Isocyanines and Pseudoisocyanines as a Novel Class of Potent Noradrenaline Transport Inhibitors: Synthesis, Detection, and Biological Activity

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Noradrenaline, the neurotransmitter of the sympathetic nervous system, is removed from the extracellular space by both neuronal and extraneuronal transport mechanisms. In the past, further investigation of the extraneuronal type of noradrenaline transporter was severely hampered by the lack of potent inhibitors. Here, we describe the synthesis of various novel noradrenaline transport inhibitors which belong to the chemical class of isocyanine and pseudoisocyanine dyes. The biological activity of these compounds was investigated in a tissue culture system (Caki-1 cells). 1,1′-Diisopropyl-2,4′-cyanine, 1,1′-diethyl-2,2′-cyanine, and 1-ethyl-1′-isopropyl-2,2′-cyanine turned out as the most potent inhibitors of the extraneuronal noradrenaline transport known so far. At 100 nmol/L, these compounds diminished extraneuronal noradrenaline transport by about 95%. Their IC_{50}'s were below 20 nmol/L. In addition, a rapid and sensitive method (based on HPLC with fluorometric detection) to measure these compounds in body fluids is reported.

Introduction

Various neurotransmitters, such as γ-aminobutyric acid, glycine, 5-hydroxytryptamine, and dopamine, are inactivated by transmembrane transport processes.[1] Noradrenaline, the neurotransmitter of the sympathetic nervous system, is removed from the synaptic cleft by transport into both the releasing neuron and the adjacent nonneuronal cells. The therapeutic effect of desipramine-like antidepressants is generally accepted to be due to the inhibition of the neuronal type of noradrenaline transporter in the central nervous system.[2,3] On the other hand, much less is known about the biological role of the extraneuronal noradrenaline transporter because of the lack of potent inhibitors. The availability of high-affinity ligands is an important prerequisite of various functional and biochemical methods for the investigation of membrane-bound proteins.

The extraneuronal type of noradrenaline transporter exists in the glia of the central nervous system as well as in various sympathetically innervated organs such as myocardium, vascular smooth muscle, and salivary glands.[4] The recent introduction of experimental models for the extraneuronal noradrenaline transporter which are based on tissue culture techniques greatly facilitated the search for high-affinity ligands and eventually led to the introduction of highly potent inhibitors of the extraneuronal noradrenaline transport.[5,6] These novel noradrenaline transport inhibitors belong to the chemical class of isocyanine and pseudoisocyanine dyes. On the one hand, highly potent inhibitors open new possibilities for the functional and biochemical characterization of the extraneuronal noradrenaline transport. On the other hand, inhibitors of the extraneuronal noradrenaline transport may well be of therapeutic value. They can be expected to elevate central and peripheral levels of free catecholamines. Here, we describe the synthesis, the effect on noradrenaline transport, and an analytical method for the detection in body fluids of various isocyanines and pseudoisocyanines.

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Results

Table I gives the structures of the isocyanines and pseudoisocyanines and the corresponding codes. The dimethyl-substituted compounds **1a** and **2a**, were synthesized according to known procedures.[7] The diethyl-substituted compounds **1b** and **2b** are availabile from commercial sources. The isopropyl-substituted compounds **1c**, **1d**, and **2c** are described here for the first time.

For the syntheses of **1d** and **2c**, 1-isopropylquinaldinium had to be prepared. The synthesis of 1-isopropylquinaldinium was carried out via a Grignard reaction followed by oxidation with iodine.[8] Direct substitution of the quinaldine nitrogen with an isopropyl moiety was not possible because of steric hindrance. In the presence of 2-propyl electrophiles, such as 2-iodopropane and propyl *p*-toluenesulfonate, quinaldine showed basic but not nucleophilic properties. Instead of substitution, elimination to propene and the corresponding quinolinium salt was observed.

Ethanolic solutions of isocyanines and pseudoisocyanines appeared purple and red, respectively. The maximal absorbance was observed at a wavelength of 565 nm for the former and 520 nm for the latter (spectra not shown). Acidified solutions of both isocyanines and pseudoisocyanines, however, were colorless and fluoresced. Excitation at 235 or 320 nm induced an emission in the visuable range with a maximum at 425 nm (Figure 1). The variation of the nitrogen substituent (methyl, ethyl, or isopropyl) influenced neither absorbance nor fluorescence properties. Interestingly enough, the emission was markedly quenched by chloride. The intensity of fluorescence was reduced to 15% in the presence of 100 mmol/L chloride with the maximum remaining at 425 nm (Figure 1).

In order to quantify the isocyanines and pseudoisocyanines, they were separated by HPLC on a silica gel column at low pH, which allowed a direct fluorometric detection (Figure 2). The limit of detection was 0.5 pmol. In the range from 1 to 300 pmol, a linear relation was found between the peak area and the injected amount of the cyanine compound (Figure 3). At a flow rate of 1.5 mL/min, the retention times of the isocyanines and pseudoisocyanines ranged from 5.5 min (**2a**) to 7.9 min (**1d**). **2b** was added to plasma samples from heparinized rabbit

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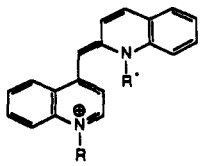
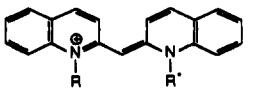
Table I. Structures and Codes of Isocyanines and Pseudoisocyanines (for corresponding anions, see Experimental Section)



isocyanines



pseudoisocyanines

R	R′	code	R	R′	code
methyl	methyl	**1a**	methyl	methyl	**2a**
ethyl	ethyl	**1b**	ethyl	ethyl	**2b**
isopropyl	methyl	**1c**	isopropyl	ethyl	**2c**
isopropyl	isopropyl	**1d**			

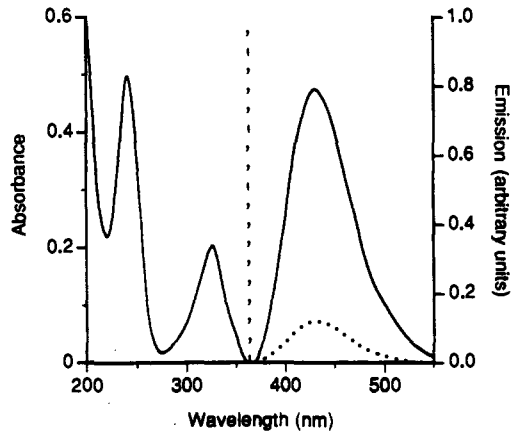


Figure 1. Absorbance and emission spectra of 10 μmol/L **1c** in 0.4 mol/L perchloric acid. The absorbance spectrum (left) was recorded with a PMQ 3 photometer (Zeiss, Oberkochen, Germany) and the emission spectrum (right) with a fluorescence spectrophotometer (excitation wavelength 320 nm, Aminco Bowman, American Instruments, Silver Springs, MD). The dashed line shows the emission spectrum in the presence of 100 mmol/L NaCl.

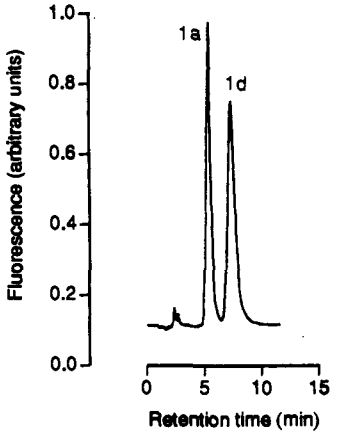


Figure 2. HPLC separation of **1a** and **1d**. A mixture (100 μL) containing 1 μmol/L both **1a** and **1d** in 0.1 mol/L perchloric acid was injected into the HPLC system (for chromatographic conditions see Experimental Section). The eluted compounds were detected by a fluorescence monitor (excitation 235 nm, emission 425 nm). **1a** was eluted with a retention time of 5.7 min and **1d** with 7.9 min at a flow rate of 1.5 mL/min.

blood (final concentration of 1 μmol/L). After deproteinization with perchloric acid, the samples were injected directly into the HPLC system. Compared to standards of **2b** dissolved in elution buffer, the recovery was (97 ± 7)% (n = 3).

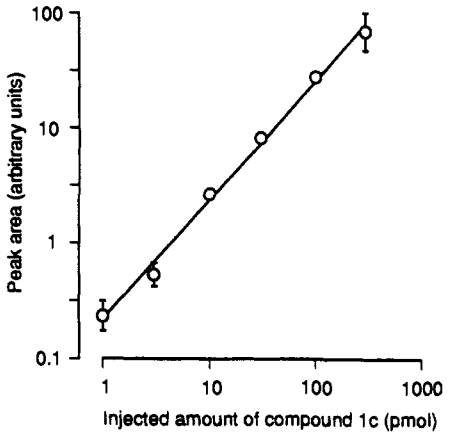


Figure 3. Relation between the amount of **1c** and the area of the resulting peak after separation (for chromatographic conditions see Experimental Section). Shown are the geometric means with 95% confidence limits (n = 4).

Table II. Partition Coefficients in an Octanol/Phosphate Buffer System and Potencies for the Inhibition of Extraneuronal Noradrenaline Uptake in Caki-1 Cells of Various Isocyanines and Pseudoisocyanines[a]

		potency		
compd	partn coef (log P)	IC_{50} (nmol/L)	n_{Hill}	n
1a	0.81 ± 0.04	100[b] (70, 150)	1.46 ± 0.17	5
1b	0.86 ± 0.03	29 (13, 61)	1.68 ± 0.19	4
1c	0.92 ± 0.02	51 (20, 130)	1.37 ± 0.20	4
1d	1.16 ± 0.02	14[b] (8, 25)	1.51 ± 0.28	5
2a	0.34 ± 0.05	94[b] (56, 160)	1.25 ± 0.09	5
2b	0.65 ± 0.03	12 (8, 17)	1.25 ± 0.21	4
2c	0.84 ± 0.04	13 (6, 24)	1.23 ± 0.14	4

[a] The log P-values were calculated from the absorbance of the corresponding isocyanine or pseudoisocyanine (20 μmol/L) in phosphate buffer (10 mmol/L, pH 7.4) before and after extraction with 1-octanol. log P-values are given ±SEM (n = 4). The IC_{50}'s for the inhibition of the specific [^{3}H]noradrenaline uptake in Caki-1 cells are given with 95% confidence intervals and the corresponding Hill coefficients (n_{Hill}) with SEM. n represents the number of separately analyzed inhibition curves. [b] Data taken from Russ et al., 1993.[6b]

Isocyanines and pseudoisocyanines were sparingly soluble in aqueous buffer at physiological pH and virtually insoluble in apolar solvents such as toluene and heptane. On the other hand, they were highly soluble in polar organic solvents such as ethanol, ethyl acetate, and, in particular, dimethyl sulfoxide. The octanol/water partition coefficient (log P) indicates the degree of lipid solubility relative to that of aqueous solubility and, thus, mirrors the ability to permeate biological membranes by simple diffusion. The P-values increased with the size of the nitrogen substituent in the order methyl < ethyl < isopropyl. The isocyanines were more lipophilic than the corresponding pseudoisocyanines (Table II). Compound **2a** was the least and **1d** was the most lipophilic compound.

The clonal cell line Caki-1 possesses the extraneuronal transport mechanism for noradrenaline. After inhibition of intracellular breakdown of [^{3}H]noradrenaline, initial rates of [^{3}H]noradrenaline transport were measured. Caki-1 cells were incubated with 100 nmol/L [^{3}H]noradrenaline for 15 min in the absence (control) and presence of various isocyanines and pseudoisocyanines. At a concentration of 100 nmol/L, all tested compounds inhibited the [^{3}H]noradrenaline uptake in Caki-1 cells (Figure 4). Interestingly enough, **1d**, **2b**, and **2c** turned out to be much more potent inhibitors than corticosterone which was the most potent inhibitor known so far. At a concentration of 100 nmol/L, they diminished specific [^{3}H]-

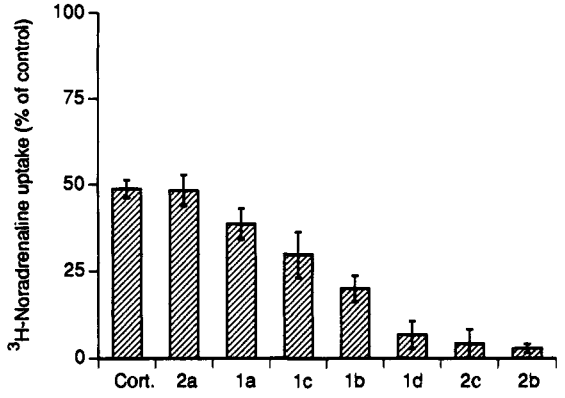


Figure 4. Inhibition of specific [^{3}H]noradrenaline uptake in Caki-1 cells by various isocyanines and pseudoisocyanines. Caki-1 cells were incubated with 100 nmol/L [^{3}H]noradrenaline for 15 min in the presence of 100 nmol/L of the tested compounds. Cort. = corticosterone. Shown are the means ± SEM (n = 4–5) of specific uptake, relative to controls in the absence of an inhibitor.

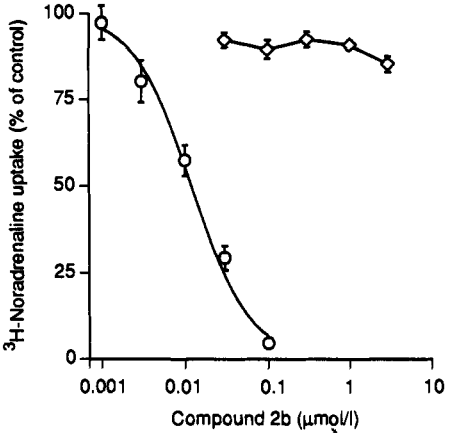


Figure 5. Effect of compound **2b** on specific [^{3}H]noradrenaline uptake in Caki-1 cells (○) and in PC12 cells (◇). The specific [^{3}H]noradrenaline uptake in Caki-1 cells was brought about via the extraneuronal noradrenaline transporter and was defined as that fraction of total uptake which was sensitive to 100 μmol/L *O*-methylisoprenaline. Specific uptake in PC12 cells was brought about via the neuronal noradrenaline transporter and was defined as that fraction which was sensitive to 1 μmol/L desipramine. Given are the means ± SEM (n = 4) of the specific uptake in the presence of **2b**, relative to controls in the absence of an inhibitor.

noradrenaline uptake by about 95%. In addition, IC_{50}'s for the inhibition of the extraneuronal [^{3}H]noradrenaline uptake in Caki-1 cells were determined (Table II). The compounds **1d**, **2b**, and **2c** had IC_{50}'s below 20 nmol/L. The other isocyanines and pseudoisocyanines had IC_{50}'s not above 100 nmol/L. The Hill coefficients were near unity.

In order to clarify the question of whether the isocyanines and pseudoisocyanines selectively inhibit the extraneuronal noradrenaline transport, the effect of compound **2b** on the neuronal noradrenaline transporter was investigated. Therefore, clonal PC12 cells which possess the neuronal transport mechanism for noradrenaline were incubated for 60 s with 10 nmol/L [^{3}H]noradrenaline. Intracellular breakdown and vesicular storage of [^{3}H]-noradrenaline were avoided. Even at a concentration of 3 μmol/L—which is about 200-fold IC_{50} for the inhibition of the extraneuronal noradrenaline uptake—compound **2b** inhibited the neuronal noradrenaline uptake by a small margin only (Figure 5).

Discussion

In the past, the lack of high-affinity ligands severely hampered the investigation of the extraneuronal transport mechanism for noradrenaline. The affinity of corticosterone which in the past was generally accepted as the most potent inhibitor is too low for various biochemical methods such as ligand-binding studies and affinity chromatography. Moreover, corticosterone is not suitable for *in vivo* investigations on extraneuronal noradrenaline transport because of multiple side effects mediated by the stimulation of intracellular steroid receptors. Thus, a recent report from our laboratory is promising. Cyanine dyes that belong to the group of isocyanines and pseudoisocyanines inhibit extraneuronal noradrenaline transport.[6a]

Numerous cyanine dyes were synthesized in the past. The first description dates back to 1856, when C. G. Williams prepared the blue dye *cyanine* which later was identified as 1,1′-diisopentyl-4,4′-cyanine.[9] Some decades later, the first members of the class of isocyanine dyes were synthesized by W. Spalteholz and by S. Hoogewerff and W. A. van Dorp.[10] 2,2′-Condensed cyanines—the so-called pseudoisocyanines—were first isolated and identified by O. Fischer and G. Scheibe in 1919.[11] Cyanine dyes have widely been used as photosensitizers of silver halogenide emulsions of photographic films.[12]

Biological effects have not been described until recently, when the inhibition by some isocyanines and pseudoisocyanines of the extraneuronal noradrenaline transporter and the renal transporter for organic cations was reported.[6a,13] The renal transporter for organic cations eliminates a variety of endogenous and exogenous weak bases by excretion into renal tubules.[14] Recent evidence suggests a close relationship between the renal transport system for organic cations and the extraneuronal transport system for noradrenaline.[5a,15]

The effect of various isocyanines and pseudoisocyanines on extraneuronal transport of noradrenaline was determined and compared to the effect of corticosterone. The experiments were carried out on isolated cells. Clonal Caki-1 cells which stem from a human renal cell carcinoma express the extraneuronal transport mechanism for noradrenaline. After the inhibition of intracellular noradrenaline breakdown, it is possible to determine initial rates of noradrenaline transport into Caki-1 cells.[5a] The isocyanines and pseudoisocyanines turned out to be potent inhibitors of the extraneuronal transport mechanism for noradrenaline. While corticosterone—at a concentration of 100 nmol/L—inhibited extraneuronal noradrenaline uptake by about 50%, the same concentration of **1d**, **2b**, and **2c** inhibited noradrenaline uptake by about 95% (Figure 4). The same concentration of **1a**, **1b**, **1c**, and **2a** inhibited extraneuronal noradrenaline uptake by values between 50% and 85%. In order to measure the inhibitory potencies more precisely, IC_{50}'s for the inhibition of the extraneuronal noradrenaline uptake in Caki-1 cells were determined. The compounds **1d**, **2b**, and **2c** are the most potent inhibitors of the extraneuronal transport mechanism for noradrenaline known so far. The IC_{50}'s were below 20 nmol/L (Table II). In other words, the affinity of these inhibitors to the transporter protein is 1 order of magnitude higher than that of corticosterone. The IC_{50}'s agree with those described elsewhere.[6b]

In order to test the selectivity of this class of inhibitors, the effect of a representative compound, **2b**, on the

desipramine-sensitive neuronal type of noradrenaline transporter was investigated. Therefore, we used PC12 cells which are a well-established tissue culture model for the investigation of the neuronal transport mechanism of noradrenaline.[2b] Up to the highest concentration tested (3 μmol/L), **2b** hardly affected the neuronal type of noradrenaline transporter. By contrast, the IC_{50} for the inhibition of the extraneuronal noradrenaline uptake in Caki-1 cells was 12 nmol/L. This finding excludes a nonspecific cytotoxic action of the isocyanines and pseudoisocyanines and indicates a high degree of selectivity for the inhibition of the extraneuronal noradrenaline transporter.

The compounds **1d**, **2c**, and **1c** are novel, and the present report is the first description of their synthesis. Compounds **1a**, **1b**, **2a**, and **2b** are known cyanine dyes.[7,11] The inhibitory potency of these compounds indicates a strong interaction with the extraneuronal noradrenaline transporter. Regarding the chemical structure, the interaction is most probably electrostatic. Hydrogen bonds as the cause of the interaction can be excluded because of the lack of functional groups which can act as either electron donors or acceptors. In addition, a typical charge–charge interaction does not seem to be involved since the positive charge of the isocyanines and pseudoisocyanines delocalizes over the whole molecule. The presence of aromatic ring systems, on the other hand, suggests that multiple weakly polar interactions are responsible for the strong binding. The importance for both protein–protein and ligand–protein interactions of weakly polar interactions with aromatic ring systems was realized only recently.[16] Two principal types of interaction have been described. Firstly, the polar attraction between a δ^- atom (oxygen, nitrogen, sulfur) and the δ^+ ring hydrogen atoms. Secondly, the interaction of a δ^+ hydrogen on an aromatic ring or on an amino group with the δ^- π-electron cloud of another aromatic ring. The finding that the affinity of the isocyanines and pseudoisocyanines increases with the electron-donating effect of the nitrogen substituents supports the view that the δ^+ hydrogen atoms on the transporter protein interact with the δ^- π-electron cloud of the isocyanines and pseudoisocyanines. The isopropyl-substituted cyanine derivatives have higher affinities than the corresponding methyl and ethyl derivatives. Accordingly, a *tert*-butyl substitution can be expected to further increase the affinity. Unfortunately, synthesis of such a compound was not successful—most probably because of steric hindrance of the bulky *tert*-butyl moiety.

The value of a ligand is determined not only by its affinity to the target protein but also by its hydrophilicity. Marked lipophilicity favors accumulation in lipid bilayers, which in binding experiments causes a high degree of nonspecific binding. The octanol/water partition coefficient (log *P*) reflects the lipophilicity of a compound. Corticosterone is quite lipophilic. Its log *P*-value was about 2. By contrast, the log *P*-values of the isocyanines and pseudoisocyanines were much lower, ranging from 0.34 to 1.16. There was a positive correlation between the log *P*-values and the size of the nitrogen substituents.

The availability of an analytical method to detect the isocyanines and pseudoisocyanines increases the usefulness of this new class of noradrenaline transport inhibitors for *in vivo* experiments. The separation by HPLC with fluorometric detection turned out to be fast and convenient. The method is based on the finding that the isocyanines and pseudoisocyanines emit a strong fluorescence in acidified solutions when excited at 235 nm, which guarantees a high degree of sensitivity as well as selectivity. The limit of detection was as low as 0.5 pmol. Quantification worked well over a concentration range of at least 2 orders of magnitude. The measurement of isocyanines and pseudoisocyanines in biological materials was feasible after deproteinization of the samples with perchloric acid. The recovery of this method was almost 100%.

The introduction of highly potent inhibitors of the extraneuronal transport mechanism for noradrenaline can be expected to facilitate work in this field significantly. The isocyanines and pseudoisocyanines described in the present study are the most potent inhibitors of extraneuronal noradrenaline transport known so far. The availability of a sensitive analytical method for detection of these compounds in biological material is essential for future *in vivo* studies.

Experimental Section

A. General Methods. All melting points were taken in open capillary tubes and are corrected values. ^{1}H-NMR spectra (TMS = 0 ppm) were measured on a 200-MHz apparatus (model AC200, Bruker, Karlsruhe, Germany). Elemental analyses were carried out in the Microanalytical Laboratory at the Department of Chemistry, University of Würzburg. Protein was determined as described by Lowry et al.[17]

B. Syntheses. 1. Intermediates. 2-Iodo-1-methylquinolinium iodide and 1-ethyl-2-iodoquinolinium iodide were prepared as described by Hamer.[7] 1-Methylquinolinium iodide and 1-isopropylquinolinium iodide were prepared by heating purified quinoline with iodomethane and 2-iodopropane, respectively.[18] 1-Methylquinaldinium iodide was synthesized by boiling quinaldine with an excess of iodomethane. 1-Isopropylquinaldinium iodide was obtained as described by Bradley and Jeffrey.[8]

2. Isocyanines. 1,1′-Dimethyl-2,4′-cyanine Iodide (1a). **1a** was prepared in accordance to Hamer.[7] 1-Methylquinolinium iodide (3.0 g) and 1.8 g of 1-methylquinaldinium iodide were dissolved in 100 mL of boiling water and treated dropwise with 6.5 mL of 1 mol/L NaOH. After 1 h of stirring, the deposited dye was collected, washed twice with diethyl ether, and recrystallized from ethanol: 0.61 g (yield 23%, not optimized) of green needles, mp 276 °C. ^{1}H NMR (DMSO-d_6): δ 3.97 (3 H, s, CH_3), 4.06 (3 H, s, CH_3), 6.48 (1 H, s, =CH), 7.4-8.6 (12 H, m, H_{arom}).

1-Methyl-1′-isopropyl-2,4′-cyanine Iodide (1c). 1-Isopropylquinolinium iodide (6.0 g) and 3.0 g of 1-methylquinaldinium iodide were dissolved in 200 mL of boiling ethanol. After the addition of 1.5 of mL of triethylamine, the color changed to purple. The solution was refluxed for 3 h. Subsequently, half of the ethanol was removed by distillation. Distilled water (200 mL) and 200 mL of toluene were added. After the solution was vigorously shook, a dark viscous mass deposited which was collected, washed several times with diethyl ether, and recrystallized from ethanol: 3.1 g of green needles (yield 65%), mp 148 °C. ^{1}H NMR (DMSO-d_6): δ 1.57 (6 H, d, $CH(C\underline{H}_3)_2$), 4.02 (3 H, s, CH_3), 5.33 (1 H, sept, $C\underline{H}(CH_3)_2$), 6.48 (1 H, s, =CH), 7.4–8.6 (12 H, m, H_{arom}).

1,1′-Diisopropyl-2,4′-cyanine Perchlorate (1d). A mixture of 7.8 g of 1-isopropylchinolinium iodide and 4.0 g of 1-isopropylquinaldinium iodide was refluxed for 1 h in 150 mL of ethanol and treated dropwise with 1.8 mL of triethylamine. Since it was not possible to crystallize the product, the reaction mixture was extracted with 0.4 mol/L perchloric acid. The acidic extract was washed several times with diethyl ether/ethyl acetate (1:1), alkalinized with NaOH, and subsequently extracted with ethyl acetate. After evaporation, the residue of the collected ethyl acetate phases was recrystallized from ethanol: 0.23 g of a bronze-colored solid (yield 4%, not optimized), mp 181 °C. ^{1}H NMR (DMSO-d_6): δ 1.56 (6 H, d, $CH(C\underline{H}_3)_2$), 1.80 (6 H, d, $CH(C\underline{H}_3)_2$), 5.30 (2 H, sept, 2 × $C\underline{H}(CH_3)_2$), 6.42 (1 H, s, =CH), 7.4–8.5 (12 H, m, H_{arom}). The NMR data were unequivocal and indicated purity of the substance.

3. Pseudoisocyanines. 1,1'-Dimethyl-2,2'-cyanine Iodide (2a). According to Hamer (1928), a solution of 3.4 g of 2-iodo-1-methylquinolinium iodide and 2.5 g of 1-methylquinaldinium iodide in 150 mL of boiling ethanol was treated dropwise with 2.0 mL of sodium methoxide (30% in methanol).[7] After 3 h, the hot mixture was filtered. A solid separated from the solution on cooling. It was filtered off and recrystallized from ethanol: 0.78 g of gold-colored needles (yield 21%), mp 259 °C (lit.[7] mp 245–246 °C). ^{1}H NMR (DMSO-d_6): δ 4.02 (6 H, s, 2 × CH_3), 5.85 (1 H, s, =CH), 7.5–8.2 (12 H, m, H_{arom}).

1-Ethyl-1'-isopropyl-2,2'-cyanine Iodide (2c). To a solution of 2.0 g of 1-ethyl-2-iodoquinolinium iodide and 2.0 g of 1-isopropylquinaldinium iodide in 80 mL of boiling ethanol was added 1.5 mL of sodium methoxide (30% in methanol) dropwise. After 30 min, the dark red solution was concentrated to 20 mL by distillation. Hot water (20 mL) was added. On cooling, a green viscous mass separated which was extracted several times with diethyl ether. The residue was recrystallized from ethanol/diethyl ether: 0.4 g of dark green needles (yield 18%, not optimized), mp 215 °C. ^{1}H NMR (DMSO-d_6): δ 1.52 (3 H, t, $CH_2\underline{CH_3}$), 1.80 (6 H, d, $CH(\underline{CH_3})_2$), 4.55 (2 H, q, $\underline{CH_2}CH_3$), 5.33 (1 H, sept, $\underline{CH}(CH_3)_2$), 5.70 (1 H, s, =CH), 7.5–8.2 (12 H, m, H_{arom}).

C. **Detection. 1. Spectrometry.** Ethanolic solutions of isocyanines and pseudoisocyanines (1 mmol/L) were diluted to 10 μmol/L with 0.4 mol/L perchloric acid. The absorbance spectra were recorded (10-nm intervals) with a PMQ 3 photometer (Zeiss, Oberkochen, Germany). Fluorescence was induced by excitation at 235 or 320 nm, and emission spectra were recorded in the range from 350 to 800 nm with an Aminco Bowman spectrophotometer (American Instruments, Silver Springs, MD) (Figure 1).

2. Chromatographic Separation. The HPLC system consisted of a P400 pump (Latek, Eppelheim, Germany), a 7125 injection valve with a 100-μL loop (Rheodyne, Berkeley, CA), a RF-535 fluorescence monitor (excitation 235 nm, emission 425 nm, Shimadzu, Kyoto, Japan), and a chromatopac C-R6A integrator unit (Shimadzu). Separation was performed by using a Nucleosil 100 7-μm column (250- × 4-mm i.d., Macherey-Nagel, Düren, Germany). The mobile phase contained 10% (v/v) 2-propanol and 6 mmol/L tetraethylammonium acetate in 0.1 mol/L perchloric acid and was pumped at a flow rate of 1.5 mL/min. Quantification of the isocyanines and pseudoisocyanines was done on the basis of peak areas by external standardization (Figure 2).

For thin-layer chromatography, a mixture of equal parts of acetone, 2-propanol, water, and acetic acid was used as the mobile phase. The stationary phase was a silica-gel-coated layer with a fluorescent indicator (Polygram Sil G/UV_{254}, Macherey-Nagel, Düren, Germany). After separation, the layer was dried and subsequently developed by exposure to ammonia vapor.

D. Octanol/Water Partition Coefficients. Ethanolic solutions of the tested compounds (2 mmol/L) were diluted to 20 μmol/L with 1-octanol-saturated sodium phosphate buffer (10 mmol/L, pH 7.4). Subsequently, the absorbance A_o was determined with a PMQ 3 photometer (Zeiss, Oberkochen, Germany) at 520 nm for the pseudoisocyanines and at 565 nm for the isocyanines. Buffer-saturated 1-octanol (250 μL) was added to 5.0 mL of the solution. After the solution was vigorously shook for 2 h at 21 °C, the phases were separated by centrifugation (20 min at 3000g) and the octanol layer was discarded. The absorbance A_x of the aqueous phase was determined, and the octanol/water partition coefficient P (Table II) was calculated according to eq 1:

$$P = (A_o - A_x)/A_x \times f \quad (1)$$

where f is the ratio of buffer volume over 1-octanol volume.

E. Biological Methods. 1. Cell Culture. Caki-1 cells (ATCC HTB 46[19]) were grown at 37 °C in a humidified atmosphere (5% CO_2) on plastic culture flasks (Falcon, 175 cm^2, Becton Dickinson, Heidelberg, Germany) essentially as described by Schömig and Schönfeld.[5a] The culture medium was McCoys medium with 16.7 mmol/L D-(+)-glucose and 10% fetal calf serum (Gibco, Eggenstein, Germany). The culture medium was changed every 3–4 days, and the culture was split every 7 days. For the experiments, the Caki-1 cells were seeded on plastic culture dishes (60-mm o.d., Nunc, Roskilde, Denmark). After 3–4 days, the cells formed a monolayer and each culture dish contained about 2 mg of cell protein. PC12 cells (ATCC CRL 1721[19]) were grown in suspension culture as described by Harder and Bönisch.[20] The culture medium was composed of 85% RPMI 1640 medium, 10% heat-inactivated horse serum, and 5% fetal calf serum (Gibco, Eggenstein, Germany) and was buffered with $NaHCO_3$ (24 mmol/L). For the experiments, the PC12 cells were seeded on plastic culture dishes (60-mm o.d., Nunc, Roskilde, Denmark) coated with poly(L-ornithine). Reserpine (10 μmol/L) was added 24 h prior to the experiments to inhibit vesicular uptake of noradrenaline and to deplete the intracellular catecholamine stores. Each culture dish contained about 1 mg of cell protein.

2. Uptake of [^{3}H]Noradrenaline in Caki-1 Cells. Caki-1 cells were preincubated at 37 °C for 20 min with buffer A (NaCl 125 mmol/L, KCl 4.8 mmol/L, $CaCl_2$ 1.2 mmol/L, KH_2PO_4 1.2 mmol/L, $MgSO_4$ 1.2 mmol/L, HEPES–NaOH 25 mmol/L pH 7.4, and D-(+)-glucose 5.6 mmol/L).[5a] Intracellular metabolism of [^{3}H]noradrenaline was avoided by the inhibition of catechol-*O*-methyl transferase (COMT) and monoamine oxidase (MAO) with U-0521 (10 μmol/L) and pargyline (10 μmol/L), respectively. Oxidative degradation of [^{3}H]noradrenaline was prevented by the addition of L-(+)-ascorbic acid (1 mmol/L). The cells were incubated at 37 °C for 15 min with 100 nmol/L [^{3}H]noradrenaline (in 3 mL of buffer A). When tested, inhibitors of [^{3}H]-noradrenaline transport were present during both the preincubation and incubation period. Incubation was stopped by rinsing the cells four times with 3 mL of ice-cold buffer A. The cells were solubilized by 0.1% (v/v) Triton X-100 (in 5 mmol/L Tris–HCl, pH 7.4), and radioactivity was determined by liquid scintillation counting. Specific uptake of [^{3}H]noradrenaline was defined as that fraction of total uptake which was sensitive to 100 μmol/L *O*-methylisoprenaline or 1 μmol/L **2b**. Nonspecific uptake was less than 35% of the total [^{3}H]noradrenaline uptake.

3. Uptake of [^{3}H]Noradrenaline in PC12 Cells. As described previously, PC12 cells were preincubated at 37 °C for 20 min with buffer B (NaCl 125 mmol/L, K_2SO_4 2.4 mmol/L, KH_2PO_4 1.2 mmol/L, $MgSO_4$ 1.2 mmol/L, HEPES–NaOH 25 mmol/L pH 7.4, D-(+)-glucose 5.6 mmol/L, L-(+)-ascorbic acid 1 mmol/L, pargyline 10 μmol/L, and U-0521 10 μmol/L).[2b] Subsequently, the cells were incubated for 60 s with 10 nmol/L [^{3}H]noradrenaline (in 3.5 mL of buffer B). When tested, compound **2b** was present during both the preincubation and incubation period. Incubation was stopped by rinsing the cells four times with 3 mL of ice-cold buffer B. The intracellular radioactivity was determined as described above. Specific uptake of [^{3}H]noradrenaline was defined as that fraction of total uptake which was sensitive to 1 μmol/L desipramine. Nonspecific uptake was less than 5% of the total [^{3}H]noradrenaline uptake.

F. Calculations. For the calculation of the IC_{50}'s, the parameters of the Hill equation for multisite inhibition were fitted to the experimental data by a nonlinear computer-assisted least-squares quasi-Newton method.[21] The IC_{50}'s are virtually identical with the K_i-values, since nonsaturating concentrations of [^{3}H]noradrenaline were used.[22] The IC_{50}'s are given as geometric means with 95% confidence limits, and the Hill coefficients are given as arithmetic means with SEM's.

G. Materials and Drugs. The following were obtained commercially: acetone p.a. (Riedel-de Haen, Seelze, Germany); 2-chloroquinoline (ICN Biomedicals, Meckenheim, Germany); triethylamine (Merck-Schuchardt, Hohenbrunn, Germany); corticosterone (Fluka, Neu-Ulm, Germany); pargyline hydrochloride and reserpine (Sigma, Deisenhofen, Germany); U-0521 (3,4-dihydroxy-2-methylpropiophenone; Upjohn, Kalamazoo, MI); desipramine hydrochloride (Ciba Geigy, Basel, Switzerland); *O*-methylisoprenaline (Boehringer, Ingelheim, Germany); Tris (tris(hydroxymethyl)aminomethane) and HEPES (*N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid) (Roth, Karlsruhe, Germany); iodoethane, iodomethane, 2-iodopropane, quinaldine, quinoline, sodium methoxide (30% in methanol), tetraethylammonium acetate (Aldrich, Steinheim, Germany); 1,1'-diethyl-2,2'-cyanine iodide (**2b**) and 1,1'-diethyl-2,4'-cyanine iodide (**1b**) (Atlanta Chemie, Heidelberg, Germany); and (−)-[7-^{3}H]noradrenaline (NET-377, 0.51 10^{15} Bq/mol) (DuPont de Nemours/NEN, Bad Homburg, Germany).

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