane. After heating under reflux for 48 h, the 5-(cyanomethyl)dibenz[*b,f*]azepine was separated by flash chromatography (silica, petroleum ether (PE):ethyl acetate, 10:1). A solution of 494.5 mg of 5-(cyanomethyl)dibenz-[*b,f*]azepine in 10 mL of THF was added to 161.5 mg of lithium aluminum hydride in 20 mL of THF. After heating under reflux for 2 h, the cooled solution was treated slowly with 0.3 mL of water. This mixture was heated under reflux for 2 h, after cooling the precipitated solid was filtered off, and the 5-(aminoethyl)dibenz[*b,f*]azepine was purified by chromatography, eluting with ethanol. The guanidinium sulfate was prepared from 181.0 mg of this amine by the procedure described above to give 163.5 mg (65%) of **2** as a light-yellow solid. Mp: 157−158 °C. ^{1}H NMR (200 MHz, DMSO-d_6): 3.10 (2 H, t, CH_2NAr_2), 3.75 (2 H, t, CH_2NH), 6.8−7.3 (10 H, m, arom), 7.10 (4 H, s, $(H_2N)_2C$), 8.00 (1 H, s, HNC_2). ^{13}C NMR (50.3 MHz, DMSO-d_6): 31.38 (1 C, CH_2NAr_2), 49.00 (1 C, CH_2-NH), 120.48−13.,30 (12 C, arom), 147.80 (1 C, CN_3), 149.75 (2 C, C−N).

1-(*trans*-Stilben-4-ylmethyl)guanidinium Sulfate (3). 571.2 mg of 4-(chloromethyl)-*trans*-stilbene was treated with 463.8 mg of potassium phthalimide in 10 mL of dimethylformamide. After stirring for 1 h, the mixture was heated (10 °C/min) to 120 °C. The solution was then extracted with trichloromethane, which was washed with aqueous sodium bicarbonate. The trichloromethane solution was dried, and the solvent was removed in vacuo. Crystallization from ethyl acetate gave 4-(phthalimidomethyl)-*trans*-stilbene (758.5 mg) as a white solid, which was dissolved in 20 mL of methanol, treated with 0.434 mL of hydrazine monohydrate, and then heated under reflux for 4 h. After removal of methanol and addition of 50 mL of water and 8 mL of 6 M HCl, the solution was heated under reflux for 3 h. Basification and extraction with trichloromethane gave 425.1 mg of the amine, which was transformed into the guanidinium sulfate by the procedure described above to yield 274.2 mg (45%) of **3** as a white solid. Mp: 260−262 °C. ^{1}H NMR (200 MHz, CD_3OD/TFA-*d*): 4.07 (2 H, s, CH_2), 7.03−7.60 (11 H, m, arom). ^{13}C NMR (50.3 MHz, CD_3OD/TFA-*d*): 39.83 (1 C, CH_2), 122.31−129.79 (11 C), 132.96 (2 C, C^1), 135.13 (1 C, C^4), 154.74 (1C, CN_3).

1-[4-(4-Phenyl-1,3-butadienyl)benzyl]guanidinium Sulfate (4). 6.10 g of (4-cyanobenzyl)triphenylphosphonium bromide was prepared by heating 4.09 g of triphenylphosphine and 2.84 g of 4-(brommethyl)benzonitrile in 110 mL of *p*-xylene at 130 °C for 24 h followed by washing with diethyl ether. The phosphonium bromide was added at −78 °C to a solution of

3.28 mL of diisopropylamine, 17.47 mL of butyllithium−hexane solution (1.6 M), and 25 mL of THF. After the resulting red solution was stirred for 1 h, 1.68 mL of cinnamaldehyde in 9 mL of THF was added slowly. The reaction was quenched with 1 mL of water the next morning, and the product was purified by flash chromatography (silica, cyclohexane:ethyl acetate, 10:1) to afford 2.66 g of the light-yellow 1-(4-cyanophenyl)-4-phenyl-1,3-butadiene. This was reduced with a 3-fold excess of diisobutylaluminum hydride in 30 mL of THF under reflux for 1.5 h. The resulting amine was purified by flash chromatography (silica, dichloromethane:ethanol, 30:1) to yield 2.06 g. Finally, the amine was transformed into the guanidinium sulfate by the procedure described above to give 2.24 g (40%) of **4** as a light-yellow solid. Mp: 241−243 °C. ^{1}H NMR (200 MHz DMSO-d_6/TFA-d): 4.01 (2 H, s, CH_2), 6.4−6.7 (4 H, m, =CH), 7.1−7.5 (9 H, m, arom). ^{13}C NMR (50.3 MHz, DMSO-d_6/TFA-d): 41.9 (CH_2), 125.9 (2 CH), 126.1 (2 CH), 126.2 (2 CH), 127.0, 128.0 (2 CH), 128.1 (2 CH), 128.2 (2 CH), 137.3, 138.6, 139.4, 172.2 (CN_3). Compound **4** is a mixture of *E*- and *Z*-isomers. From the gas chromatography data one can see that the *all-trans*,*E*-configurated isomer is predominant (longer retention times). One can also see that the amount of the *all-trans*-configurated product increases with time. Therefore, clear NMR assignments to the different isomers are not possible.

1-[4-(6-Phenyl-1,3,5-hexatrienyl)benzyl]guanidinium Sulfate (5). 15.88 g of 2-(bromomethyl)-1,3-dioxolane and 25.10 g of triphenylphosphine were heated over calcium chloride for 48 h at 100 °C. After the crude orange product was washed with diethyl ether, the light-yellow solid was dried in vacuo and added under nitrogen to a solution of 0.6 g of lithium in 50 mL of dried methanol. After addition again of 50 mL of dried methanol, a clear red solution was obtained to which 9.14 mL of cinnamaldehyde was added dropwise. The solution was then heated under reflux for 1 h and stirred at room temperature for a further 12 h. Reaction with 20 mL of water followed by removing the solvent resulted in a brown oil, which was turned into the corresponding aldehyde by reaction with a 10% solution of hydrochloric acid for 16 h. After basification and extraction with PE/trichloromethane (10:1), the aldehyde was purified by flash chromatography (silica, PE: trichloromethane, 1:1). The aldehyde thus obtained was then added dropwise to a solution of 0.26 g of lithium and 14.55 g of (4-cyanobenzyl)triphenylphosphonium bromide (starting material for compound **4**) in 80 mL of dried methanol. After heating under reflux for 3 h and stirring 16 h at room temperature, a yellow-green solid was filtered off, washed with methanol, and purified by flash chromatography (silica, PE: trichloromethane, 1:3) affording 3.84 g of the nitrile. This was reduced by diisobutylaluminum hydride as in the case of the nitrile precursor of compound **4** to give 3.19 g of the amine. This was purified and finally transformed into the guanidinium sulfate by the procedure described above to afford 1.60 g (74%) of **5** as a light-yellow solid. Mp: 271−274 °C. ^{1}H NMR (200 MHz, DMSO-d_6 /TFA-d): 3.98 (2 H, s, CH_2), 6.5−6.7 (6 H, m, =CH), 7.0−7.5 (9H, m, arom). ^{13}C NMR (50.3 MHz, DMSO-d_6/TFA-d): 42.9 (CH_2), 127.0 (2 CH), 127.1 (2 CH), 128.3 (2 CH), 129.0, 129.3 (2 CH), 129.6 (2 CH), 129.8 (2 CH), 130.0 (2 CH), 135.0, 137.8, 138.2, 172.0 (CN_3). Compound **5** is a mixture of *E*- and *Z*-isomers. From the gas chromatography data one can see that the *all-trans*,*E*-configurated isomer is predominant (longer retention times). One can also see that the amount of the *all-trans*-configurated product increases with time. Therefore, clear NMR assignments to the different isomers are not possible.

(*2R,3S*)-2β-Ethoxycarbonyl-3β-tropanyl 5-(Dimethylamino)naphthalene-1-sulfonate (6). (−)-(*2R,3S*)-Cocaine hydrochloride (400 mg, 1.18 mmol) was dissolved in 5 mL of ethanol and 2 mL of 6 M HCl and refluxed under a continuous stream of dry gaseous HCl for 8 h. After removing the solvent the residue was made alkaline with aqueous sodium bicarbonate and extracted with trichloromethane. (*2R,3S*)-Ethyl-3β-tropanol-2β-carboxylate (218 mg, 1.02 mmol) was isolated by flash chromatography (silica, ethanol). It was dissolved in 7 mL of dichloromethane. Dansyl chloride (158.2 mg, 1.02 mmol) and dry pyridine (0.17 mL, 2.08 mmol) were added to this solution at 0 °C, and then the entire mixture was slowly warmed to reflux and heated at reflux for 2 h followed by stirring 16 h at room temperature. A small amount of silica was added, and the solvent was removed. The crude product adsorbed on silica was purified by flash chromatography (silica, first ethyl acetate followed by ethyl acetate:acetone, 1:5) to afford **6** (207.8 mg, 46%) as a yellow fluorescent oil. ^{1}H NMR (200 MHz, $CDCl_3$/CD_3OD, 1:1): 1.29 (7.1 Hz, 3 H, t, CH_3), 1.92−2.08 (6 H, m, CH_2), 2.94 (3 H, s, NCH_3), 3.43 (6 H, s, $N(CH_3)_2$), 3.61 (1 H, m, CHCOOR), 4.04 (7.1 Hz, 2 H, q, OCH_2), 4.12 (2 H, m, $CHNR_2$), 5.83 (1 H, m, $CHOSO_2$), 6.9−7.3 (3 H, m, arom. C6, C7, C8), 7.8−8.4 (3 H, m, arom. C2, C3, C4). ^{13}C NMR (50.3 MHz, $CDCl_3$/CD_3OD, 1:1): 18.42 (CH_3), 21.36 (CH_2), 26.27 (CH_2), 33.83 (CH_2), 36.98 (NCH_3), 43.52 ($N(CH_3)_2$), 46.01 (CHCOOR), 58.31 (CHN), 61.43 (CHN), 62.75 ($CHSO_3$), 63.11 (OCH_2), 117.06, 120.17, 123.85, 124.62, 126.09, 126.83, 130.21, 131.12, 136.19 (CSO_3), 146.24 ($CN(CH_3)_2$), 170.06 (COOR).

(*2R,3S*)-*O*-(2β-Ethoxycarbonyl-3β-tropanyl)-4-(dimethylamino)azobenzole-4′-thiocarbamide (7). Under a nitrogen atmosphere, ethyl 3β-tropanol-2β-carboxylate (205 mg, 0.97 mmol) was treated with sodium hydride (29 mg, 1.2 mmol) in dry THF. After 15 min, 4-(dimethylamino)azobenzene 4′-isothiocyanate (273 mg, 0.97 mmol) was added, and the solution was stirred 16 h at room temperature. Then, 1 mL of water was added, the THF was removed, and the solution was extracted with trichloromethane. Purification by flash chromatography (silica, first dichlomethane followed by dichloromethane:ethanol, 100:1 then 25:1) afforded **7** (180 mg, 37%) as a red oil. ^{1}H NMR (200 MHz, $CDCl_3$/CD_3OD, 1:1) 1.27 (7.0 Hz, 3 H, t, CH_3), 1.85−2.99 (6 H, m, CH_2), 3.08 (3 H, s, NCH_3), 3.12 (6 H, s, $N(CH_3)_2$), 3.59 (1 H, m, CHCOOR), 4.09 (7.0 Hz, 2 H, q, OCH_2), 4.19 (2 H, m, $CHNR_2$), 5.82 (1 H, m, CHOCS), 7.8−6.8 (8 H, m, arom). ^{13}C NMR (50. 3 MHz, $CDCl_3$/CD_3OD, 1:1): 14.25 (CH_3), 24.11 (CH_2), 25.28 (CH_2), 35.27 (CH_2), 37.77 (NCH_3), 40.29 ($N(CH_3)_2$), 41.08 (CHCOOR), 49.67 (CHN), 60.12 (CHN), 61.65 (OCH_2), 63.11 (CHOCS), 111.47, 121.00, 122.93, 124.68, 143.61, 150.21, 152.33, 152.39, 170.48 and 171.50 (COOR or CS).

***rac*-*N*-[(3-Methylamino-1-phenyl)propyl]-5-(dimethylamino)-1-naphthalenesulfonamide (8).** 3-Chloro-1-phenylpropanone (2 g, 11.9 mmol) was reduced by a borane−THF solution (1 M, 35.7 mL) to give the respective alcohol (1.98 g, 11.7 mmol), which was dissolved in 15 mL of ethanol and dropped into 57 mL of aqueous 40% methylamine solution. After 16 h of stirring, methylamine and ethanol were removed in vacuo, and the resulting 3-(methylamino)-1-phenylpropanol (1.89 g, 11.46 mmol) was extracted with dichloromethane. The protecting group was introduced by dissolving the secondary amine in 20 mL of methanol, treatment with 2.75 mL of di-*tert*-butyl dicarbonate and 1.6 g of sodium monocarbonate, and sonication for 8 h. Methanol was removed in vacuo, dichloromethane was added, and the salt was filtered off. Then, 35 mL of water were added, and the protected aminopropanol (2.54 g, 9.61 mmol) was separated by extraction with dichloromethane. The hydroxy group of this compound was converted into an amino group by a Mitsunobu reaction. Thus, under a nitrogen atmosphere, the aminopropanol was dissolved in 10 mL of dry THF, and diethyl azodicarboxylate (1.51 mL, 9.61 mmol), triphenylphosphine (2.52 g, 9.61 mmol), and phthalimide (1.41 g, 9.61 mmol) were added. After 16 h of stirring at room temperature, the solvent was removed in vacuo, and the product was separated by flash chromatography (silica, first PE:dichloromethane, 3:1, followed by dichloromethane) to give the phthalimide (822 mg, 2.09 mmol). This was dissolved in ethanol, treated with 8.88 mL of hydrazine monohydrate, and heated for 1 h under reflux to give a light-yellow suspension. Ethanol was removed in vacuo, diluted sodium bicarbonate solution was added, and the mixture was extracted three times with dichloromethane. The organic solvent was twice extracted with 5% citric acid. To the aqueous citric acid solution was added dichloromethane followed by basification with sodium

bicarbonate under cooling with ice and vigorous stirring. The dichloromethane phase was separated and dried with sodium sulfate. By removing the dichloromethane in vacuo, *rac*-3-(*N*-methyl-*N*-(*tert*-butyloxycarbonyl)amino)-1-phenylpropylamine, the starting material for the formation of **8**−**10**, was obtained as a colorless oil (1.03 g, 3.84 mmol). This oil was dissolved in 40 mL of dichloromethane, and then 5.4 mL of dry pyridine and 1.04 g (3.84 mmol) of dansyl chloride were added. After 16 h of stirring, dichloromethane was removed in vacuo and the residual yellow fluorescent oil was purified by flash chromatography (silica, first dichloromethane followed by dichloromethane:ethanol, 100:1 then 25:1) to give 1.07 g (2.14 mmol) of the product. The BOC group was removed by reaction with 150 mL of TFA at 0 °C. The mixture was allowed to reach room temperature within 6 h, followed by basification with aqueous sodium bicarbonate solution. After extraction with dichloromethane, the product was separated by flash chromatography (silica, first ethanol followed by ethanol: dichloromethane, 1:1) to give **8** (567 mg, 1.4 mmol, 65%) as a yellow fluorescent oil. ^{1}H NMR (200 MHz, $CDCl_3/CD_3OD$ 1:1): 1.25 (2 H, m, CH_2), 2.09 (3 H, s, NCH_3), 2.22 (5.9 Hz, 2 H, t, CH_2N), 3.21 (6 H, s, $N(CH_3)_2$), 4.64 (5.8 Hz, 1 H, t, $CHNHSO_2$), 6.9−7.3 (8 H, m, arom), 7.7−8.5 (3 H, m, arom). ^{13}C NMR (50.3 MHz, $CDCl_3/CD_3OD$, 1:1): 33.11 (NCH_3), 38.03 (CH_2), 40.97 ($N(CH_3)_2$), 46.32 (CH_2N), 47.31 (CHN), 116.53, 122.24, 122.65, 124.72, 125.04, 126.22, 126.98, 129.17 (2 CH), 129.50 (2 CH), 129.87, 132.34, 137.31 (CSO_2), 140.01, 147.13 ($CN(CH_3)_2$).

***rac*-4-[(3-Methylamino-1-phenyl)propyl]amino-7-nitro-2,1,3-benzoxadiazole (9).** *rac*-3-(*N*-Methyl-*N*-(*tert*-butoxycarbonyl)amino)-1-phenylpropylamine (110 mg, 0.42 mmol) was dissolved in 15 mL of dichloromethane followed by addition of 89 mg of sodium bicarbonate and 83.3 mg (0.42 mmol) of 4-chloro-7-nitro-2,1,3-benzoxadiazole under a nitrogen atmosphere. The mixture was stirred 16 h at room temperature. The dichloromethane was removed in vacuo, and the product precipitated. After the residue was dissolved again in dichloromethane, the sodium bicarbonate was filtered off. Purification by flash chromatography as described for the precursor of compound **8** gave the product (127.3 mg, 0.30 mmol) as a yellow-green fluorescent oil. The BOC group was removed, and the product was purified according to the procedure described above to give **9** (71.6 mg. 0.22 mmol, 73%) as a yellow-green fluorescent oil. ^{1}H NMR (200 MHz, $CDCl_3/CD_3OD$, 1:1): 1.20 (2 H, m, CH_2), 1.98 (3 H, s, NCH_3), 2.21 (5.9 Hz, 2 H, t, CH_2N), 4.86 (5.7 Hz, 1 H, t, CHNH), 7.1−7.35 (6 H, m, arom), 8.22 (9 Hz, 1 H, d, $CHCNO_2$). ^{13}C NMR (50. 3 MHz, $CDCl_3/CD_3OD$, 1:1): 35.32 (NCH_3), 35.67 (CH_2), 48.45 (CH_2N), 58.19 (CHN), 101.28 (CNH, CNO_2), 123.06 (CH), 126.93 (2 CH), 128.68 (2 CH), 129.72 (2 CH), 137.53 (C), 145.32 (2 CN).

***rac*-*N*-[4-(4-(Dimethylamino)phenylazo)phenyl]-*N*′-[(3-methylamino-1-phenyl)propyl]thiourea (10).** *rac*-3-(*N*-Methyl-*N*-(*tert*-butoxycarbonyl)amino)-1-phenylpropylamine (108 mg, 0.41 mmol) was dissolved in 15 mL of dry THF followed by addition of sodium hydride (10.8 mg, 0.45 mmol). After 15 min, 4-(dimethylamino)azobenzole 4′-isothiocyanate (116 mg, 0.41 mmol) was added under a nitrogen atmosphere. After 16 h of stirring at room temperature, 1 mL of water was added, THF was removed, and the solution was extracted three times with dichloromethane. Purification by flash chromatography as described for the precursor of compound **8** gave the product (109.2 mg, 0.20 mmol) as a red oil. The BOC group was removed, and the product was purified according to the procedure described above to give **10** (58.8 mg, 0.13 mmol, 66%) as a red oil. ^{1}H NMR (200 MHz, $CDCl_3/CD_3OD$, 1:1): 1.28 (2 H, m, CH_2), 2.00 (3 H, s, NCH_3), 2.19 (6 Hz, 2 H, t, CH_2N), 4.68 (6 Hz, 1 H, t, CHNH), 6.8−7.8 (13 H, m, ArH). ^{13}C NMR (50.3 MHz, $CDCl_3/CD_3OD$, 1:1): 29.05 ($NHCH_3$), 39.23 (CH_2), 40.43 ($N(CH_3)_2$), 46.77 (CH_2NH), 53.45 (CHNH), 112.09, 125.66, 125.91, 126.20, 128.04, 129.25, 129.58, 140.09, 143.70, 152.20, 153,0.1, 153.48, 170.31 (CS).

C. Biological Methods. Cell culture: The human neuroblastoma SK-N-SH-SY5Y cells (ATCC, HTB 11) were plated on 24-multiwells (Falcon) and grown at 37° C (5% CO_2 incubator) in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Before use for uptake experiments, the culture medium was removed, and the cells were washed three times with Krebs−Ringer−Hepes buffer (KRH) of the following composition (mM): 125 NaCl, 2.4 K_2SO_4, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 5.6 D-(+)-glucose, 1 ascorbic acid, 25 Hepes/Tris (pH 7.4).

Affinity assay: To test the affinity for the NET of the synthesized compounds, IC_{50} values for inhibition of uptake of [^{3}H]norepinephrine ([^{3}H]NE) into the neuroblastoma cells were determined essentially as described earlier.[17] Cells were preincubated for 10 min in KRH containing 10 μM pargyline (to inhibit monoamine oxidase), 10 μM U-0521 (3,4-dihydroxy-2-methylpropiophenone (Upjohn); to inhibit catechol *O*-methyltransferase), 1 g/L bovine serum albumin, and various concentrations of the test compound (except for controls). Thereafter, cells were incubated for 5 min in the additional presence of 10 nM [^{3}H]NE (NEN-DuPont; specific activity: 52 Ci/mmol). Nonspecific uptake of [^{3}H]NE was determined in parallel control experiments carried out in the presence of 10 μM nisoxetine. At the end of the experiment, cells were rinsed three times with ice-cold KRH and then solubilized in 1.5 mL of 0.1% Triton-X-100. Radioactivity of the solubilized cells was determined by scintillation counting. Specific uptake was defined as the difference between total [^{3}H]NE uptake and uptake in the presence of nisoxetine. Data for specific uptake were related to cell protein which was determined according to the Lowry method.[20] Experiments were carried out in duplicate and are presented as means ± SEM. IC_{50} values were determined using the commercial software program GRAPHPAD INPLOT.

Fluorescent labeling: The fluorescent compounds (Table 1) were examined for their ability to label human SK-N-SH-SY neuroblastoma cells. Cells were grown until confluent in small tissue culture flaks (25 cm^2) under the conditions descibed above. After removal of the culture medium and after two washes with KRH (10 mL each), cells were preincubated for 10 min (at 37 °C) in KRH (in the absence or presence of 10 μM nisoxetine) and then incubated for 30 min (at 37 °C) in 5 mL of KRH containing either 10 μM nisoxetine (to obtain blank values) or 10 μM of the fluorescent compound (to obtain total retention) or 10 μM of the fluorescent compound together with 10 μM nisoxetine (to measure nonspecific retention). Thereafter the incubation medium was completely aspirated, and the cells were washed with 3 × 10 mL of 0.9% sodium chloride solution. After removal of the washing solution, one set of cells was inspected under the fluorescence microscope. To the other set of cells was added 400 μL of distilled water, and the cells (in closed flasks) were frozen at −70 °C for 24 h and thereafter sonicated for 10 s. The resulting suspension was transferred to small centrifuge tubes and centrifugated for 10 min at 10000*g*. From the clear supernatant excitation and fluorescence spectra were recorded using a Shimadzu RF-5000 spectrofluorophotometer. Specific cell retention of the fluorescent compound was determined from the difference between the maximum peak of substance-specific fluorescence intensity obtained in the absence and presence of the specific NET inhibitor nisoxetine.

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Supporting Information Available: FAB-MS respectively MALDI-TOF-MS of compounds **1**−**15**. This material is available free of charge via the Internet at http://pubs.acs.org.

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