Articles

Transformation of a κ-Opioid Receptor Antagonist to a κ-Agonist by Transfer of a Guanidinium Group from the 5′- to 6′-Position of Naltrindole

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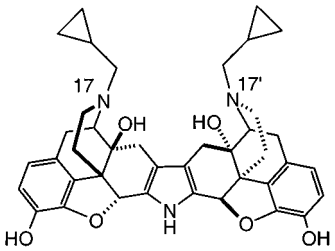
The importance of the indole scaffold of GNTI **3** in directing its address (5′-guanidinium group) to associate with the Glu297 residue of the κ-opioid receptor was investigated by the synthesis and biological evaluation of its 4′- (**4a**), 6′- (**4b**), and 7′- (**4c**) regioisomers. The finding that only the 5′-regioisomer (GNTI) possessed potent κ-opioid antagonist activity and high affinity at κ-receptors illustrates the importance of the 5′-position in orienting the guanidinium group to the proper recognition locus (Glu 297) for potent κ-antagonist activity. The discovery that the 6′-regioisomer of GNTI was a potent κ-agonist, together with the results of site-directed mutagenesis studies that are consistent with association between the 6′-guanidinium group and Glu297, suggest that the transition from an inactive to an active state of the κ-receptor involves a conformational change of TM6. We propose that association of the 6′-guanidinium group of **4b** with Glu297 promotes axial rotational motion of transmembrane helix VI which leads to receptor activation via a conformational change of inner loop 3.

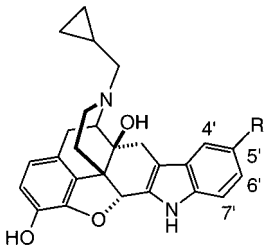
Introduction

The κ-opioid receptor is one of the three major opioid receptors that are found in the central nervous system and in the periphery.[1] The precise roles of κ-receptors have not yet been established, but it appears that κ-selective endogenous opioid peptides (e.g., dynorphin A) function both as neuro- and immunomodulators. A large number of nonpeptide κ-agonists have been developed as promising potential analgesics, and some of them have found wide use as pharmacological tools in opioid research.[2] In particular, norbinaltorphimine (*nor*BNI **1**), a bivalent ligand, which contains two naltrexone-derived pharmacophores, is the only nonpeptide ligand that is highly selective and widely used as a κ-opioid receptor antagonist.[3] The structure−activity relationship of *nor*BNI **1** has been extensively investigated[4] and it has been recently demonstrated that the basic group (N17′) in the second pharmacophore of *nor*BNI acts as an "address"[5] to confer selectivity by interacting with a nonconserved acidic residue (Glu297)[6] located at the top of the transmembrane spanning helix-VI (TM6) of the κ-receptor.[6] Moreover, it has also been shown that incorporation of a glutamate residue into the corresponding position of the μ-receptor leads to high affinity binding of *nor*BNI to the mutant μ-receptor, which illustrates the importance of this glutamate residue.[7]



1 *nor*-BNI

In light of the structural requirements of *nor*BNI, 5′-guanidinonaltrindole **3** (GNTI) was synthesized as it has been reported to be a highly potent and selective κ-opioid receptor antagonist.[7a−c] The design of GNTI utilized the indole moiety of the δ-selective antagonist, naltrindole[8] (NTI, **2**), as a rigid scaffold[9] for the attachment of a guanidinium group at the 5′-position to permit interaction with Glu297 of the κ-opioid receptor. This position orients the guanidinium group to a location similar to that of the N17′ basic group of *nor*BNI. It has been established that the cationic 5′-guanidinium group functions as an "address" to confer selectivity through interaction with the anionic Glu297 residue of the κ-receptor.[7c]



2 R = H (NTI)
3 R = NHC(=NH)NH$_2$ (GNTI)

In the present study, we have investigated the interaction of the 4′-, 6′-, and 7′-regioisomers (**4a**−**c**) of

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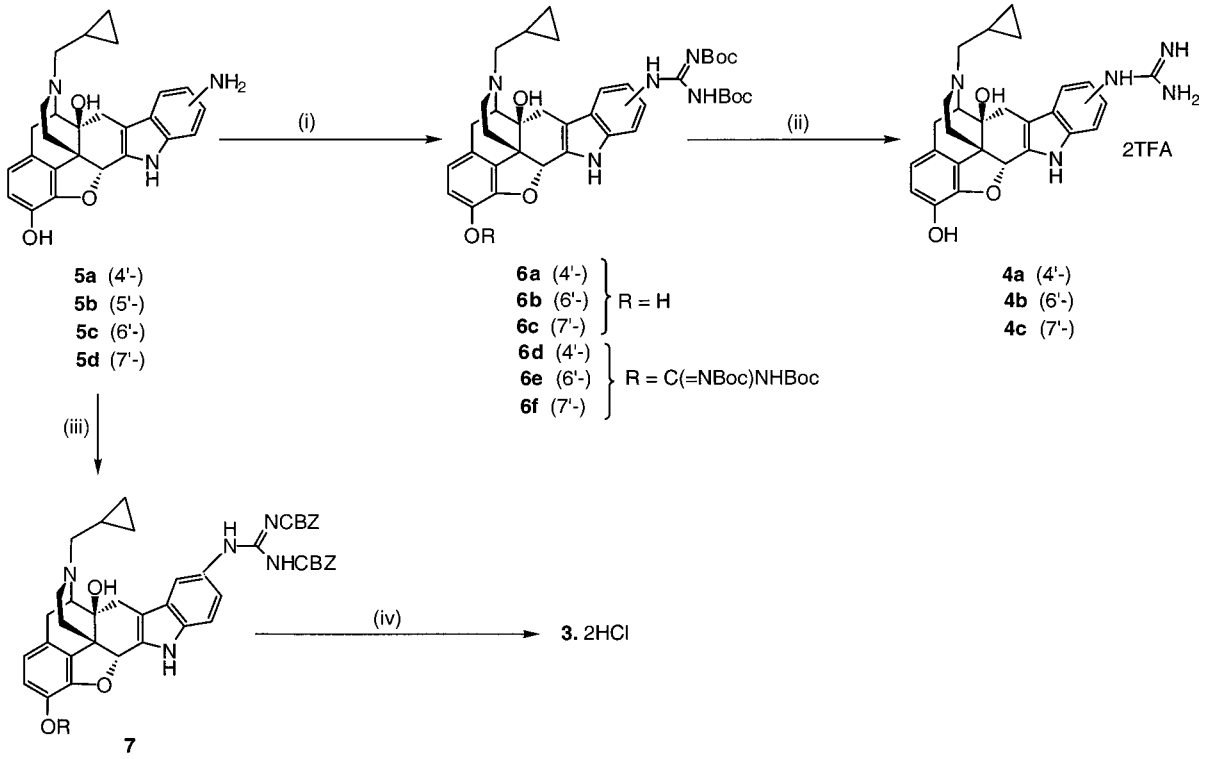
Scheme 1. Synthetic Route to Guanidinonaltrindole Regioisomers[a]



[a] Reagents: (i) (BocNH)(BocN=)CSMe, $HgCl_2$, CH_2Cl_2, NEt_3, rt, 18−24 h; (ii) TFA, CH_2Cl_2, N_2, rt, 36 h; (iii) (CBZ-NH)(CBZ-N=)CSMe, $HgCl_2$, CH_2Cl_2, NEt_3, rt, 2 h or (CBZ-NH)(CBZ-N=)C-$NHSO_2CF_3$, CH_2Cl_2, NEt_3, rt, 4 days; (iv) Pd/C, H_2, MeOH−dil. HCl, 65 psi, 4 h.

GNTI with opioid receptors in order to evaluate the relationship between the position of the guanidinium group and its interaction with Glu297 of the κ-receptor. The data reveal that movement of the guanidinium group from the 5′- to the 6′-position changes the activity from a κ-antagonist **3** to a κ-agonist **4b**, and that Glu297 of the κ-receptor may be involved in this transition. The significance of these results is discussed in terms of the conformational change that may occur in receptor activation.

Chemistry

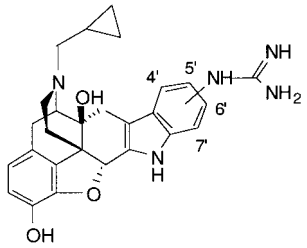
The synthetic route to the target compounds **4a**−**c** is outlined in Scheme 1. The 5′- and 7′-nitronaltrindole intermediates were prepared as reported previously.[8,10] Fisher-indole cyclization[11] of naltrexone with 3-nitrophenylhydrazine yielded a mixture of regioisomers consisting of the 4′-nitro (minor) and 6′-nitro (major) derivatives of naltrindole. Separation of the regioisomers was accomplished by repeated column chromatography. Raney nickel-catalyzed reduction[12] of the nitro function in all of the regioisomers using hydrazine hydrate in ethanol afforded the corresponding amino derivatives **5a**−**d** in 70−86% yields.[10] Mercury(II) chloride-assisted coupling[13] of the regioisomers **5a**,**c**,**d** with 1,3-bis(*tert*-butoxycarbonyl)-2-methyl-2-thiopseudourea in dry CH_2Cl_2 yielded the Boc-protected guanidinonaltrindole **6a**−**c** (major product, 78−81%) and a small amount (2.6−3.8%) of the esterified side product **6d**−**f**. Trifluoroacetic acid (TFA) deprotection of the Boc groups in intermediates **6a**−**c** afforded regioisomers of GNTI **4a**−**c** as bis-TFA salts in good yield after reverse-phase HPLC.

A new procedure to synthesize GNTI **3** as its dihydrochloride salt in multigram quantities without the reported[7c] tedious chromatographic separation has been developed. 5′-Amino-NTI **5b** was allowed to react with 3.3 equiv of di-*N*′,*N*″-carboxybenzyloxy-*N*‴-trifluoromethanesulfonylguanidine[14] in the presence of triethylamine in CH_2Cl_2 for 4 days to give bis(benzyloxycarbonyl)- protected intermediate **7**. This intermediate **7** was also prepared by an alternate route that involved the reaction of 5′-amino-NTI **5b** with 1,3-bis(benzyloxycarbonyl)-2-methyl-2-thiopseudourea using a $HgCl_2$-assisted coupling reaction.[13] Deprotection of intermediate **7** was carried out by catalytic hydrogenation (10% Pd/C) to afford **3**·HCl. The overall yield from **5b** by either of the modified routes was 65−70%.

Biological Results

Smooth Muscle Preparations. The in vitro pharmacological data for GNTI **3** and its regioisomers **4a**−**c**, as well as the standard κ-antagonist, *nor*BNI **1**, are listed in Table 1. All of the compounds were tested on the electrically stimulated guinea pig ileal longitudinal muscle (GPI)[15] and the mouse vas deferens (MVD)[16] preparations for their agonist and antagonist activities as previously described.[17] The compounds were incubated with the aforementioned preparations 15 min prior to testing with standard selective agonists. Morphine (M), (−)-ethylketazocine (EK), and [D-Ala^2,D-Leu^5]-enkephalin (DADLE) were employed as μ-, κ-, and δ-selective agonists, respectively. Morphine and EK were used in the GPI, and DADLE was employed in the MVD studies. Concentration−response curves were obtained in the absence (control) and presence of the antagonist in the same preparation. Antagonist potency is expressed as an IC_{50} ratio (IC_{50} of the agonist in the presence of antagonist divided by the control IC_{50} of the

Table 1. Opioid Antagonist Potency of GNTI Regioisomers in Smooth Muscle Preparations



		EK (κ)[a,c]		DADLE (δ)[b,c]		M (μ)[a,c]	
	regioisomers	K_e (nM)	IC_{50} ratio[d]	K_e (nM)	IC_{50} ratio[d]	K_e (nM)	IC_{50} ratio[d]
1 (*nor*BNI)		0.41	49.8 ± 7.8 (10)[e]	10.6	10.4 ± 2.9 (3)	12.5	2.6 ± 0.6 (12)[e]
3 (GNTI)	5′	0.16	139 ± 33 (11)[e]	115	1.9 ± 0.5 (3)	30.3	4.3 ± 0.7 (5)
4a	4′	80	2.3 (2)	263	1.4 (2)	*f*	0.34 (2)
4b	6′	*g*	*g*	*f*	0.2 (2)	*g*	*g*
4c	7′	100	2.0 (2)	0.96	105 ± 30 (4)	238	1.4 ± 0.9 (3)

[a] Determined in the guinea pig ileum (GPI) preparation. [b] Determined in the mouse vas deferens (MVD) preparation. [c] All antagonists were tested at a concentration of 100 nM unless otherwise noted. [d] Values are expressed mean ± standard error of the mean with the number of determinations in parentheses. The agonists employed were ethylketazocine (EK), morphine (M), and [D-Ala^2-D-leu^5]enkephalin (DADLE). [e] Tested at 20 nM. [f] Not calculated because IC_{50} ratio was <1. [g] Not determined due to full agonist activity.

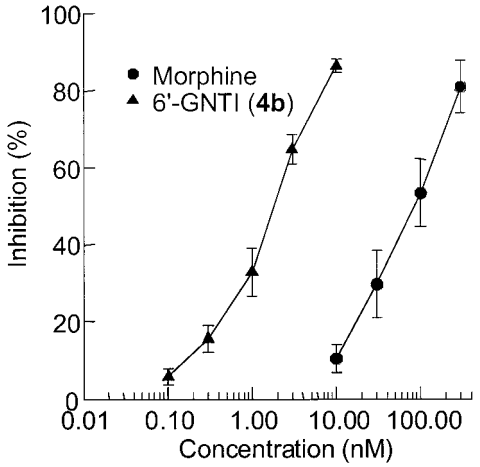


Figure 1. Concentration−response curves of **4b** and morphine in the guinea pig ileal preparation.

agonist in the same preparation) and as a K_e value derived from the relationship, K_e = [antagonist]/(IC_{50} ratio − 1).

The data presented in Table 1 clearly illustrate that the 5′-regioisomer GNTI **3** is a highly potent and selective κ-opioid receptor antagonist as reported previously.[7a,b] In this regard, GNTI was approximately 4-fold more potent than *nor*BNI **1**, with δ/κ and μ/κ selectivity ratios that are 718 and 188, respectively. The 4′-regioisomer **4a** was virtually inactive at all opioid receptor types. While the 7′-regioisomer **4c** was inactive at μ- and κ-receptors, it was a potent δ-opioid receptor antagonist. This is inconsistent with the previously reported[8] steric tolerance for δ-antagonist activity when the 7′-position of naltrindole is substituted. Surprisingly, the 6′-regioisomer **4b** was found to be an agonist with a 51-fold greater potency relative to that of morphine (Figure 1) in the GPI assay. The finding that the agonist effect of **4b** was only partially reversed by naltrexone (500 nM) suggested that it was not a μ-selective agonist. However, the κ-selective antagonist, *nor*BNI (20 nM), completely reversed the agonist effect. In the MVD assay, **4b** was inactive as an agonist at 1 μM and did not antagonize DADLE (IC_{50} ratio = 0.2). These results strongly suggest that **4b** is a κ-selective agonist.

Table 2. Binding Affinity of GNTI Regioisomers to Cloned Opioid Receptors

		K_i (nM)[a]		
	regioisomer	κ	δ	μ
3 (GNTI)	5′	0.14 ± 0.03	24.8 ± 11.3	99.7 ± 8.7
4a	4′	>1000	>1000	>1000
4b	6′	1.15 ± 0.39	20.3 ± 6.7	81.8 ± 20.7
4c	7′	69.1 ± 25	2.75 ± 0.48	181 ± 20

[a] The K_i values were determined in competition binding using [^{3}H]diprenorphine in transiently expressed rat HEK-293 cells and analyzed by whole cell binding. K_i values were derived from the Cheng−Prusoff equation, $K_i = IC_{50}/(1 + [L]/K_d)$, where IC_{50} is the concentration of competing ligand producing 50% inhibition of the specific binding of radioligand [L] and having dissociation constant K_d. The values are the mean ± SE of three experiments in duplicate.

Receptor Binding. Receptor binding was determined in triplicate using [^{3}H]diprenorphine on HEK-293 cells that were transiently transfected with plasmids encoding rat κ-, rat μ-, or mouse δ-opioid receptors (Table 2). GNTI **3** possessed greater than 2 orders of magnitude higher affinity for the κ-receptor relative to μ- and δ-receptors. Consistent with the smooth muscle data, substitution at the 4′-position (**4a**) eliminated binding to all three receptor types. Like GNTI, the 6′-regioisomer **4b** was κ-selective but possessed one-tenth the affinity of GNTI for κ-receptors. The results are inconsistent with the κ-agonist selectivity of **4b** observed in the GPI. The δ-selectivity of regioisomer **4c** also corresponded well with the functional data. Binding to μ-receptors was uniformly low for all regioisomers.

In view of the finding[6,7a] that Glu297 at the top of TM6 is important for high κ-antagonist affinity, the binding of the 5′-, 6′-, and 7′- regioisomers (**3**, **4b**, **4c**) to mutant κ-receptor was determined (Table 3). It is noteworthy that the 5′- and 6′-regioisomers displayed a significant decrease in affinity for the Glu297Ala mutant while the binding of the 7′-regioisomer was virtually unchanged.

Discussion

The importance of Glu297, located at the top of transmembrane helix VI (TM6), for the antagonist

Table 3. Effect on Binding to the κ-Opioid Receptor upon Mutational Exchange of Glu297

		K_i (nM)[a]	
	regioisomer	wild-type κ	κ (Glu297Ala)
3 (GNTI)	5′	0.21 ± 0.05	3.93 ± 0.58
4b	6′	1.13 ± 0.56	12.3 ± 2.5
4c	7′	32.7 ± 5.1	47.3 ± 6.7

[a] The K_i values were determined in competition binding using [^{3}H]diprenorphine in transiently expressed rat HEK-293 cells and analyzed by whole cell binding.

activity of *nor*BNI **1** and GNTI **3** has been reported previously.[7a,c] The results of the present study on the GNTI regioisomers **4a**−**c** clearly support the proposal that the indole moiety functions as a rigid scaffold for directing the guanidinium group to this nonconserved glutamate residue (Glu297). In this regard, it is likely that the 4′-, 6′-, and 7′-regioisomers (**4a**−**c**) exhibit greatly reduced binding relative to the 5′-regioisomer, GNTI **3**, in part as a consequence of the less favorable alignment of the guanidinium group with the glutamate carboxylate group (Glu297) of the κ-receptor.

Although GNTI **3** and its regioisomers bind uniformly poorly to μ-receptors, this is not the case for δ-receptors, where **4b** and **4c** have moderate affinity. These data are inconsistent with the reported structure−activity relationship[8] of the parent ligand, naltrindole **2**, which is known to tolerate substitution in the order 7′ > 6′ > 5′ ≫ 4′ for δ-receptor antagonist potency. Accordingly, it is not surprising that the 7′-regioisomer **4c** has the greatest affinity for δ-receptors.

Significantly, the 6′-regioisomer **4b** functions as a potent κ-opioid receptor agonist in the guinea pig ileum preparation. This represents a remarkable transition from κ-antagonist (**3**) to a κ-agonist (**4b**), simply by moving the guanidinium group from the 5′- to 6′-position. The finding that the mutant (Glu297Ala) κ-receptor exhibited reduced affinity for GNTI **3** and its 6′-regioisomer **4b** suggests that the guanidinium group of each of these ligands interacts with Glu297 (Table 3). Thus, one possible explanation for the antagonist−agonist transition may involve stabilization of the receptor via salt bridge formation between the carboxylate group of Glu297 and the 5′- or the 6′-guanidinium group in either the inactive or active state of the receptor, respectively.

Interaction with the Glu297 residue on TM6 may have special relevance to the receptor function, as this helix is contiguous with an inner loop 3 (IL3) which is the major domain involved in G protein activation. Conformational differences in TM6 may therefore be a key feature that distinguishes an inactive state from an agonist state of the receptor. Such a conformational change could involve rotation and/or tilting of TM6, which could promote a conformational change of IL3. Conceivably, interaction of **3** or **4b** with Glu297 may therefore lead to different conformational transitions of TM6 due to the regioisomeric relationship of the 5′- or 6′-guanidinium group.

Our structure−activity results are consistent with recent studies that implicate movement of TM6 in the activation of rhodopsin and the β_2 adrenergic receptor.[18−24] Transfer of the guanidinium group from the 5′- to the 6′-position could result in a counterclockwise rotation of the TM6 in order to facilitate interaction with Glu297 (Figure 2). Evidence for a counterclockwise rotation of TM6 has been reported to be a consequence of activation of the β_2-adrenergic receptor.[23−27]

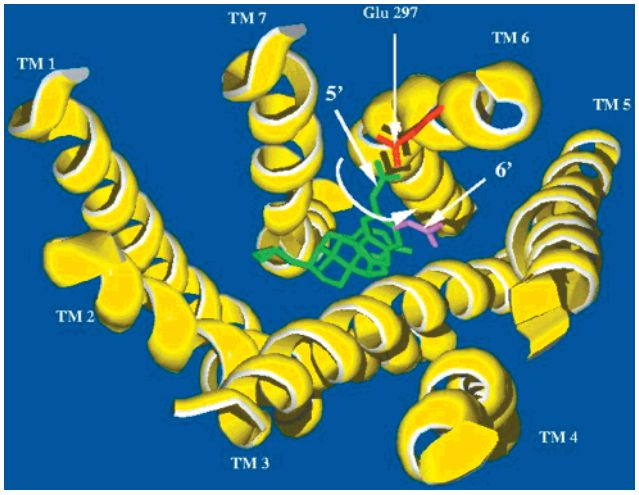


Figure 2. Depiction of the interaction of the GNTI **3** (green) with Glu297 of the κ-opioid receptor. A counterclockwise rotation of helix 6 (curved arrow) is required to enable the interaction of the glutamate side chain (red) with the 6′-guanidinium group (magenta). Transmembrane helix backbones are shown as ribbons in yellow.

Conclusions

The critical nature of the position of attachment of the guanidinium group to the indole moiety in naltrindole **2** illustrates the principle of a rigid scaffold in directing an "address" to a specific subsite (Glu297) on the κ-opioid receptor. The remarkable transition from potent κ-antagonist (**3**) to potent κ-agonist (**4b**) upon moving the guanidinium group from the 5′- to 6′-position may be due to salt bridge-induced stabilization of the receptor in an inactive state with the former and an active state with the latter. If both **3** and **4b** interact with Glu297 on TM6, a possible explanation for this phenomenon may involve differential axial rotational motion of TM6 by the 6′-guanidinium group to promote a conformational change of inner loop 3, which is involved in G protein coupling and activation.

Experimental Section

General. Materials. Naltrexone was obtained from Mallinckrodt & Co. All reactions were carried out under an inert atmosphere of nitrogen. Triethylamine (NEt_3) was distilled from KOH, while dichloromethane and acetonitrile were distilled from calcium hydride prior to their use. Dry dimethylformamide (DMF) was obtained by storing reagent grade material over 3 Å sieves for at least 24 h. All other chemicals were either HPLC or reagent grade and used without further purification. Thin-layer chromatography (TLC) was performed on analytical Uniplate silica gel GF plates (250 μm by 2.5 × 20 cm^2), and preparative thin-layer chromatography was performed on 1.0 or 0.5 mm silica gel plates purchased from Analtech. Gravity and low pressure column chromatography were performed over silica gel (200−400 mesh, 60 Å, Aldrich) as the stationary phase under N_2. The reverse phase high pressure liquid chromatography (HPLC) was performed with Beckman model 110A pumps, a Beckman Analytical Optical Unit (fixed wavelength UV), and a Hewlett-Packard HP 3390A integrating recorder. Purification was performed with a Ranin Dynamax macro HPLC column (C18, 5 μm, 1 × 28 cm^2) or an Alltech Altima C18 column (10 μm, 250 × 22 mm^2). Chromatographic elution solvent systems are reported as vol:vol

ratios. Infrared (IR) spectra were recorded on a Perkin-Elmer PE-281 spectrophotometer or a Nicolet 5DXC FT-IR spectrometer as potassium bromide (KBr) disks. Low resolution (LRMS) and high resolution (HRMS) mass spectra were obtained on a Finnigan 4000 or VG-707EHF spectrometer by the Chemistry Mass Spectrometry Laboratory at the Department of Chemistry, University of Minnesota. All ^{1}H and ^{13}C spectra were recorded on a Varian 300 MHz or a GE 300 MHz spectrometer, and the chemical shifts are reported as δ values with units of parts-per-million (ppm). ^{1}H NMR spectra are referenced to tetramethylsilane (TMS) at 0.00 ppm as an internal standard, and ^{13}C NMR spectra are referenced to either $CDCl_3$ (77.00 ppm) or DMSO-d_6 (40.00 ppm) and are recorded at room temperature (20 ± 1 °C). All recorded spectra are for the free base unless otherwise stated. Elemental analyses were performed by the M-H-W Laboratory in Phoenix, AZ, and are within ±0.4% of theoretical values. Melting points were determined in open capillary tubes on a Thomas-Hoover melting point apparatus and are uncorrected. All of the tested regioisomers of GNTI (**3**, **4a**−**c**) were purified by silica gel flash chromatography (CH_2Cl_2−MeOH−NH_4OH, 78:20:2), then by silica gel preparative TLC (CH_2Cl_2−MeOH−NH_4OH, 78:20:2), and finally by reverse-phase HPLC (CH_3CN−H_2O, 65:35 containing 0.1% of TFA) to afford as their TFA salts.

Transient Transfection. HEK-293 in DMEM (Gibco, BRL) supplemented with 10% bovine calf serum (Hyclone) and 1% penicillin/streptomycin (Gibco, BRL) were maintained at 37 °C and in 5% CO_2. Cells were seeded at 16% for 24 h prior to transfection. Fresh media was added 2 h prior to transfection. Cells were transfected with plasmid DNA (20 μg/100 mm plate) of either wild-type or mutant receptor cDNA using the calcium phosphate precipitation method.[28] Media was changed 5 h after transfection. Transfected cells were harvested 48−72 h after tranfection for binding studies.

Receptor Binding Assays. Sixty to 72 h after transfection, HEK cells were washed three times with 25 mM HEPES buffer (pH 7.4) and were resuspended with 8−12 mL of 25 mM HEPES/100 mm plate. Saturation binding assays were performed in triplicate. Nonselective binding was determined using 10 μM naltrexone. Assays were incubated at room temperature for 90 min in a total binding volume of 0.5 mL and were terminated by filtration through a Whatman GF/B filter that had been presoaked in 0.25% poly(ethyleneimine) immediately prior to filtration. Filters were washed three times with 4 mL of ice-cold 25 mM HEPES buffer, and scintillation counting was performed with a Beckman 3801 LS scintillation counter. Protein concentrations were determined by the method of Bradford.[29] Raw binding data was analyzed with RADLIG and LIGAND (G. A. McPherson, Biosoft, Cambridge, U.K.). Inhibition constants (K_i) were determined from IC_{50} values with the Cheng−Prusoff equation.[30]

Site-Directed Mutagenesis. Rat κ-opioid receptor cDNA was subcloned into pcDNA3 (Invitrogen). Point mutation Glu297Ala was introduced in the opioid receptor genes by polymerase chain reaction (QuikChange Site Directed Mutagenesis Kit, Stratogene). Primers were designed to incorporate an Eco47 III restriction site that was used for screening of mutant DNA. Mutations were then confirmed by DNA sequencing.

4′-*N*′-(*N*″,*N*‴-Bis(*tert*-butoxycarbonyl)guanidino-17-(cyclopropylmethyl)-6,7-didehydro-4,5α-epoxy-3,14-hydroxyindolo[2′,3′:6,7]morphinian (6a). In a 250 mL flask was stirred a mixture of **5a** (325 mg, 0.75 mmol) and $HgCl_2$ (400 mg, 1.33 mmol) in HPLC grade CH_2Cl_2 (50 mL) for few minutes, and 1,3-bis(*tert*-butoxycarbonyl)-2-methyl-2-thiopseudourea (320 mg, 1.1 mmol) was added, followed by few drops of NEt_3. The mixture was allowed to stir at room temperature under a sealed N_2 atmosphere. The progress of the reaction was monitored by TLC. After completion the reaction (24 h), mixture was filtered through Celite under vacuum to remove mercuric sulfide, and the residue was washed throughly with methanol. The combined filtrate was concentrated to give a solid product which was subjected to column chromatography (CH_2Cl_2−MeOH−NH_4OH, 94.5:5.0:0.5) to give two products. Major product **6a** (410 mg, 79%): mp 255 °C (dec); ^{1}H NMR (DMSO-d_6) δ 11.57 (s, 1H, NH), 11.33 (s, 1H, NH), 10.00 (s, 1H, NH), 8.93 (s, 1H, Ar−OH), 7.17−7.11 (m, 2H, ArH), 7.09−6.97 (m, 1H, ArH), 6.46 (m, 2H, ArH), 5.46 (s, 1H, 5-H), 4.70 (b, 1H, 14-OH), 3.24−3.02 (m, 1H), 2.84−2.79 (m, 1H), 2.64−2.59 (m, 2H), 2.45−2.38 (m, 1H), 2.36 (m, 2H), 2.27−2.25 (m, 2H), 2.16−2.12 (m, 1H), 1.49 (s, 9H, ^{t}Bu), 1.27 (s, 9H, ^{t}Bu), 0.99 (m, 1H), 0.45 (m, 2H), 0.09 (m, 2H); HRMS (FAB) *m*/*z* 672.3386 (M + H)$^+$, $C_{37}H_{45}N_5O_7$ requires 671.3397. Minor product **6d** (26 mg, 3.8%): mp >280 °C; ^{1}H NMR (DMSO-d_6) δ 11.46 (s, 1H, NH), 11.22 (s, 1H, NH), 10.70 (s, 1H, NH), 9.83 (s, 1H, NH), 7.36 (s, 1H, ArH), 7.25 (d, 1H, J = 8.1 Hz, ArH), 7.16 (d, 1H, J = 8.1 Hz, ArH), 6.70 (d, 1H, J = 8.1 Hz, ArH), 6.66 (d, 1H, J = 8.4 Hz, ArH), 5.61 (s, 1H, 5-H), 4.76 (b, 1H, 14-OH), 3.14 (d, 1H, J = 18.9 Hz), 2.81−2.63 (m, 5H), 2.39−2.46 (m, 3H), 2.12 (m, 1H), 1.53 (d, 1H, J = 13.2 Hz), 1.47 (s, 9H, ^{t}Bu), 1.38 (s, 9H, ^{t}Bu), 1.31 (s, 9H, ^{t}Bu), 1.25 (s, 9H, ^{t}Bu), 0.84 (m, 1H), 0.47 (m, 2H), 0.12 (m, 2H); HRMS (FAB) *m*/*z* 914.4717 (M + H)$^+$, $C_{48}H_{63}N_7O_{11}$ requires 913.4585.

6′-*N*′-(*N*″,*N*‴-Bis(*tert*-butoxycarbonyl)guanidino-17-(cyclopropylmethyl)-6,7-didehydro-4,5α-epoxy-3,14-hydroxyindolo[2′,3′:6,7]morphinian (6b). A mixture of **5c** (216 mg, 0.5 mmol), $HgCl_2$ (250 mg, 0.83 mmol), 1,3-bis(*tert*-butoxycarbonyl)-2-methyl-2-thiopseudourea (200 mg, 0.7 mmol) in freshly dried CH_2Cl_2 containing few drops of NEt_3 was allowed to stir for 18 h under a sealed N_2 atmosphere at room temperature. After work up of the reaction according to the above procedure for **6a**, it gave two products which were separated by column chromatography (CH_2Cl_2-MeOH−NH_4-OH, 94.5:5.0:0.5); Major product **6b** (260 mg, 78%)**:** mp 270 °C (dec); ^{1}H NMR (DMSO-d_6): δ 11.43 (s, 1H, NH), 11.20 (s, 1H, NH), 10.00 (s, 1H, NH), 8.88 (s, 1H, Ar−OH), 7.69 (s, 1H, ArH), 7.25 (d, 1H, J = 8.7 Hz, ArH), 6.90 (d, 1H, J = 8.7 Hz, ArH), 6.47 (d, 1H, J = 8.1 Hz, ArH), 6.42 (d, 1H, J = 8.1 Hz, ArH), 5.44 (s, 1H, 5-H), 4.70 (b, 1H, 14-OH), 3.24−3.07 (m, 2H), 3.02 (m, 1H), 2.60 (m, 2H), 2.48−2.08 (m, 5H), 1.53 (m, 1H), 1.48 (s, 9H, ^{t}Bu), 1.38 (s, 9H, ^{t}Bu), 0.80 (m, 1H), 0.45 (m, 2H), 0.12 (m, 2H);^{13}C NMR (DMSO-d_6): δ 153.35, 152.81, 143.56, 140.31, 136.98, 131.64, 131.44, 130.87, 124.73, 124.24, 118.73, 117.37, 114.99, 110.56, 106.23, 84.34, 72.66, 62.11, 59.11, 47.74, 43.80, 39.15, 31.79, 29.18, 28.40, 23.14, 9.71, 4.35, 3.97. HRMS (FAB) *m*/*z* 672.3405 (M + H)$^+$, $C_{37}H_{45}N_5O_7$ requires 671.3397. Minor product **6e** (12 mg, 2.6%): mp >280 °C; ^{1}H NMR (DMSO-d_6): δ 11.44 (s, 1H, NH), 11.28 (s, 1H, NH), 10.70 (s, 1H, NH), 10.02 (s, 1H, NH), 7.37 (s, 1H, ArH), 7.27 (d, 1H, J = 7.8 Hz, ArH), 6.89 (d, 1H, J = 8.1 Hz, ArH), 6.72 (d, 1H, J = 8.4 Hz, ArH), 6.68 (d, 1H, J = 8.1 Hz, ArH), 5.60 (s, 1H, 5-H), 4.76 (b, 1H, 14-OH), 3.12 (d, 1H, J = 18.1 Hz), 2.78−2.56 (m, 3H), 2.48−2.36 (m, 5H), 2.16−2.03 (m, 1H), 1.52 (m, 1H), 1.49 (s, 9H, ^{t}Bu), 1.36 (s, 18H, ^{t}Bu), 1.26 (s, 9H, ^{t}Bu), 0.86 (m, 1H), 0.47 (m, 2H), 0.12 (m, 2H); HRMS (FAB) *m*/*z* 914.4670 (M + H)$^+$, $C_{48}H_{63}N_7O_{11}$ requires 913.4585.

7′-*N*′-(*N*″,*N*‴-Bis(*tert*-butoxycarbonyl)guanidino-17-(cyclopropylmethyl)-6,7-didehydro-4,5α-epoxy-3,14-hydroxyindolo[2′,3′:6,7]morphinian (6c). A mixture of **5d** (420 mg, 1 mmol), $HgCl_2$ (430 mg, 1.44 mmol), 1,3-bis(*tert*-butoxycarbonyl)-2-methyl-2-thiopseudourea (380 mg, 1.3 mmol), and a few drops of NEt_3 in dried CH_2Cl_2 was allowed to stir for 18 h at room temperature under a sealed N_2 atmosphere and worked up according to the procedure for **6a**. On subjecting the mixture to column chromatography (CH_2Cl_2−MeOH−NH_4-OH 94.5:5.0:0.5), it gave major product **6c** (530 mg, 81%): mp 230 °C (dec); ^{1}H NMR (DMSO-d_6) δ 11.64 (s, 1H, NH), 11.28 (s, 1H, NH), 9.67 (s, 1H, NH), 8.89 (s, 1H, Ar−OH), 7.26 (d, 1H, J = 7.5 Hz, ArH), 6.97−6.89 (m, 2H, ArH), 6.50−6.43 (m, 2H, ArH), 5.70 (s, 1H, 5-H), 4.70 (b, 1H, 14-OH), 3.27 (m, 2H), 3.06 (d, 1H, J = 18.3 Hz), 2.73−2.65 (m, 2H), 2.44−2.03 (m, 5H), 1.51 (s, 9H, ^{t}Bu), 1.26 (s, 9H, ^{t}Bu), 0.86 (m, 1H), 0.46 (m, 2H), 0.11 (m, 2H); ^{13}C NMR (DMSO-d_6) δ 155.93, 143.54, 140.48, 133.38, 131.28, 130.74, 128.24, 124.52, 121.09, 118.95, 118.82, 117.98, 117.36, 111.11, 84.25, 72.72, 62.11, 59.02, 47.77, 43.89, 31.80, 29.29, 28.69, 28.31, 23.21, 9.52, 4.42, 3.95; HRMS (FAB) *m*/*z* 672.3406 (M + H)$^+$, $C_{37}H_{45}N_5O_7$ requires

671.3397. Minor product **6f** (34 mg, 3.7%): mp 165 °C (dec); ^{1}H NMR (DMSO-d_6) δ 11.63 (s, 1H, NH), 11.37 (s, 1H, NH), 10.70 (s, 1H, NH), 9.66 (s, 1H, NH), 7.26 (d, 1H, J = 8.1 Hz, ArH), 6.98 (d, 1H, J = 6.60 Hz, ArH), 6.91 (d, 1H, J = 7.5 Hz, ArH), 6.73 (d, 1H, J = 8.1 Hz, ArH), 6.66 (d, 1H, J = 7.2 Hz, ArH), 5.61 (s, 1H, 5-H), 4.70 (b, 1H, 14-OH), 3.44 (m, 1H), 3.39 (m, 1H), 3.14 (d, 1H, J = 18.9 Hz), 2.85−2.68 (m, 3H), 2.45−2.29 (m, 4H), 2.18−2.13 (m, 1H), 1.51 (s, 9H, ^{t}Bu), 1.37 (s, 9H, ^{t}Bu), 1.32 (s, 9H, ^{t}Bu), 1.30 (s, 9H, ^{t}Bu), 0.88 (m, 1H), 0.49 (m, 2H), 0.12 (m, 2H); HRMS (FAB) *m/z* 914.4630 (M + H)$^+$, $C_{48}H_{63}N_7O_{11}$ requires 913.4585.

4′-Guanidino-17-(cyclopropylmethyl)-6,7-didehydro-4,5α-epoxy-3,14-hydroxyindolo-[2′,3′:6,7]morphinian (4a). Compound **6a** (400 mg, 0.6 mmol) was dissolved in HPLC grade CH_2Cl_2, and the contents were cooled in an ice bath. TFA (3.0 mL) was added in divided portions over a period of 10 min, and the flask was sealed under N_2 atmosphere and allowed to stir at room temperature. The reaction was monitored by TLC, and after 36 h, CH_2Cl_2 and TFA were removed with a stream of N_2, leaving a residue which was subjected to column chromatography (CH_2Cl_2−MeOH−NH_4OH, 78:20:2) to afford **4a** along with $CF_3CO_2^-NH_4^+$. Further purification was accomplished by preparative TLC (CH_2Cl_2−MeOH−NH_4OH, 78:20:2) to give **4a** (211 mg, 75%); IR KBr disk υ (cm^{-1}): 3400−3150 (br), 1675 (s), 1507, 1463, 1432, 1332, 1202, 1134; ^{1}H NMR (DMSO-d_6): δ 11.64 (s, 1H, NH), 9.84 (s, 1H, NH), 9.32 (s, 1H, NH), 8.92 (s, 1H, Ar−OH), 7.33−7.06 (m, 4H, ArH and NH_2), 6.78 (d, 1H, J = 7.20 Hz, ArH), 6.59 (d, 1H, J = 8.1 Hz, ArH), 6.52 (d, 1H, J = 8.1 Hz, ArH), 5.64 (s, 1H, 5-H), 4.11 (b, 1H, 14-OH), 3.44 (m, 1H), 3.12−2.99 (m, 4H), 2.87 (m, 1H), 2.70−2.55 (m, 4H), 1.76 (d, 1H, J = 11.7 Hz), 1.07 (m, 1H), 0.64−0.56 (m, 2H), 0.37−0.44 (m, 2H); HRMS (FAB) *m/z* 472.2357 (M + H)$^+$, $C_{27}H_{29}N_5O_3$ requires 471.2270). Anal. ($C_{27}H_{29}N_5O_3$·2TFA·2H_2O) C, H, N.

6′-Guanidino-17-(cyclopropylmethyl)-6,7-didehydro-4,5α-epoxy-3,14-hydroxyindolo-[2′,3′:6,7]morphinian (4b). Compound **6b** (500 mg, 0.75 mmol) was dissolved in a mixture of TFA (3.0 mL) and dried CH_2Cl_2 (28 mL) and allowed to stir under N_2 atmosphere at room temperature for 36 h. The reaction was worked up according to the procedure for **4a** and purified by preparative TLC to give **4b** (260 mg, 74%) as a free base: IR KBr disk υ (cm^{-1}) 3450−3150 (br), 1683 (s), 1506, 1463, 1433, 1330, 1202, 1132; ^{1}H NMR (DMSO-d_6) δ 11.50 (s, 1H, NH), 9.96 (s, 1H, NH), 9.29 (s, 1H, NH), 8.95 (s, 1H, Ar-OH), 7.36−7.09 (m, 3H, ArH and NH_2), 6.77 (d, 1H, J = 8.10 Hz, ArH), 6.59−6.52 (m, 2H, ArH), 6.39 (s, 1H), 5.67 (s, 1H, 5-H), 4.05 (b, 1H, 14-OH), 3.43−3.23 (m, 3H), 3.18−3.06 (m, 2H), 2.96−2.91 (m, 2H), 2.68−2.57 (m, 2H), 2.50 (m, 1H), 1.78 (d, 1H, J = 11.7 Hz), 1.05 (m, 1H), 0.68 (m, 1H), 0.58 (m, 1H), 0.40 (m, 2H); HRMS (FAB) *m/z* 472.2356 (M + H)$^+$, $C_{27}H_{29}N_5O_3$ requires 471.2270. Anal. ($C_{27}H_{29}N_5O_3$·2TFA·2H_2O) C, H, N.

7′-Guanidino-17-(cyclopropylmethyl)-6,7-didehydro-4,5α-epoxy-3,14-hydroxyindolo-[2′,3′:6,7]morphinian (4c). Intermediate **6c** (530 mg, 0.78 mmol), TFA (3 mL), and CH_2Cl_2 (28 mL) were subjected to conditions similar to those employed in the procedure for **4a** to give **4c** (251 mg, 67%) as a free base: IR KBr disk υ (cm^{-1}) 3450−3150 (br), 1676 (s), 1506, 1462, 1431, 1324, 1202, 1134; ^{1}H NMR (DMSO-d_6): δ 11.65 (s, 1H, NH), 9.88 (s, 1H, NH), 9.31 (s, 1H, NH), 8.96 (s, 1H, Ar−OH), 7.34−7.03 (m, 4H, ArH and NH_2), 6.60 (d, 1H, J = 8.10 Hz, ArH), 6.53 (d, 1H, J = 8.7 Hz, ArH), 6.39 (s, 1H), 5.62 (s, 1H, 5-H), 4.07 (b, 1H, 14-OH), 3.43−3.23 (m, 4H), 3.18−3.06 (m, 2H), 2.98−2.90 (m, 2H), 2.67−2.52 (m, 2H), 1.78 (d, 1H, J = 15.7 Hz), 1.05 (m, 1H), 0.68 (m, 1H), 0.59 (m, 1H), 0.41 (m, 2H); HRMS (FAB) *m/z* 472.2338 (M + H)$^+$, $C_{27}H_{29}N_5O_3$ requires 471.2270. Anal. ($C_{27}H_{29}N_5O_3$·2TFA·3H_2O) C, H, N.

5′-*N*′-(*N*″,*N*‴-Bis(benzyloxycarbonyl)guanidino-17-(cyclopropylmethyl)-6,7-didehydro-4,5α-epoxy-3,14-hydroxyindolo[2′,3′:6,7]-morphinian (7). Method A. A mixture of *N*,*N*′-dicarboxybenzyloxy-*N*″-trifluoromethanesulfonylguanidine (3.80 g, 8.27 mmol, 3.3 equiv), 5′-amino-NTI **5b** (1.06 g, 2.5 mmol), and triethylamine (1.5 mL) in dry CH_2Cl_2 (50 mL) was stirred at room temperature until all of **5b** was consumed (by TLC). After completion of the reaction (4 days), the mixture was diluted with CH_2Cl_2 (100 mL) and washed with 2 M of sodium bisulfate, saturated $NaHCO_3$, and brine. After drying with $MgSO_4$ and filtering, the solvent was removed under reduced pressure, and the crude product was subjected to flash column chromatography on silica gel (CH_2Cl_2−MeOH−NH_4OH, 94.5:5.0:0.5) to give 1.41 g (76%) of **7**.

Method B. 5′-Amino-NTI **5b** (2.68 g, 6.24 mmol) was dissolved in freshly distilled dry CH_2Cl_2, and 3.0 mL of triethylamine was added. To this mixture was added $HgCl_2$ (2.70 g, 10 mmol), followed by l,3-bis(benzyloxycarbonyl)-2-methyl-2-pseudothiourea (2.64 g, 7.33 mmol) in portion. The flask was sealed under N_2 atmosphere and allowed to stir at room temperature, and the progress of the reaction was monitored by TLC (CH_2Cl_2−MeOH−NH_4OH, 89:10:1). After completion of the reaction (2 h), it was filtered through Celite under vacuum to remove $HgSO_4$, and the residue was washed throughly with methanol. The combined filtrate was concentrated under reduced pressure to give a brownish material which was subjected to column chromatography (CH_2Cl_2−MeOH−NH_4OH, 97.5:2.0:0.5) to give **7** (3.84 g, 78%): ^{1}H NMR (DMSO-d_6) δ 11.35 (s, 1H, NH), 11.09 (s, 1H, NH), 9.83 (s, 1H, NH), 8.82 (s, 1H, 3-OH), 7.38 (s, 1H, Ar), 7.16−7.04 (m, 13H), 7.01 (m, 1H, ArH), 6.38−6.31 (m, 2H), 5.62 (s, 1H), 5.08 (b, 2H), 4.93 (b, 2H), 4.62 (b, 1H, 14-OH), 3.34−3.24 (m, 1H), 3.01 (d, 1H, J = 19.2 Hz), 2.70−2.56 (m, 2H), 2.46−2.24 (m, 4H), 2.21 (m, 1H), 1.54 (d, 1H, J = 11.1 Hz), 0.86 (m, 1H), 0.48 (m, 2H), 0.12 (m, 2H). ^{13}C NMR (DMSO-d_6) δ 153.78, 143.79, 143.59, 135.40, 131.77, 131.58, 129.10, 128.84, 128.73, 128.49, 127.31, 127.11, 126.73, 124.92, 119.30, 119.02, 117.67, 113.98, 112.04, 110.93, 84.53, 72.85, 63.66, 62.39, 59.30, 55.60, 48.04, 43.98, 31.83, 29.36, 23.31, 9.87, 4.54, 4.22; HRMS (FAB) *m/z* 740.3093 (M + H)$^+$, $C_{43}H_{41}N_5O_7$ requires 739.8355.

5′-Guanidino-17-(cyclopropylmethyl)-6,7-didehydro-4,5α-epoxy-3,14-hydroxyindolo-[2′,3′:6,7]morphinian Hydrochloride (3·2HCl). Di-Cbz-protected GNTI **7** (820 mg, 1.10 mmol) was dissolved in MeOH (100 mL). To a Parr hydrogenating bottle was added 100 mg of Pd/C (10% wt), followed by anhydrous MeOH (care was taken in order to avoid a possible fire; for a precaution, it is suggested to use a nitrogen atmosphere in the bottle). Di-Cbz-protected GNTI solution was added to the reaction bottle, stirred well, and added dilute HCl dropwise. The reaction bottle was subjected to hydrogenation at a pressure of 65 psi. After completion of the reaction (4 h), it was left overnight at room temperature. The soluble part was decanted, and the residue was stirred two times with 150 mL of hot MeOH (containing 5% of water) for a few minutes and filtered off. The combined filtrate were concentrated to a volume of ca. 5 mL under reduced pressure. Lyophilization gave the dihydrochloride salt of **3** (520 mg, 90%). **3·2HCl**: IR KBr disk υ (cm^{-1}) 3400−3200 (br), 1675 (s), 1502, 1461, 1430, 1325, 1202, 1136; ^{1}H NMR (DMSO-d_6) δ 11.56 (s, 1H, NH), 9.92 (s, 1H, NH), 9.35 (s, 1H, NH), 9.00 (s, 1H, 3-OH), 7.40−7.33 (m, 3H, NH_2), 7.15 (s, 1H, ArH), 6.90 (d, 1H, J = 8.10 Hz, ArH), 6.87 (d, 1H, J = 5.7 Hz, ArH), 6.64−6.50 (m, 2H, ArH), 5.68 (s, 1H, 5-H), 4.14 (d, 1H, J = 5.7 Hz, 14-OH), 3.43 (m, 1H), 3.26 (m, 1H), 3.16−3.06 (m, 2H), 2.94−2.90 (m, 2H), 2.69−2.57 (m, 2H), 2.54−2.45 (m, 2H), 1.77 (d, 1H, J = 15.7 Hz), 1.10 (m, 1H), 0.71−0.66 (m, 1H), 0.63−0.59 (m, 1H), 0.50−0.47 (m, 1H), 0.43−0.39 (m, 1H); HRMS (FAB) *m/z* 472.2349 (M + H)$^+$, $C_{27}H_{29}N_5O_3$ requires 471.2270.

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