oxidation, stability, and preliminary in vivo studies support this statement and justify further studies in different species. Their β-lactamase inhibitory properties can be also exploited in order to protect β-lactamase-susceptible penicillin CDS's.

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A Probe for Octopamine Receptors: Synthesis of 2-[(4-Azido-2,6-diethylphenyl)imino]imidazolidine and Its Tritiated Derivative, a Potent Reversible-Irreversible Activator of Octopamine-Sensitive Adenylate Cyclase

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In order to develop an irreversible ligand for octopamine receptors, a highly potent azido-substituted 2-(phenylimino)imidazolidine (NC-5Z, 8) and its tritiated derivative (^{3}H-NC-5Z, 11) have been designed and synthesized. Under reversible-binding conditions, NC-5Z is 50–100-fold more potent than octopamine in activating octopamine-sensitive adenylate cyclase in a variety of tissues. After photolysis, ^{3}H-NC-5Z binds irreversibly to cell membranes, and this binding is reduced by preincubation with octopamine agonists and antagonists but not by norepinephrine, dopamine, serotonin, or histamine. NC-5Z should be useful both as a potent reversible octopamine agonist and as an affinity probe for characterizing and isolating octopamine-receptor proteins.

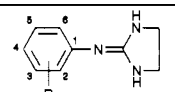
Octopamine is a physiologically significant neurotransmitter in invertebrates, having both neurohumoral and transmitter actions.[1-3] Octopamine is also found in vertebrates, although it is as yet unclear whether unique receptors exist for this amine in the higher phyla.[2] Because exogenously applied octopaminergic agonists can disrupt insect behavior and interfere with feeding, potent octopamine analogues have pesticidal and pestistatic activity.[4-7]

Recent evidence suggests that the toxic and behavioral actions of octopamine agonists in insects are, at least in part, mediated by a subclass of octopamine receptors that activate adenylate cyclase and lead to the synthesis of cyclic AMP.[7,8] The pharmacology of cyclic AMP associated octopamine receptors has been studied through physiological experiments[9] and through measurements of adenylate cyclase activation.[4,8,10,11] However, direct labeling of these receptors has been difficult because of their relatively low (20 μM) affinity for octopamine, resulting in an extremely rapid off-time for existing agonists. Because of this and because there presently exist no irreversible ligands for octopamine receptors, little is known about octopamine-receptor structure or biochemistry. It is for these reasons that we have developed a new agonist of octopamine-sensitive adenylate cyclase, 2-[(4-azido-2,6-diethylphenyl)imino]imidazolidine (NC-5Z, 8). Under conditions of dim light, this compound is 50–100 times more potent than octopamine itself and is the most potent octopamine agonist yet described. Furthermore, when photolyzed, NC-5Z and its tritiated derivative bind irreversibly to membrane octopamine receptors associated with the activation of adenylate cyclase. NC-5Z thus constitutes the first example of a covalent octopamine receptor label which should be useful in the eventual isolation of octopamine-receptor proteins.

Chemistry

The strategy for developing an irreversible octopamine agonist was influenced by recent studies of the 2-(phenylimino)imidazolidines (PIIs), a potent class of reversible octopamine receptor agonists.[7,8] In addition to being potent, a number of PII derivatives exhibit a high degree of octopamine-receptor selectivity which is clearly distinct from activity at mammalian adrenergic, dopaminergic, and serotonergic receptors.[8] Prior studies have shown that

Table I. Octopamine-Receptor Activity of Compounds[a]

compd		V_{max} (% OCT)	K_a(OCT)/K_a compd
11	2,6-$(CH_2CH_3)_2$, 3,5-$(^3H)_2$, 4-N_3	100	86
8	2,6-$(CH_2CH_3)_2$, 4-N_3	100	70
5	2,6-$(CH_2CH_3)_2$	100	12
octopamine (OCT)		100	1
7	2,6-$(CH_2CH_3)_2$, 4-NH_2	82	1.3
9	2,6-$(CH_2CH_3)_2$, 3,5-$(Br)_2$, 4-NH_2	24	15
1	2,6-$(Cl)_2$, 4-$NCH_3(CH_2CH_2Cl)$	5	6

[a] Activity (stimulation of firefly light organ adenylate cyclase) is expressed relative to the activity of octopamine (V_{max} = 100%; K_a ratio = 1) run in the same experiment. Typically, basal adenylate cyclase activity was 20–40 pmol/mg of protein per min, and octopamine stimulation at V_{max} was 50-fold.

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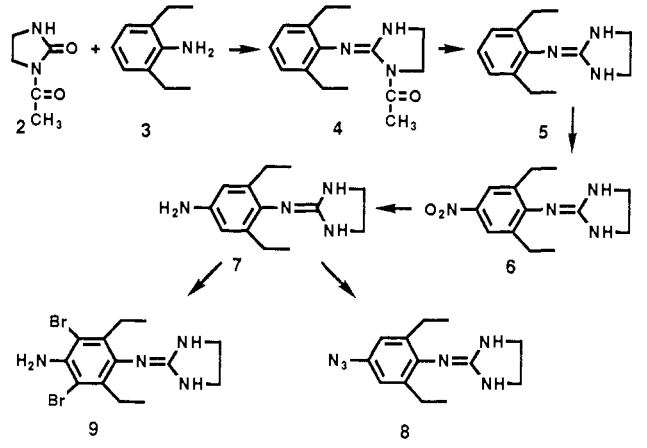
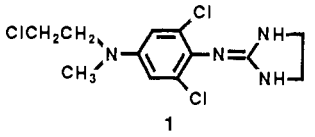
Scheme I



2,6-diethyl-PII (NC-5) is a full agonist, about 15–20-fold more potent than octopamine in activating the octopamine-sensitive adenylate cyclase present in the firefly light organ. (The light organ, highly enriched in octopamine-sensitive adenylate cyclase, but not other amine-activated adenylate cyclases, is a highly specific and sensitive assay system for detecting compounds capable of activating octopamine receptors.[11-13]) Studies with the PIIs have also shown that 4-phenyl substitutions, as exemplified by 2-chloro-4-methyl-PII (NC-7), enhance potency for octopamine-stimulated adenylate cyclase in the nerve cord of certain invertebrate species such as the tobacco hornworm (*Manduca sexta*). To develop an affinity probe which would have the range of activity of both NC-5 and NC-7, we focused on compounds with combined substitutions in the 2-, 4-, and 6-positions, one of these substitutions having the potential to bind irreversibly to octopamine-receptor proteins.

Results and Discussion

Reversible Activation of Octopamine-Sensitive Adenylate Cyclase. Experiments were first carried out with 2,6-dichloro-[*N*-(chloroethyl)-*N*-methylamino]-PII (1).



The chloroethyl substitution of the PII derivative was of interest, since it is similar to that on certain other compounds (such as phenoxybenzamine and some alkylating agents) which are known to exhibit irreversible binding to membrane proteins.[14] Disappointingly, however, in experiments testing the reversible activation of adenylate cyclase in firefly membranes (Table I), compound 1, although potent, was only a poor partial agonist of light-organ adenylate cyclase, possibly because of the bulkiness of the 4-substitution. Furthermore, other experiments (not shown) indicated that the activation of adenylate cyclase by 1 was entirely reversed by three 30-min washes.

Because of the known ability of azido-substituted ligands to photolabel receptors and because our prior experiments had indicated that reducing the bulkiness of 4-substituents could increase the potency of PIIs in activating octopamine-sensitive adenylate cyclase, a 4-azido derivative of NC-5 was synthesized (Scheme I). The procedure was adapted from a reported method.[15] Although the yield for the nitration of **5** was low (20%, unoptimized), the other steps proceeded in good to excellent yields and the overall scheme was quite short. The resulting compound, designated NC-5Z (**8**), was first tested under conditions (in dim light) in which the azido group remains intact and in which binding is reversible. Data in Figure 1 and Table I indicate that NC-5Z was not only a full agonist but was extremely potent, indeed the most potent octopamine agonist yet described in this assay system. In various experiments, the calculated K_a for NC-5Z ranged from 350 to 700 nM, which therefore is about 50–100-fold more potent than octopamine itself, and 5 times more potent than NC-5. In other experiments (not shown), NC-5Z was about 100-fold more potent than octopamine in stimulating octopamine-sensitive adenylate cyclase in cockroach thoracic ganglia, cockroach leg muscle, and *Manduca* nerve cord.

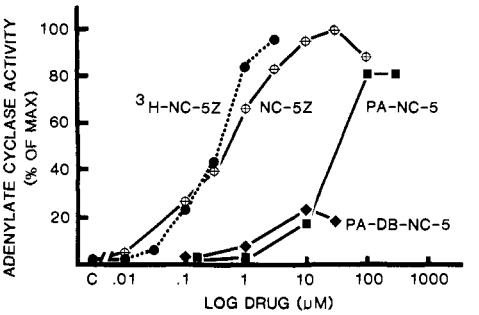
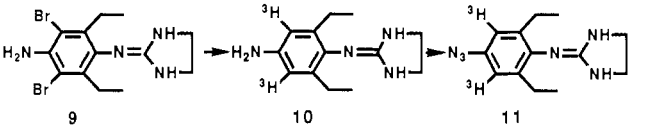


Figure 1. Effects of NC-5Z (**8**), ^{3}H-NC-5Z (**11**), 2-[(4-amino-2,6-diethylphenyl)imino]imidazolidine (**7**) (PA-NC-5), and 2-[(4-amino-2,6-diethyl-3,5-dibromophenyl)imino]imidazolidine (**9**) (PA-DB-NC-5) on firefly light organ adenylate cyclase activity. Enzyme activity is expressed as a percentage of the maximal stimulation (100%) caused by octopamine in the same experiment. Values shown are mean of replicate samples, each assayed for cyclic AMP content in duplicate. Typically, basal enzyme activity was 20–50 pmol/mg of protein per min in the light organ, and at optimal concentrations, enzyme activity was stimulated by octopamine 30–60-fold.

Scheme II



Tritiated NC-5Z. The UV absorption spectrum of NC-5Z showed peaks at 210 and 255 nm. As with other azido-substituted ligands, it was hoped that exposure of NC-5Z to intense UV in these wavelengths would yield an active nitrene compound which could then react covalently with sites on the octopamine-receptor protein. In order to detect irreversible binding, a tritiated derivative of NC-5Z was prepared. Unfortunately, because of the lability of the azido group, we could not label NC-5Z itself. Instead, Scheme II was devised, in which 3,5-dibromo-4-amino-NC-5 (**9**) was reduced with tritium gas to form 4-amino-NC-5 (**10**) labeled with tritium in the 3- and 5-positions. This compound was then converted to the corresponding azido analogue by the same procedure as

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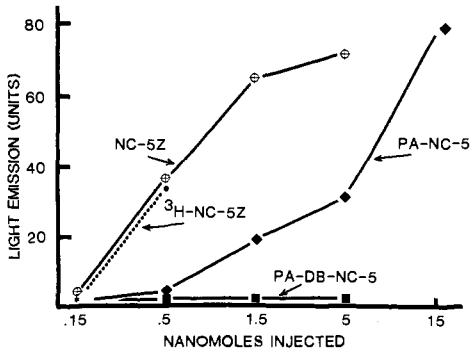

Figure 2. Effects of NC-5Z (**8**), ^{3}H-NC-5Z (**11**), 2-[(4-amino-2,6-diethylphenyl)imino]imidazolidine (**7**) (PA-NC-5), and 2-[(4-amino-2,6-diethyl-3,5-dibromophenyl)imino]imidazolidine (**9**) (PA-DB-NC-5) on eliciting light emission in the isolated firefly tail. Shown is the maximal illumination (in units as defined in ref 8) resulting from injection of the indicated dose of drug. Values are the means of data from six animals for NC-5Z, from four animals for ^{3}H-NC-5Z, from five animals for PA-NC-5, and from four animals for PA-DB-NC-5Z.

for the unlabeled amine **7** to form ^{3}H-NC-5Z (**11**).

Before performing receptor-labeling experiments, the bioactivity of ^{3}H-NC-5Z was verified by two separate methods. In the first, we measured the ability of the labeled compound to activate adenylate cyclase in light-organ membranes. Figure 1 shows that the activity profile of labeled NC-5Z was virtually identical with that of unlabeled NC-5Z. Figure 1 also shows that the other possible compounds, **9** and **7** (the nontritiated derivative of **10**), which might have been present at the end of the synthesis, were markedly different (and much less potent) in their activity profiles, indicating that, had they contaminated ^{3}H-NC-5Z substantially, this would have been easily detected by a decrease in the apparent activity of ^{3}H-NC-5Z.

In the second verification procedure, we carried out a bioassay and measured the effect of ^{3}H-NC-5Z on eliciting light emission when injected into isolated firefly tails. (As mentioned, it is known that the light organ located in the firefly tail is highly enriched in postsynaptic octopamine receptors which control initiation of light emission.[11-13]) Figure 2 shows that injection of 0.5 nmol of the labeled compound (the highest dose tested) caused a light response identical with that due to injection of the same dose of genuine unlabeled NC-5Z. As with the adenylate cyclase experiment in Figure 1, the other possible compounds **9** and **7** (the nontritiated derivative of **10**), which might have been present at the end of the synthesis, were much less active. Taken together, these two experiments provided strong evidence that the labeled NC-5Z was biologically identical with unlabeled NC-5Z.

Irreversible Binding of ^{3}H-NC-5Z. To demonstrate irreversible binding to light-organ membranes, washed membranes were preincubated with the labeled probe under various conditions in dim light, and then the mixture was photolyzed in quartz microcuvettes with shortwave UV light. The free (unbound) label was removed by either five cycles of trichloroacetic acid (7.5%) precipitation and NaOH solubilization or by repeated high-speed centrifugation. Figure 3 shows that exposure to UV light resulted in a 20-fold increase in the amount of label permanently bound.

To determine the specificity of binding, various displacing agents were added during the preincubation with ^{3}H-NC-5Z, and the mixture was then photolyzed. As shown in Figure 3, octopamine or the synthetic octopaminergic agonist NC-7 reduced ^{3}H-NC-5Z binding by about 35–40%. Cyproheptadine, a known octopaminergic antagonist,[16] reduced ^{3}H-NC-5Z binding about 35% at 1 mM, and one of the most potent octopaminergic antagonists known, mianserin, reduced binding by 38% at 0.01 mM and by 51% at 1 mM. Binding was not reduced with the nonoctopaminergic compounds, dopamine, serotonin, norepinephrine, or histamine, which were also much less effective than NC-5Z, octopamine, or NC-7 in activating light-organ adenylate cyclase under reversible conditions. The ineffectiveness of dopamine, serotonin, norepinephrine, and histamine in reducing photoaffinity labeling makes it unlikely that the presence of a UV-absorbing chromophore was responsible for the decrease in binding caused by octopamine, NC-7, cyproheptadine, and mianserin. In other experiments (not shown), we found that UV photolysis of light-organ membranes with NC-5Z, followed by extensive washing (to remove free and reversibly bound NC-5Z), resulted in a persistently activated adenylate cyclase (due to irreversibly bound NC-5Z). This activation could be blocked by octopamine (which displaced the NC-5Z prior to photolysis and was later removed by washing), thereby providing additional evidence that affinity labeling of light-organ-membrane proteins by NC-5Z occurs, at least in part, at the octopamine binding site of octopamine receptors associated with activation of adenylate cyclase.

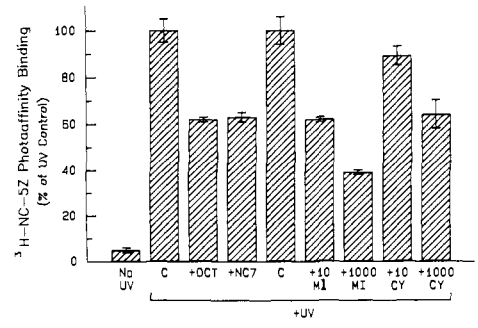

Figure 3. Photoaffinity labeling of ^{3}H-NC-5Z (**11**) to light-organ membranes. Values (mean ± range of duplicate samples) are for irreversible binding of 1 μM ^{3}H-NC-5Z and are expressed as a percentage of UV binding seen in the absence of displacing agents. Procedure was as described in the Experimental Section. C = control; OCT = octopamine (1 mM); NC-7 (1 mM); MI = mianserin (10 or 1000 μM); CY = cyproheptadine (10 or 1000 μM).

NC-5Z and its tritiated derivative, in addition to being among the most potent octopaminergic compounds yet described, are also the first examples of irreversible ligands for octopamine receptors. Although we have not tested these compounds on octopamine receptors not associated with adenylate cyclase, Evans has shown that the related reversible ligands NC-5 and NC-7 are effective in activating several octopamine-receptor subtypes; i.e., octopamine$_1$, octopamine$_{2A}$, and octopamine$_{2B}$ receptors.[17] Accordingly, it is likely that NC-5Z, in addition to labeling octopamine receptors associated with adenylate cyclase activation, may also be useful in labeling other types of octopamine receptors. Such labeling should be helpful in the eventual biochemical isolation and purification of octopamine-receptor proteins and in histochemically localizing octopamine receptors in tissues.

Experimental Section

Melting points were determined on a Mettler FP61 melting point apparatus. NMR spectra were recorded on a Varian EM390 or a Bruker AM300 spectrometer with tetramethylsilane as internal standard. The mass spectra were recorded on a MAT CH7

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(17) Evans, P. D. *J. Exp. Biol.* **1987**, *129*, 239.

or MAT 8230B spectrometer. The IR spectrum was recorded on a Perkin-Elmer 298 spectrometer. Elemental analyses were provided by the Physical and Analytical Chemistry Department, The Upjohn Company. Catalytic reduction of **9** by tritium and subsequent conversion to **11** were carried out at New England Nuclear Corp. (Boston, MA).

1-Acetyl-*N*-(2,6-diethylphenyl)-4,5-dihydro-1*H*-imidazol-2(3*H*)-imine (4). To 14.92 g (0.100 mol) of 2,6-diethylaniline and 12.81 g (0.100 mol) of 1-acetyl-2-imidazolidone[18] was added 100 mL of $POCl_3$. The mixture was refluxed for 3 h, most of the $POCl_3$ was removed by distillation, the residue was treated with ice and water, the solution was made basic with 50% NaOH solution and extracted twice with CH_2Cl_2, and the extracts were dried over Na_2SO_4 and evaporated to dryness to yield 18.12 g (69.9%) of product. The analytical sample, mp 141.7 °C, was obtained by recrystallization from cyclohexane. ^1H NMR ($CDCl_3$): δ 1.10 (t, 6 H), 2.43 (q, 4 H), 2.68 (s, 3 H), 3.20 (m, 2 H), 3.85 (t), and 4.0 (br s, 3 H), 6.9 (m, 3 H). Anal. ($C_{15}H_{21}N_3O$) C, H, N.

***N*-(2,6-Diethylphenyl)-4,5-dihydro-1*H*-imidazol-2(3*H*)-imine (5).** To 1.630 g (6.28 mmol) of **4** was added 30 mL of water, and the suspension was refluxed until a clear solution was obtained (3 h). The solution was cooled in ice and made basic with 50% NaOH solution. The precipitate was filtered, washed with water, and dried to yield 1.244 g (91.1%); mp 157.6 °C. ^1H NMR ($CDCl_3$): δ 1.17 (t, 6 H), 2.53 (q, 4 H), 3.33 (s, 4 H), 4.46 (br s, 2 H), 6.9 (m, 3 H). Anal. ($C_{13}H_{19}N_3$) C, H, N.

***N*-(2,6-Diethyl-4-nitrophenyl)-4,5-dihydro-1*H*-imidzol-2-(3*H*)-imine (6).** To 1.845 g (8.49 mmol) of **5** in 25 mL of 50% v/v aqueous acetic acid at 5 °C was added 1.172 g (17.0 mmol) of sodium nitrite in 8 mL of H_2O. After 1.5 h at 5 °C, 10 mL of 35% nitric acid was added. After 0.5 h at 5 °C, the solution was refluxed for 1 h, cooled to room temperature, and made strongly basic with 50% NaOH solution. The mixture was extracted twice with $CHCl_3$, and the extracts were chromatographed on 150 g of silica gel with 2% triethylamine in MeOH to yield 440 mg (19.8%); mp 217.6 °C dec. ^1H NMR ($CDCl_3$): δ 1.23 (t, 6 H), 2.60 (q, 4 H), 3.53 (s, 4 H), 5.23 (br s, 2.8 H), 7.80 (s, 2 H). Anal. ($C_{13}H_{18}N_4O_2 \cdot {}^2/_3H_2O$) C, H, N.

1-[(4,5-Dihydro-1*H*-imidazol-2(3*H*)-ylidene)amino]-2,6-diethylbenzen-4-amine (7). To 468 mg (1.78 mmol) of **6** suspended in 5 mL of 50% w/w aqueous EtOH and 558 mg (10.0 mmol) of iron filings at reflux was added 2.1 mL (4.1 mmol) of 1.9 N aqueous ethanolic HCl dropwise. The aqueous ethanolic HCl was prepared from 5 mL of concentrated HCl which was diluted to 31 mL with 50% w/w aqueous ethanol. The mixture was then refluxed for 0.5 h, cooled to room temperature, made strongly basic with 50% NaOH solution, extracted three times with CH_2Cl_2, and the extracts were dried over Na_2SO_4 and evaporated to dryness. The residue was dissolved in $CHCl_3$, saturated with gaseous HCl, and evaporated to dryness. The residue was chromatographed on 50 g of silica gel with 800 mL of 20% MeOH in EtOAc and then with 30% MeOH in EtOAc. The product (519 mg, 95.2%) was eluted between 400 and 1200 mL as the dihydrochloride salt. ^1H NMR ($CDCl_3$, MeOH-d_4): δ 1.10 (t, 6 H), 2.50 (q, 4 H), 3.60 (m, 4 H), 4.25 (br s, 6 H), 6.43 (s, 2 H).

To 97 mg of the product in water was added 50% NaOH solution, the mixture was extracted with Et_2O, and the extract was dried over Na_2SO_4 and evaporated to dryness to yield 56 mg (76%) of the free base, mp 169.7 °C. ^1H NMR ($CDCl_3$): δ 1.10 (t, 6 H), 2.30 (q, 4 H), 3.37 (s, 4 H), 4.0 (br s, 4 H), 6.33 (s, 2 H).

***N*-(4-Azido-2,6-diethylphenyl)-4,5-dihydro-1*H*-imidazol-2(3*H*)-imine (8).** To 166 mg (0.715 mmol) of **7** in 10 mL of 3 M acetic acid at 4 °C was added 50 mg (0.72 mmol) of sodium nitrite in 1 mL of water. After 3 min, a solution of 55 mg (0.85 mmol) of sodium azide in 1 mL of water was added, the solution was stirred at 2–4 °C for 10 min, made basic with 50% sodium hydroxide, and diluted with ether. The organic layer was washed three times with water and once with saturated salt solution, dried over Na_2SO_4, and evaporated to dryness to yield 148 mg of crude product. The material was chromatographed to two 1-mm Analtech silica gel GF plates with 2% Et_3N in MeOH to yield 115 mg (62.2%). The analytical sample was recrystallized from ether to yield pale yellow crystals, mp 157.6 °C dec. ^1H NMR ($CDCl_3$): δ 1.16 (t, 6 H), 2.50 (q, 4 H), 3.40 (s, 4 H), 5.10 (br, 2 H), 6.66 (s, 2 H). IR ($CHCl_3$): 3450, 2105, 1675 cm^{-1}. MS (EI) *m/e* 258 (M^+). Anal. ($C_{13}H_{18}N_6$) H, N, C: calcd 60.44; found 59.93.

2,6-Dibromo-4-[(4,5-dihydro-1*H*-imidazol-2(3*H*)-ylidene)amino]-3,5-diethylbenzenamine (9). To 100 mg (0.328 mmol) of **7** in 10 mL of glacial acetic acid and 1.5 mL of trifluoroacetic acid at 8 °C was added 2.20 mL (0.76 mmol) of bromine in acetic acid (55 mg/mL). The solution was stirred in an ice bath for 1.5 h, allowed to warm to room temperature, and filtered, and the filtrate was evaporated to dryness. The residue was chromatographed on 60 g of silica gel with 10% MeOH in ethyl acetate to yield 115 mg of material, which was dissolved in aqueous MeOH, made strongly basic with 50% NaOH solution, diluted with H_2O, and filtered. The solids were dissolved in $CHCl_3$, dried over Na_2SO_4, and evaporated to dryness to yield 72 mg (56.3%). ^1H NMR ($CDCl_3$): δ 1.02 (t, 6 H), 2.65 (m, 4 H), 3.36 (br s, 4 H), 4.36 (s, 4 H). ^{13}C NMR ($CDCl_3$): δ 13.05, 25.96, 42.35, 109.50, 136.47, 137.53, 138.02, 157.64. MS (EI) *m/z* 390. The analytical sample, mp 245.8 °C, was obtained by recrystallization from EtOAc/cyclohexane. Anal. ($C_{13}H_{18}N_4Br_2$) C, H, N.

***N*-(4-Azido-2,6-diethyl[3,5-$^3H_2$]phenyl)-1*H*-imidazol-2-(3*H*)-imine (11).** Compound **9** (0.8 mg) was reduced under a T_2 atmosphere in the presence of 0.8 mg of 10% Pd/C. The sealed mixture was stirred for 2 hours. With use of the appropriate radiological procedures, the mixture was filtered and labiles were removed and trapped. The product was taken up in 10 mL of ethanol. An assay showed the crude yield to be 37 mCi. The solvent was removed by rotary evaporation on a modified apparatus to trap all labiles. The product was taken up in 2 mL 2 of N HCl and cooled to 0 °C. After 10 min, 2 mg of $NaNO_2$ in 0.1 mL of water was added and the solution stirred for another 10 min. At this point a test of the solution gave a positive starch–iodide test. NaN_3 (200 mg) was added and stirring continued at 0 °C. After 1 h, 10 mL of water was added and the product was extracted with 10 mL of CH_2Cl_2. The aqueous solution was made basic with 10 drops of NH_4OH and extracted with another 10 mL of CH_2Cl_2. An assay of the organic phase showed 25 mCi to be present. The product was purified by TLC. The specific activity was determined by UV and the product was found to be 99% radiochemically pure by TLC (silica gel, $CHCl_3$MeOHNH$_4$OH (9:1:0.1) and MeOH with 2% NH_4OH).

Biological Methods. Octopamine-Activated Adenylate Cyclase Assay. For biological measurement of octopaminergic activity, adenylate cyclase activity was measured as the rate of synthesis of cyclic AMP from ATP according to the method and with the tissues previously described.[18] Cyclic AMP formed during the reaction was subsequently measured with the protein-binding assay of Brown and co-workers.[19] For assaying the effects of ^3H-NC-5Z (but not the nonradioactive compounds) on cyclic AMP production, the Brown assay (which utilizes tritiated cyclic AMP) was preceded by a purification procedure to remove ^3H-NC-5Z, since the radioactivity of this compound would otherwise interfere with the binding assay. For this purification, samples were loaded on a 0.5 × 9 cm Dowex AG50×8, 200–400-mesh column, washed with 4 mL of 1 mM K_2HPO_4, pH 7.0, and eluted with 6 mL of H_2O. The eluted fraction was concentrated to dryness, rechromatographed on a second Dowex column, concentrated, and assayed for cyclic AMP content. The assayed values were corrected for column recovery, which was 70%. NC-5Z was initially solubilized in 50% polyethylene glycol 400, which was diluted to a final concentration of 5% in the adenylate cyclase assay.

To determine the reversibility of adenylate cyclase activation by the described compounds, tissue homogenates were first preincubated with 3 μM drug (or control), ±1 mM octopamine, according to the conditions used for the adenylate cyclase assay, except that ATP was omitted. Homogenates were transferred to open-well plastic tissue-culture dishes, photolyzed as described below, and then diluted 100-fold with 6 mM Tris maleate (pH 7.4) and centrifuged at 120 000*g* for 30 min. The pellet was resuspended in 20 mL of buffer and the washing was repeated twice. The final pellet was resuspended (2–4 mg wet weight/mL)

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and assayed for adenylate cyclase to determine if basal activity was elevated.

Effects of Compounds on Light Emission. The procedures were as previously described,[8] except that drugs were dissolved in insect saline containing no calcium and 20 mM manganese chloride. These salt conditions increased the specificity of the assay by blocking any potential effects of drugs on endogenous release of octopamine from nerve terminals.

Determination of Irreversible Binding of ^{3}H-NC-5Z. Washed light-organ membranes (0.04 mL of 100 mg wet wt/mL) were added to 0.16 mL of a mixture containing (final concentration): 80 mM Tris maleate, pH 7.4; 10 mM theophylline; 8 mM $MgCl_2$; 0.5 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; 2 mM ATP; and 0.1 mM GTP. Displacing agents were added and the mixture was incubated at 4 °C for 15 min, after which ^{3}H-NC-5Z (17 Ci/mmol) was added to a final concentration of 1 μM and the incubation continued for an additional 60 min. The mixture was then transferred to quartz minicuvettes (1-mm path length, total volume 0.25 mL) and photolyzed for 15 min at a distance of 2 cm from a bank of three 15-W Sylvania GTE germicidal lamps (G15T8). Cuvettes were turned over every 2 min during the exposure and, following photolysis, the contents of the cuvettes were transferred to tubes containing 10 mL of 6 mM Tris maleate, pH 7.4. The tubes were centrifuged at 120 000*g* for 30 min, the pellet was resuspended in 10 mL of buffer and washed twice more. The final pellet was solubilized in 1 mL of Protosol (New England Nuclear) for 15 h at 30 °C, transferred to a scintillation vial, and neutralized with 1 mL of 1N HCl. Eighteen milliliters of Aquasol-2 (New England Nuclear) was then added and the radioactivity was measured by scintillation counting in a Serle Delta 300. In some experiments, membranes (along with 0.2 mL of 0.63% bovine serum albumin was carrier protein) were washed, instead, by five cycles of precipitation with 7.5% cold trichloroacetic acid and solubilization of the centrifuged pellet with 0.2 mL of 1 M NaOH.

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Synthesis and Benzodiazepine Receptor Affinities of Rigid Analogues of 3-Carboxy-β-carbolines: Demonstration That the Benzodiazepine Receptor Recognizes Preferentially the s-Cis Conformation of the 3-Carboxy Group

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1*H*-Indolo[3',2':4,5]pyrido[3,2-*b*]-2-penten-5-olide (**6**) and 1*H*,5*H*-indolo[3',2'-*c*]-6,7-dihydro-2-pyridone (**7**), rigid analogues of methyl 4-ethyl-β-carboline-3-carboxylate (**8**) and *N*-methyl-4-ethyl-β-carboline-3-carboxamide (**9**), respectively, were synthesized and their in vitro binding affinities to the central type benzodiazepine receptors were compared. The IC_{50} values of **6** and **8** were approximately equivalent (42 and 27 nM, respectively). The amide derivative **9**, for which theoretical energy calculations indicate that the s-trans carbonyl conformation is the preferred one, displayed very low affinity ($IC_{50} > 10^4$ nM). However, when the carbonyl group of **9** was forced to adopt the s-cis conformation as in lactam **7**, binding to the benzodiazepine receptor was largely restored (IC_{50} = 150 nM), indicating that the s-cis carboxy conformation at C-3 of β-carbolines is preferentially recognized by this receptor. In vivo, compound **6** showed neither convulsant, proconvulsant, nor anticonvulsant activity in mice. Moreover, **6** did not antagonize methyl β-carboline-3-carboxylate induced convulsions in mice. This lack of activity of **6** was attributed to its inability to cross the blood–brain barrier since no significant displacement of [^{3}H]Ro 15-1788 from mouse brain benzodiazepine receptors by **6** could be observed in vivo.

β-Carbolines possessing a carboxyl group at the 3-position [for example, ethyl β-carboline-3-carboxylate (β-CCE, **1**; see Chart I)], are known to bind with high affinity to the central benzodiazepine receptors (BZR)[1,2] and have been proposed as endogenous ligands of these receptors.[1,3] Moreover, structure–activity studies in this series have shown that a carboxyl group implicated in an ester linkage [e.g., **1**, IC_{50} (concentration of ligand inhibiting 50% of tritiated benzodiazepine binding) = 7 nM][1] demonstrates a higher affinity for the BZR than one that is part of an amide linkage [e.g., FG 7142 (**2**); IC_{50} = 657 nM].[4,5] On the basis of the X-ray crystal structures of a homologous ester methyl β-carboline-3-carboxylate (β-CCM, **3**)[6,7] and amide [*N*-ethyl-β-carboline-3-carboxamide (β-CEA, **4**)],[8] it has been proposed by Codding that one possible reason for this observed difference in binding affinities of the

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