The reaction mixture was diluted into methylene chloride (200 mL) and washed with cold 0.5 N HCl (3 × 100 mL), saturated $NaHCO_3$ (2 × 100 mL), and saturated NaCl (1 × 100 mL). The organic phase was dried over magnesium sulfate, suction filtered, and concentrated in vacuo to obtain a white solid (2.54 g, 8.9 mmol, 89%): R_f(4) 0.42; mp 127–129 °C; $[\alpha]_D$ −23.5° (c 1.01, MeOH); 1H NMR ($CDCl_3$) δ 0.93 (12 H, m), 1.57 (9 H, s), 1.23–1.77 (3 H, m), 2.10 (1 H, m), 3.23 (2 H, br q, J = 9 Hz), 3.83 (1 H, dd, J = 9, 6 Hz), 5.17 (1 H, br d, J = 9 Hz), 6.23 (1 H, br m); mass spectrum, 286 (M^+), 213, 172, 116, 72.

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Probes for Narcotic Receptor Mediated Phenomena. 15.[1] (3S,4S)-(+)-*trans*-3-Methylfentanyl Isothiocyanate, a Potent Site-Directed Acylating Agent for the δ Opioid Receptors in Vitro

Chong-Ho Kim,[†] Richard B. Rothman,[‡] Arthur E. Jacobson,[†] Mariena V. Mattson,[†] Victor Bykov,[‡] Richard A. Streaty,[§] Werner A. Klee,[§] Clifford George,[∥] Joseph B. Long,[⊥] and Kenner C. Rice*,[†]

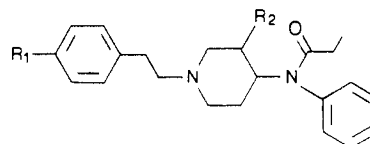
Laboratory of Medicinal Chemistry, National Institute of Diabetes, Digestive and Kidney Diseases, National Institutes of Health, Laboratory of Clinical Science, Laboratory of Molecular Biology, National Institute of Mental Health, Bethesda, Maryland 20892, Laboratory for the Structure of Matter, Naval Research Laboratory, Washington, D.C. 20375, and Neuropharmacology Branch, Walter Reed Army Institute of Research, Washington, D.C. 20307-5100. *Received May 13, 1988*

Recently we reported the synthesis of the first enantiomeric pair of irreversible opioid ligands [(3S,4R)-(−)- and (3R,4S)-(+)-*cis*-4, SUPERFIT] and specific interaction of the latter with the δ receptor. Here we report another enantiomeric pair of irreversible opioid ligands, (+)-*trans*- and (−)-*trans*-3-methylfentanyl isothiocyanates [(3S,4S)-(+)-*trans*- and (3R,4R)-(−)-*trans*-4]. A single-crystal X-ray analysis of the 2,4,6-trinitrobenzenesulfonic acid salt of (+)-*trans*-3-methyl-N-phenyl-4-piperidinamine [(+)-*trans*-8] revealed it (and, therefore, 4) to have the trans configuration and the absolute configuration of (+)-*trans*-8 to be 3S,4S. The (+)-trans enantiomer of 4 was shown to be highly potent and about 10-fold more selective as an acylating agent than (−)-*trans*-4 for the higher affinity [^{3}H]DADL (δ) binding site in rat brain membranes. In that assay, (+)-*trans*-4 and (+)-*cis*-4 were essentially equipotent as affinity ligands, and the levo enantiomers were considerably less potent. (+)-*trans*-4 was, thus, a potent, subtype-selective acylating agent for the δ opioid receptor in vitro. With membranes from NG108-15 neuroblastoma × glioma hybrid cells, containing only δ receptors, (+)-*cis*-4 was found to be a little more potent than (+)-*trans*-4. Similarly, (+)-*cis*-4 is the most effective inhibitor of adenylate cyclase in these membranes, (+)-*trans*-4 has weak activity, and the levo enantiomers are inactive. Only (+)-*cis*-4 was found to have antinociceptive activity in vivo.

Since the discovery of saturable, high-affinity, stereospecific receptors for opioid drugs and their antagonists in the mammalian central nervous system[2] and the identification of endogenous peptide ligands[3] for these receptors, much effort has been devoted toward elucidating their structure and function. These efforts have firmly established[4] the existence of distinct μ, δ, and κ subtypes and validated the hypothesis of heterogeneity of opioid receptors advanced more than two decades ago.[5] Additionally, on the basis of a variety of in vitro and in vivo evidence, an "opioid receptor complex" consisting of distinct yet interacting μ and δ binding sites has been postulated.[6-8]

A number of groups have synthesized irreversible ligands as tools for the study of opiod receptor subtypes.[9-13] Some of these ligands were used for the purification to homogeneity of μ[14] and δ[15] receptor subtypes. Other irreversible ligands have facilitated the study of the mechanisms of tolerance and dependence,[16] interactions among opioid receptor subtypes,[17] and examination of the hypothesis of multiple μ receptors,[18] as well as developing conditions for the autoradiographic visualization and mapping of the anatomical distributions of opioid receptor subtypes.[19-21]

As part of our ongoing opioid receptor studies, we described recently the μ-selective acylating agent BIT based on etonitazene and the δ-specific ligand FIT (2), a derivative of the potent opioid agonist fentanyl (1).[22,23] We also



1: $R_1 = R_2 = H$
2: $R_1 = NCS, R_2 = H$
3: $R_1 = H, R_2 = CH_3$
4: $R_1 = NCS, R_2 = CH_3$

reported the synthesis, absolute configuration, and bio-

† NIDDK.
‡ Laboratory of Clinical Science, NIMH.
§ Laboratory of Molecular Biology, NIMH.
∥ Naval Research Laboratory.
⊥ Walter Reed Army Institute for Research.

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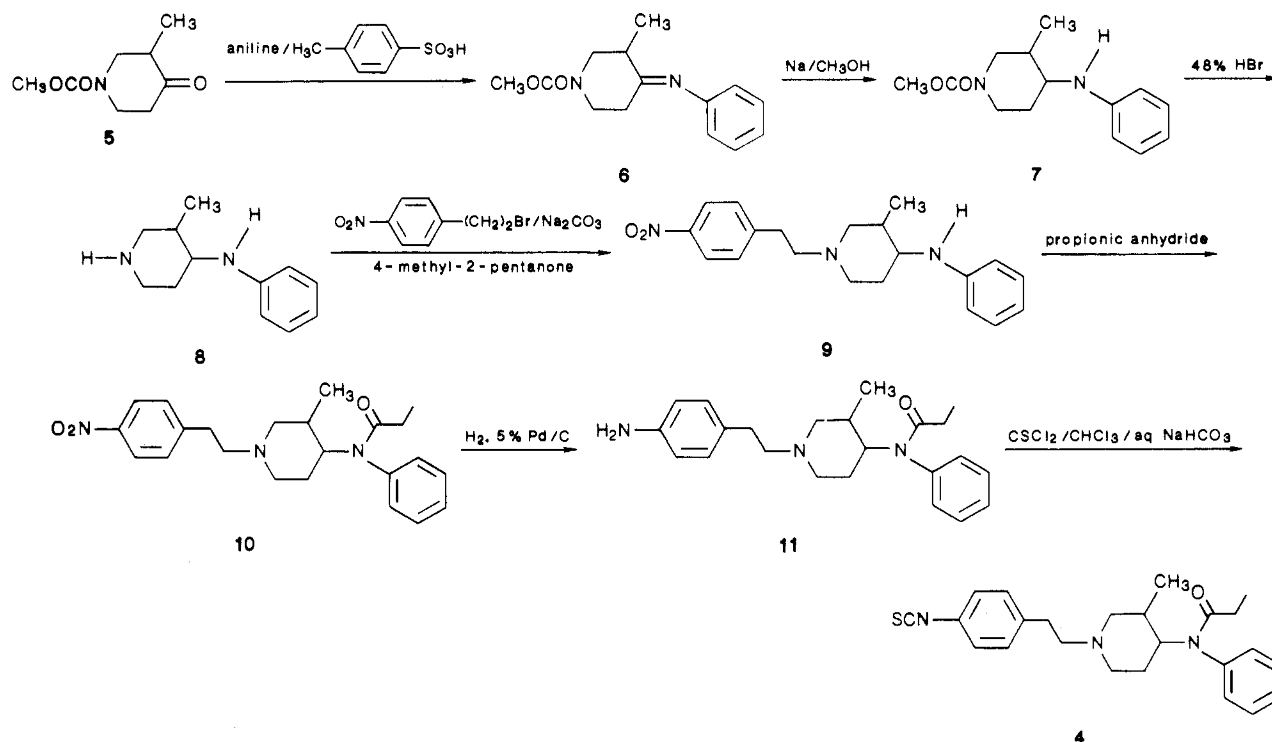
Scheme I



logical characterization of (3*R*,4*S*)-(+)-*cis*-3-methylfentanyl isothiocyanate [(+)-*cis*-**4**, SUPERFIT] and its enantiomer, as the first enantiomeric pair of irreversible opioid ligands.[24] Tritiated (+)-*cis*-**4** was essential for purification to homogeneity of δ opioid receptors from NG108-15 neuroblastoma × glioma hybrid cells,[15] and has provided insight into the roles of opioid receptor subtypes in analgesia.[25] In order to obtain further insight into the steric requirements for acylating opioids in the fentanyl series, we have synthesized the enantiomers of *trans*-**4**, determined their absolute configurations by single-crystal X-ray analysis, and studied them in vitro and in vivo. Although racemic *trans*-3-methylfentanyl [(±)-*trans*-**3**] has been noted to have only one-fifth the antinociceptive potency of the corresponding racemic *cis*-**3** in a rat tail withdrawal assay after systemic administration,[26] we did not feel that this observation was necessarily predictive of the value of enantiomeric acylating derivatives of *trans*-**4** for studies at the receptor level.

Synthesis

The synthesis is outlined in Scheme I. Piperidone **5** was obtained essentially as previously described[24,26] and by use of an improved procedure was converted to Schiff base **6** in 96% distilled yield. Analytical conditions were developed for gas chromatographic determination of the ratio of *trans*-:*cis*-**7** resulting from reduction of **6**. This reaction was studied in considerable detail to obtain a more favorable ratio of *trans*-:*cis*-**7** since the $NaBH_4$ originally used[26] for reduction of Schiff base **6** was found to give a 3:7 mixture of trans:cis. Clearly, a more favorable ratio would greatly simplify preparation of the substantial amount of (±)-*trans*-**8** required for optical resolution and further transformation to gram amounts of the enantiomers of *trans*-**4**.

Catalytic hydrogenation of **6** over PtO_2 in MeOH (3 h, 45 psig) gave only a 1:4 mixture of *trans*-:*cis*-**7**. However, reduction of **6** with sodium in MeOH reversed the ratio and afforded a 3:2 mixture of *trans*-:*cis*-**7**. When ethanol was utilized as solvent substantial amounts of the ethyl

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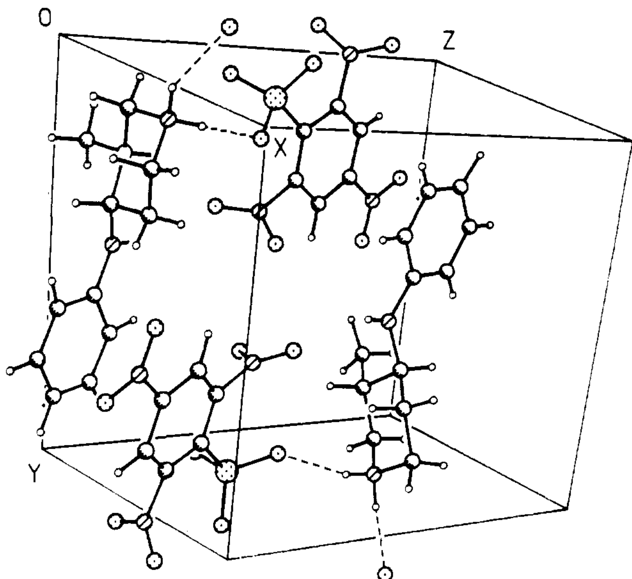

Figure 1. Packing plot for the 2,4,6-trinitrobenzenesulfonic acid salt of (+)-*trans*-3-methyl-*N*-phenyl-4-piperidinamine. The unit cell contains two crystallographically independent ion pairs. Hydrogen bonds are indicated with dashed lines. Only the hydrogen bonded oxygen atoms of the 2,4,6-trinitrobenzenesulfonate outside the unit cell are shown.

piperidine-1-carboxylate was produced. Acid hydrolysis of the isomeric mixture of **7** with 48% HBr gave an 83% yield of a mixture of *trans*- and *cis*-**8**. Treatment of the mixture of bases with fumaric acid in MeOH gave pure *cis*-**8**·fumarate after three crystallizations. The bases from the combined filtrates were converted to the oxalates, which after three crystallizations gave pure *trans*-**8**·oxalate. With methodology available to obtain a satisfactory ratio of racemic *trans*-:*cis*-**7** and -**8** and to separate the isomers of **8**, we next studied optical resolution of (±)-*trans*-**8** and were successful via fractional crystallization of the tartrate salts. After two crystallizations, the resulting amines, (+)-*trans*-**8** [obtained by crystallization of the L-(+)-tartrate salt] and (-)-*trans*-**8** [obtained by crystallization of the D-(-)-tartrate salt], were shown to be optically pure (>99.9%) by conversion to their diastereoisomers, the α-methylbenzylcarbamates, and analysis by HPLC. The absolute configuration of (+)-*trans*-**8** was established as 3*S*,4*S* as decribed below.

Alkylation of (3*S*,4*S*)-(+)-*trans*-**8** with 4-nitrophenethyl bromide[24,26] afforded (3*S*,4*S*)-*trans*-**9** (63%), which was acylated with propionic anhydride to give (3*S*,4*S*)-*trans*-**10** (62%). Catalytic hydrogenation of this material followed by treatment of the resulting (3*S*,4*S*)-*trans*-**11** with thiophosgene and product isolation as the hydrochloride afforded one of our target compounds, (3*S*,4*S*)-(+)-*trans*-**4**·HCl (63%). Conversion of (3*R*,4*R*)-(-)-*trans*-**8** to (3*R*,4*R*)-(-)-*trans*-**4** was accomplished by the same series of reactions.

Determination of the Absolute Configuration of (+)-*trans*-8 by Single-Crystal X-ray Analysis

In the least-squares refinement the final *R* factors for the 3213 observed reflections [$F_o > 3\sigma(|F_o|)$], including 285 Friedel equivalents were $R = 0.032$, $wR = 0.038$ and $R = 0.045$, $wR = 0.060$ for the two possible enantiomorphs. Application of the Hamilton ratio test[27] to the ratios of the *R* values, 1.406 (*R*) and 1.568 (*wR*), respectively, is well above the value 1.161 required for a significance level of 0.01. The contents of the unit cell for the 2,4,6-trinitrobenzenesulfonic acid salt of (+)-*trans*-3-methyl-*N*-phenyl-4-piperidinamine are shown in Figure 1. There are two independent anion–cation pairs in the asymmetric unit whose primary difference is the torsion of the phenyl group with respect to the piperidinium ring, with C(9)–N(8)–C(4)–C(3) = −169.8° and C(9′)-N(8′)–C(4′)–C(3′) = −112.9°, respectively, for the two cations. In Figure 2, drawn from experimental coordinates for the enantiomorph with the lowest *R* values, the asymmetric carbons C3 and C4 have an *S* configuration for each of the two independent anion–cation pairs in the asymmetric unit. Bond distances and angles are normal, and the piperidinium ring has a chair conformation. The methyl group is trans with respect to the pyramidal *N*-phenyl nitrogen, which is equatorially bonded to the piperidinium ring. A pair of hydrogen bonds occurs between the quadrivalent nitrogen of each cation and the sulfonate oxygens of the two crystallographically independent anions. This forms an infinite hydrogen bonded chain with the following range for the hydrogen bond parameters: N···O = 2.81–2.88 Å, O···H = 1.86–2.05 Å, and angle N–H···O = 134.2–167.9°.

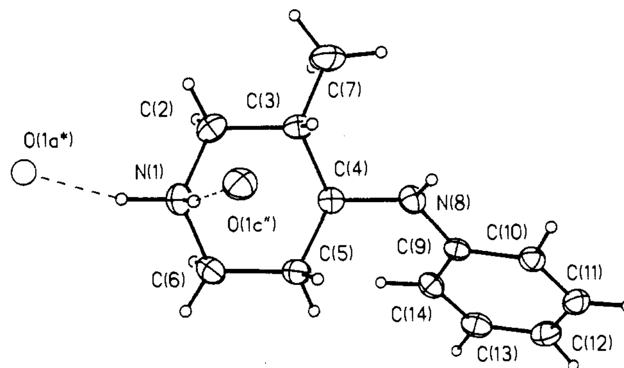

Figure 2. Thermal ellipsoid plot of one of the two independent (+)-*trans*-3-methyl-*N*-phenyl-4-piperidinium cations with thermal ellipsoids drawn at 20% probability level. The 2,4,6-trinitrobenzenesulfonate ions, except for two sulfonate oxygens, are omitted. The dashed lines are hydrogen bonds. O(1a*) is related to the asymmetric unit by the symmetry transformation ($x,y - 1.0, z$).

Results and Discussion

The ligand [^{3}H]-D-Ala^2-D-Leu^5-enkephalin ([^{3}H]DADL) is known to label two binding sites on rat brain membranes, distinguished by the inhibitor mechanism of μ ligands. μ ligands are weak, competitive inhibitors at the higher affinity [^{3}H]DADL binding site (commonly identified as δ), whereas they are potent, noncompetitive inhibitors at the lower affinity [^{3}H]DADL binding site (commonly identified as μ). Previous studies established that conducting the assay in the presence of 50 nM [D-Pen^2,L-Pen^5]enkephalin (DpLp) and 100 nM MeTyr-D-Ala-Gly-N(Et)CH(CH_2Ph)CH_2N(CH_3)$_2$ (LY164929) permit the separate assay of the lower and higher affinity [^{3}H]-DADL binding sites, respectively.[28]

Under these assay conditions, as reported in Table I, all four acylating agents [(+)-*cis*-**4**, (-)-*cis*-**4**, (+)-*trans*-**4**, (-)-*trans*-**4**] produced an almost parallel wash-resistant inhibition of μ binding sites labeled by tritiated 3,14-dihydroxy-4,5α-epoxy-6β-fluoro-17-methylmorphinan ([^{3}H]FOXY),[29] as well as the lower affinity [^{3}H]DADL binding site. However, certain concentrations of (+)-*cis*-**4**,

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Table I. Effect of SUPERFIT Analogues on Opioid Binding Sites[a]

drug	% of control 0 nM	10 nM	100 nM	1000 nM	10 000 nM
1. (-)-*cis*-4					
[³H]FOXY	100	98.6	95.9	67.8[b]	21.9[b]
[³H]DADL, lower affinity binding site	100	98.3	99.4	72.2[b]	21.7[b]
[³H]DADL, higher affinity binding site	100	96.1	59.2[b]	5.8[b]	4.8[b]
2. (+)-*cis*-4					
[³H]FOXY	100	94.3	90.3[b]	53.8[b]	15.0[b]
[³H]DADL, lower affinity binding site	100	79.5[b]	70.8[b]	49.2[b]	16.6[b]
[³H]DADL, higher affinity binding site	100	21.0[b]	2.47[b]	1.18[b]	4.63[b]
3. (-)-*trans*-4					
[³H]FOXY	100	94.7	106	100	78.1[b]
[³H]DADL, lower affinity binding site	100	93.7	93.4	89.3[b]	69.1[b]
[³H]DADL, higher affinity binding site	100	83.8[b]	21.9[b]	2.28[b]	1.33[b]
4. (+)-*trans*-4					
[³H]FOXY	100	94.5	101	100	58.6[b]
[³H]DADL, lower affinity binding site	100	84.3[b]	86.2[b]	78.3[b]	48.8[b]
[³H]DADL, higher affinity binding site	100	10.8[b]	4.3[b]	2.4[b]	1.5[b]

[a] Membranes were treated with acylating agents as described in the Experimental Section. The concentration of [³H]FOXY was 2.8 nM while that of [³H]DADL was 4.2 nM. The lower and higher affinity [³H]DADL binding sites were assayed with 50 nM DpLp and 100 nM LY164929 to block [³H]DADL binding to the higher and lower affinity binding sites, respectively. Each point is the mean of three determinations whose SDs were less than 6% of the mean. [b] $p < 0.01$ when compared to control.

(-)-*trans*-4, and (+)-*trans*-4 had apparently greater effect on the lower affinity [³H]DADL site than [³H]FOXY binding site(s), indicating that these two binding sites are similar, but not identical.

Presumptive evidence that the isomers of 4 act irreversibly was obtained from the observation that the binding lost by treatment with these isomers could not be recovered after the extensive wash procedures and that preincubation of membranes with (+)-*cis*- and (-)-*cis*-3-methylfentanyl[26] had no effect on the higher affinity [³H]DADL binding site after the washing. Irreversible binding of (+)-*cis*-4 to δ receptors of NG108-15 neuroblastoma × glioma hybrid cells has been unequivocally demonstrated earlier.[15]

The rank order of potency for the wash-resistant inhibition of [³H]DADL binding to its higher affinity binding site was (+)-*trans*-4 ≈ (+)-*cis*-4 > (-)-*trans*-4 > (-)-*cis*-4. The same rank order applies to their ability to greatly reduce binding of [³H]DADL to its higher affinity site without greatly reducing either [³H]DADL binding to its lower affinity site or [³H]FOXY binding. Thus the optical isomerism appears to play a more important role than the cis:trans configuration.

Comparing the in vitro effects of all four isomers, (+)-*trans*-4 appears to possess the highest potency and selectivity for the higher affinity [³H]DADL (δ) binding site. However, acylation of the receptor at sites outside the drug binding site, thereby altering properties of the binding, cannot be ruled out. Sulfhydryl groups in sulfhydryl-containing amino acids, which may control the conformation of opioid receptors,[30] are known to irreversibly interact with the isothiocyanate moiety in opioid

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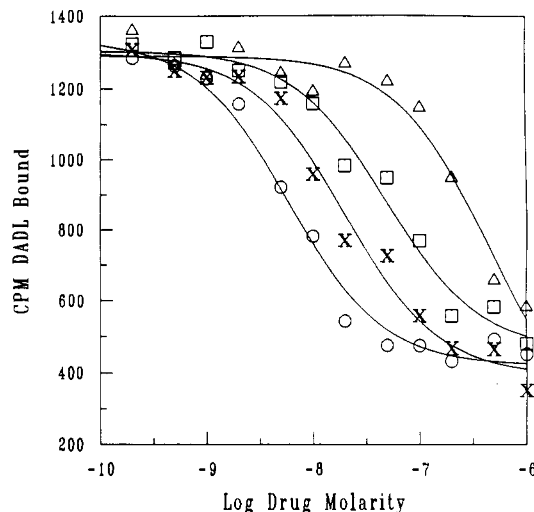

Figure 3. Binding of SUPERFIT analogues to the δ receptors of NG108-15 cell membrane. Membranes (0.62 mg of protein) were incubated with 2 nM [³H]DADL and the indicated concentrations of (+)-*cis*-4 (○), (+)-*trans*-4 (x), (-)-*trans*-4 (□), (-)-*cis*-4 (△) in 0.01 M Tris-Cl, pH 7.5, for 10 min at 37 °C. Bound radioactivity was measured after filtration through PD-10 columns as described previously.[15]

ligands. For example, although treatment of membranes with FIT almost completely eliminates the higher affinity [³H]DADL binding site, there is a concurrent increase in the B_{max} of the lower affinity [³H]DADL binding site.[31] It is interesting to note that the parent compound, fentanyl, from which these acylators are derived, binds with high affinity to μ receptors, and FIT has been found in reversible binding assays to bind to both μ and δ receptors.[32] However, FIT preferentially acylates δ receptors.[22] Thus, the in vivo pharmacology of these compounds might be expected to be complex. For example, the icv administration of (+)-*cis*-4 to rats initially produces naloxone-reversible antinociception,[25] which is followed by a period of prolonged antagonism of morphine analgesia.[33] The apparently irreversible effect of (+)-*cis*-4 on morphine analgesia is prevented when the δ selective agonist ICI 174864 is administered 15 min prior to (+)-*cis*-4. When given sc,[34] only (+)-*cis*-4 (ED_{50} = 1.2 mg/kg) displays antinociceptive activity comparable to that of morphine in mice in the hot-plate assay.

The experiments reported in Table I were designed to determine the potency of fentanyl analogues as irreversible ligands. The ability of these compounds to inhibit [³H]-DADL binding to δ receptors of the NG108-15 cells was also determined (Figure 3). These assays were conducted (10 min at 37 °C) so as to minimize covalent interactions. (+)-*cis*-4 (IC_{50} = 6 nM) was somewhat more potent than (+)-*trans*-4 (IC_{50} = 20 nM) in displacing [³H]DADL binding. (-)-*cis*-4 (IC_{50} = 300 nM) and (-)-*trans*-4 (IC_{50} = 60 nM) were less potent than the (+) isomers. Thus the potency of the fentanyl analogues as inhibitors of [³H]-DADL binding to the δ receptor of the NG108-15 cell almost parallels their ability to irreversibly inhibit [³H]-DADL binding to the δ receptor of rat brain membranes. The compounds are especially interesting when examined

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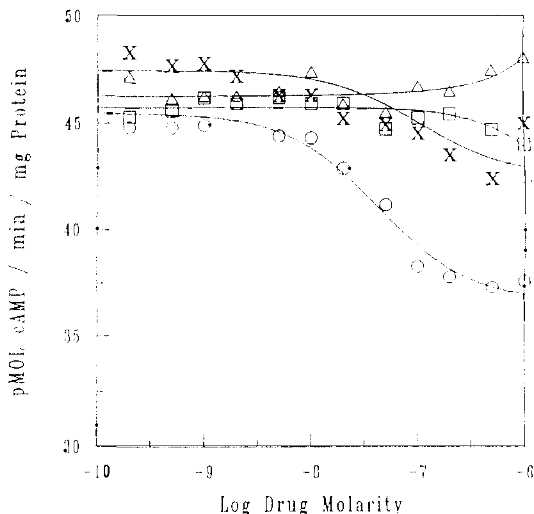


Figure 4. Inhibition of adenylate cyclase activity of NG108-15 membranes by SUPERFIT analogues. Activity was measured as described earlier[40] after a 5-min incubation with the indicated concentrations of (+)-*cis*-4 (O), (+)-*trans*-4 (x), (−)-*trans*-4 (□), (−)-*cis*-4 (△) at pH 7.5 for 5 min at 37 °C.

for their ability to inhibit adenylate cyclase of NG108-15 membranes. In experiments with 5-min incubation times, chosen to minimize covalent interactions, (+)-*cis*-4 is by far the best inhibitor (Figure 4). DADL, a pure agonist in this system, inhibits adenylate cyclase activity to an even greater extent. Thus (+)-*cis*-4 is only a partial agonist. The other compounds tested in this study are inactive, or only weakly active in the case of (+)-*trans*-4. Experiments with longer incubation times gave qualitatively similar results (data not shown). The ability of all four fentanyl analogues to bind to the δ receptor of NG108-15 cells and to weakly, if at all, inhibit adenylate cyclase suggests that these compounds might be forming a covalent bond with the receptor, producing a conformational change that prevents the coupling of the receptor with G proteins. Alternatively, these data are consistent with the observations of Law et al.[35] that μ agonists are weak, partial agonists at the δ receptor of NG108-15 cells.

Experimental Section

Melting points were determined on a Thomas-Hoover capillary melting point apparatus and uncorrected. IR spectra were determined with a Beckman IR 4230 instrument. NMR spectra were recorded with a Varian XL-300 MHz spectrophotometer. Chemical-ionization mass spectra (CIMS) were obtained with a Finnigan 1015D spectrometer with a Model 6000 data collection system, and high-resolution mass spectra were obtained with a VG Micromass 7070F spectrometer. Gas chromatographic analysis was done on a Hewlett-Packard Model 5880A instrument equipped with an SE-30 capillary column, with standard flow rates, and a flame-ionization detector. HPLC was performed with a Gilson Model 303 pumps with a Model 811 solvent mixer and a Model 620 Data Master coupled to an Apple IIe computer. Optical rotations were measured with a Perkin-Elmer 241MC polarimeter. Elemental analyses were done by Galbraith Laboratories, Inc., Knoxville, TN, or by Atlantic Microlaboratories, Inc., Atlanta, GA. All compounds gave NMR and IR spectra consistent with their assigned structures.

Methyl 3-Methyl-4-oxo-1-piperidinecarboxylate (5). This compound was prepared from commercially available 3-carbomethoxy-4-oxopiperidine hydrochloride in three steps (70% overall yield) as previously reported.[24]

Methyl 3-Methyl-4-(phenylimino)piperidinecarboxylate (6). A mixture of compound **5** (11.17 g, 65.3 mmol), aniline 6.6 mL, 72 mmol), and a catalytic amount of *p*-toluenesulfonic acid in toluene (50 mL) was stirred at reflux overnight with the removal of water by a Dean–Stark trap. The reaction mixture was cooled and distilled in vacuo to give 14.62 g (96%) of imine **6** [IR (film) 1660 cm^{-1} (C=N); bp 135–137 °C (0.5 mm) (lit.[26] bp 149–158 °C (0.1–0.4 mm))].

Methyl 3-Methyl-4-(phenylamino)-1-piperidinecarboxylate (7). (i) **Reduction by $NaBH_4$.** The imine **6** (22.68 g, 97.8 mmol) was dissolved in methanol (80 mL) and $NaBH_4$ (3.16 g, 83.5 mmol) was added in small portions. The mixture was stirred at 50 °C for 1 h, water was added, and the mixture was extracted with chloroform. The combined organic extracts were dried ($MgSO_4$) and evaporated, and the residue was distilled [bath temperature 190–210 °C (0.02 mm) (lit.[26] bp 171–172 °C (0.1–0.015 mm))] to give 9.26 g (81%) of the amine **7** as a mixture (cis:trans = 7:3 determined by capillary GC analysis). The *trans*- and the *cis*-**7** showed retention times of 14.52 and 15.28 min, respectively, at 150 °C.

(ii) **Reduction by Adam's Catalyst (PtO_2).** PtO_2 (0.1 g) was added to the imine **6** (0.5 g, 2.03 mmol) in MeOH (10 mL), and hydrogenated on a Parr apparatus at 45 psi for 3 h. The reaction mixture was filtered through a Celite bed, concentrated, and chromatographed (silica gel 60, ether) to give 0.24 g (48%) of a 3:1 cis:trans mixture of amine **7**.

(iii) **Reduction by Na/MeOH.** Na (0.22 g) was added to a solution of the imine **6** (0.54 g, 2.17 mmol) in absolute MeOH (20 mL) over 15 min, and the reaction mixture was stirred at reflux for 2 h. The reaction mixture was cooled, additional Na (0.22 g × 2) was added, and the mixture was stirred at reflux for 2 h (GC showed over 90% conversion). The reaction was quenched by pouring onto ice and the mixture was extracted with chloroform. The combined organic extracts were dried (K_2CO_3) and evaporated to give 0.38 g (71%) of a syrup (cis:trans = 2:3), which was used for the next step without further purification.

***cis*- and *trans*-3-Methyl-*N*-phenyl-4-piperidinamine (8).** A mixture of the carbamate **7** (1.58 g, 6.4 mmol) and 30 mL of 48% HBr was stirred at reflux for 4 h, cooled, basified to pH 11 with NaOH, and extracted with chloroform. The combined organic extracts were dried (K_2CO_3) and distilled [bath temperature 190–210 °C (0.05–0.1 mm) (lit.[26] bp 140–145 °C (0.4 mm))] to give 0.93 g (83%) of the amine **8**, which showed GC retention times of 8.10 min (*trans*-**8**) and 8.81 min (*cis*-**8**), respectively, at 125 °C.

Separation of *cis*- and *trans*-8. Fumaric acid (1.39 g, 12 mmol) in MeOH (10 mL) was added to an 2-propanol (10 mL) solution of the 3:2 mixture of *trans*- to *cis*-**8** (2.28 g, 12 mmol). The solution was evaporated to ca. 15 mL volume and left overnight at 0 °C. The precipitate (trans:cis = 33:67) was filtered off and the filtrate was basified with NH_4OH to give 0.63 g (3.3 mmol) of the amine **8** (trans:cis = 83:17). The amine **8** was dissolved in ether (20 mL) and mixed with oxalic acid (0.32 g, 3.5 mmol) in ether (20 mL), left at 0 °C for 2 h, filtered, and recrystallized (acetone–ether) to give 0.49 g of (±)-*trans*-**8**·oxalate, mp 167–169 °C. By repeating the same sequence three times [the purity of (±)-*trans*-**8** was assessed as >99.8% by GC analysis], 1.4 g (70%) of (±)-*trans*-**8**·oxalate was obtained. Anal. ($C_{14}H_{20}N_2O_4 \cdot 0.25\ H_2O$) C, H, N.

Optical Resolution of (±)-*trans*-8. The (±)-*trans*-**8**·oxalate (0.56 g, 2 mmol) was dissolved in water, basified to pH 11 with NH_4OH, and extracted with chloroform. The combined organic extract was dried (K_2CO_3) and evaporated. The residue in MeOH was heated to solution with L-(+)-tartaric acid (0.30 g, 2 mmol). Hot acetone (20 mL) was added and the solution was cooled and kept at 0 °C overnight to give the crystalline tartrate salt (0.276 g). The tartrate salt was dissolved in water, basified with 20% NaOH, extracted with chloroform, dried (K_2CO_3), and again treated with L-(+)-tartaric acid (0.124 g, 0.83 mmol) to give (+)-*trans*-**8**·(+)-tartrate (0.261 g, 77%), mp 165–167 °C (MeOH–acetone), $[\alpha]^{22}_D$ +75.5° (*c* 0.143, MeOH). Anal. ($C_{16}H_{24}N_2O_6$) C, H, N.

The (+)-tartrate salt was basified with 20% NaOH to give the free amine (+)-*trans*-**8**, $[\alpha]^{22}_D$ +116.3° (*c* 0.057, MeOH).

Solvent was removed from the combined filtrates, the residual solid was dissolved in water, basified with 20% NaOH, extracted with chloroform, and dried (K_2CO_3). The solvent was removed, the residual material was dissolved in MeOH, D-(−)-tartaric acid (0.30 g, 2 mmol) was dissolved in this solution with heating, and after standing at 0 °C overnight, crystalline (−)-*trans*-**8**·(−)-tartrate

(35) Law, P. Y.; Hom, D. S.; Loh, H. H. *Mol. Pharmacol.* **1982**, *23*, 26.

was obtained (0.250 g, 74% after two recrystallizations), mp 173–175 °C (MeOH–acetone), $[\alpha]^{22}_D$ −74.5° (*c* 0.145, MeOH). Anal. ($C_{16}H_{24}N_2O_6 \cdot 0.33H_2O$) C, H, N.

The (−)-tartrate salt was basified with 20% NaOH to give the free amine (−)-*trans*-8, $[\alpha]^{22}_D$ −116.7° (*c* 0.163, MeOH).

Determination of Enantiomeric Purity.[24] A sample of ca. 5 mg of either (+)-*trans*-8·(+)-tartrate or (−)-*trans*-8·(−)-tartrate was basified with 20% NaOH, extracted with chloroform, dried (K_2CO_3), and treated with ca. 1 μL of optically pure (*R*)-(+)-α-methylbenzyl isocyanate.[1] The product was driectly analyzed by HPLC as described[24] for the enantiomer of *cis*-8. The α-methylbenzylcarbamate of (+)-*trans*-8 and (−)-*trans*-8 gave peaks at 10.71 and 16.24 min, respectively (μ-Porasil column, 2-propanol–hexane = 3:97). Integration of peak areas indicated enantiomeric purities of >99.9%.

(3*S*,4*S*)-*trans*-3-Methyl-1-[2-(4-nitrophenyl)ethyl]-*N*-phenyl-4-piperidinamine [(3*S*,4*S*)-*trans*-9]. A mixture of (+)-*trans*-8 (0.4 g, 2.1 mmol), 4-nitrophenethyl bromide (0.51 g, 2.2 mmol), Na_2CO_3 (0.45 g, 4.2 mmol), and KI (catalytic amount) in 4-methyl-2-pentanone[26] (30 mL) was stirred at reflux overnight. The reaction mixture was filtered, evaporated, and purified by column chromatography (silica gel 60, CH_2Cl_2 then 3% CH_3OH/CH_2Cl_2) to give 0.45 g (63%) of (3*S*,4*S*)-*trans*-9 as an oil; high-resolution MS ($C_{20}H_{25}N_3O_2$) calcd 339.1947, found 339.1914.

(3*R*,4*R*)-*trans*-3-Methyl-1-[2-(4-nitrophenyl)ethyl]-*N*-phenyl-4-piperidinamine [(3*R*,4*R*)-*trans*-9]. Similarly, (3*R*,4*R*)-*trans*-9 was prepared from (3*R*,4*R*)-(−)-*trans*-8 in 62% yield; high-resolution MS ($C_{20}H_{25}N_3O_2$) calcd 339.1947, found 339.1945.

(3*S*,4*S*)-*trans*-*N*-[3-Methyl-1-[2-(4-nitrophenyl)ethyl]-4-piperidyl]-*N*-phenylpropanamide [(3*S*,4*S*)-*trans*-10]. To a toluene (30 mL) solution of (3*S*,4*S*)-*trans*-9 (0.21 g, 0.62 mmol) was added 0.62 mL of propionic anhydride and the mixture was stirred at reflux overnight. The solvent was evaporated and the resulting syrup was purified by column chromatography (silica gel 60, 3% $CH_3OH–CH_2Cl_2$) to give 0.21 g (81%) of (3*S*,4*S*)-*trans*-10 as an oil; high-resolution MS ($C_{23}H_{29}N_3O_3$) calcd 395.2208, found 395.2207.

(3*R*,4*R*)-*trans*-*N*-[3-Methyl-1-[2-(4-nitrophenyl)ethyl]-4-piperidyl]-*N*-phenylpropanamide [(3*R*,4*R*)-*trans*-10]. Similarly, (3*R*,4*R*)-*trans*-10 was prepared from (3*R*,4*R*)-*trans*-9 in 86% yield; high-resolution MS ($C_{23}H_{29}N_3O_3$) calcd 395.2208, found 395.2195.

(3*S*,4*S*)-*trans*-*N*-[1-[2-(4-Aminophenyl)ethyl]-3-methyl-4-piperidyl]-*N*-phenylpropanamide Hydrochloride [(3*S*,4*S*)-*trans*-11·HCl]. Pd/C (5%, 0.1 g) was added to a solution of (3*S*,4*S*)-*trans*-10·HCl (0.27 g, 0.63 mmol) in methanol (20 mL) and hydrogenated on a Parr apparatus (45 psi, 3 h). The reaction mixture was filtered through a Celite bed and concentrated to give 0.24 g (97%) of a foam; CIMS (NH_3) *m/z* 366 (M + 1).

(3*R*,4*R*)-*trans*-*N*-[1-[2-(4-Aminophenyl)ethyl]-3-methyl-4-piperidyl]-*N*-phenylpropanamide Hydrochloride [(3*R*,4*R*)-*trans*-11·HCl]. Similarly, (3*R*,4*R*)-*trans*-11·HCl was prepared from (3*R*,4*R*)-*trans*-10·HCl in 94% yield, white foam; CIMS (NH_3) *m/z* 366 (M + 1).

(3*S*,4*S*)-(+)-*trans*-*N*-[1-[2-(4-Isothiocyanatophenyl)-ethyl]-3-methyl-4-piperidyl]-*N*-phenylpropanamide Hydrochloride [(+)-*trans*-4·HCl]. A solution of (3*S*,4*S*)-*trans*-11·HCl (0.25 g, 0.62 mmol) in $CHCl_3$ (20 mL) was stirred with $NaHCO_3$ (0.37 g, 4.4 mmol) in H_2O (10 mL) for 15 min and mixed with freshly distilled thiophosgene (60 μL, 0.8 mmol). After the mixture was stirred for 30 min at 23 °C, the organic layer was separated and the aqueous layer was extracted with chloroform. The combined organic extracts were evaporated and converted to the hydrochloride salt with methanolic HCl to give 0.174 g (63%) of (+)-*trans*-4·HCl, mp 187–190 °C (2-propanol–ether); CIMS (NH_3) *m/z* 408 (M + 1); $[\alpha]^{22}_D$ +16.9° (*c* 0.133, MeOH). Anal. ($C_{24}H_{29}N_3SO \cdot HCl \cdot 1.25H_2O$) C, H, N, S.

(3*R*,4*R*)-(−)-*trans*-*N*-[1-[2-(4-Isothiocyanatophenyl)-ethyl]-3-methyl-4-piperidyl]-*N*-phenylpropanamide Hydrochloride [(−)-*trans*-4·HCl]. Similarly, (−)-*trans*-4·HCl was prepared from (3*R*,4*R*)-*trans*-11·HCl in 76% yield, mp 198–202 °C (2-propanol–ether); CIMS (NH_3) *m/z* 408 (M + 1); $[\alpha]^{22}_D$ −17.4° (*c* 0.134, MeOH). Anal. ($C_{24}H_{29}N_3SO \cdot HCl$) C, H, N, S.

X-ray Analysis for (+)-*trans*-8. The 2,4,6-trinitrobenzenesulfonic acid salt of (+)-*trans*-8 used for X-ray crystallographic analysis recrystallized from 2-propanol–ethyl acetate as reddish brown crystals, mp 214–215 °C. Anal. ($C_{18}H_{21}N_5O_9S$) C, H, N, S. A 0.06 × 0.21 × 0.62 mm crystal was used for data collection, and cell dimensions were determined with 25 centered reflections within 40 < 2θ < 50°. The space group is triclinic, *P*1, with a = 8.443 (1) Å, b = 10.646 (1) Å, c = 13.195 (2) Å, α = 82.01 (1)°, β = 75.87 (1)°, γ = 89.13 (1)°, and V = 1138.7 (3) Å^3, and D_{calcd} = 1.428 g cm^{-3}, Z = 2 (two formula units in the asymmetric unit). A computer-controlled diffractometer (Nicolet R3mμ, with Cu Kα radiation, λ = 1.54178 Å, with an incident beam graphite monochromator was used for the data collection. A total of 3289 unique reflections were measured in the θ/2θ mode to $2\theta_{max}$ = 112. The scan width was $[2\theta(K\alpha_1) - 1.0]$ to $[2\theta(K\alpha_2) + 1.0]$ while the scan rate was a function of the count rate (8/min minimum, 30/min maximum). Standard reflections were monitored every 60 reflections and the data were corrected for an 8.5% linear decay. Corrections were also applied for Lorentz, polarization, and absorption effects. The structure was solved by direct methods with the aid of the program SHELX[36] and refined with use of the full-matrix least-squares program SHELXLS.[36] The 755 parameters refined include the coordinates and anisotropic thermal parameters for all non-hydrogen atoms. Hydrogen atoms were refined isotropically. The final *R* factors for the 3213 observed reflections with $[F_o > 3\sigma(|F_o|)]$ were R = 0.032 and wR = 0.038, S (goodness of fit parameter) = 1.655. Tables of coordinates and bond distances and angles have been deposited with the Crystallographic Data Center, Cambridge CB2 1EW, England.

Binding Assay. (i) With Rat Brain Membranes. Frozen lysed-P2 membranes were prepared from male Sprague–Dawley rats (150–200 g) as previously described.[6] [^{3}H]DADL (sp act. = 29.1 and 43 Ci/mmol) binding assays were conducted in 10 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris·HCl), pH 7.4, containing 100 mM choline chloride, 3 mM $MnCl_2$, and a protease inhibitor cocktail consisting of bacitracin (0.1 mg/mL), bestatin (0.01 mg/mL), leupeptin (0.004 mg/mL), and chymostatin (0.002 mg/mL). To separate the lower and higher affinity [^{3}H]DADL binding sites, incubations were conducted in the presence of blocking concentrations of DpLp, which is highly selective for the higher affinity binding site, and LY164929, which is highly selective for the lower affinity binding site. The concentrations used, 50 nM DpLp and 100 nM LY164929, were chosen on the basis of previously published data.[28] Triplicate samples were filtered with a Brandell Cell Harvester over glass fiber filters after a 4–6-h incubation at 25 °C with less than 5% variation between triplicates. μ binding sites were labeled with [^{3}H]FOXY (sp act. = 53 Ci/mmol).[37] Assays were conducted as described above, except that they took place in 50 mM Tris·HCl at pH 7.4. Nonspecific binding was measured by incubation in the presence of 20 μM levallorphan. Protein concentrations were determined by the method of Lowry et al.[38] Each data point was the mean of three separate determinations, whose SD was less than 6% of the mean. Statistical significance was assessed by use of the Student's *t* test.

For treatment with SUPERFIT analogues, lysed-P2 membranes were resuspended with 10 mM 3-morpholinopropanesulfonic acid (MOPS), pH 7.4, containing 3 mM $MnCl_2$ and incubated for 60 min at 25 °C with varying concentrations of agents. The incubations were terminated by centrifugation (at 4 °C) at 12000 *g*. Pellets were then washed twice by centrifugation in ice-cold 10 mM Tris·HCl, pH 7.4, and then resuspended with 50 mM Tris·HCl, pH 7.4, containing 100 mM NaCl and 10 μM guanosine 5′-*o*-[3-thio]triphosphate, for a 60-min incubation at 37 °C, to facilitate the dissociation of reversibly bound drugs.[39] In other control experiments the specific binding of [^{3}H]DADL to its higher affinity binding site could be recovered after addition of 1 μM

(36) Sheldrick, G. M. *SHELXTL 80. Minicomputer Programs for Structure Determination*; University of Göttingen, 1980.
(37) Rothman, R. B.; Bykov, V.; Rice, K. C.; Jacobson, A. E.; Kooper, G. N.; Bowen, W. D. *Neuropeptides* **1988**, *11*, 1.
(38) Lowry, O. H.; Rosenbrough, N. H.; Farr, A. L.; Randall, P. J. *J. Biol. Chem.* **1951**, *193*, 275.
(39) Bowen, W. D.; Hellewell, S. B.; Kelemen, M.; Huey, R.; Stewart, D. *J. Biol. Chem.* **1987**, *262*, 13434.

of (−)- or (+)-*cis*-3-methylfentanyl and such washing. The homogenate was then washed three times by centrifugation in 50 mM Tris·HCl, pH 7.4, and then assyed as described above with [^{3}H]DADL and [^{3}H]FOXY.

(ii) **With NG 108-15 Cell Membranes.** Membranes from NG108-15 neuroblastoma × glioma hybrid cells were used to assay binding of SUPERFIT analogues (by measuring competition with [^{3}H]DADL)[15] and their effects on adenylate cyclase[40] as described. Binding data were obtained in duplicate and adenylate cyclase determinations were in triplicate. Data were fitted to the Adair equation by using the program DATAPLOT developed by J. Filliben at the National Bureau of Standards.

(40) Sharma, S. K.; Nirenberg, M.; Klee, W. A. *Proc. Natl. Acad. Sci. U.S.A.* **1975**, *72*, 590.

Registry No. (−)-*cis*-**4**, 101472-20-2; (+)-*cis*-**4**, 101472-19-9; (−)-*trans*-**4**, 120143-77-3; (−)-*trans*-**4**·HCl, 120143-75-1; (+)-*trans*-**4**, 120143-78-4; (+)-*trans*-**4**·HCl, 120143-72-8; **5**, 31633-72-4; **6**, 120059-78-1; *cis*-**7**, 120059-79-2; *trans*-**7**, 120059-82-7; *cis*-**8**, 53757-54-3; *trans*-**8**, 57444-99-2; (+)-*trans*-**8**, 120143-73-9; (+)-*trans*-**8**·(+)-tartrate, 120199-48-6; (+)-*trans*-**8**·α-methylbenzylcarbamate, 120143-79-5; (+)-*trans*-**8**·2,4,6-trinitrobenzenesulfonic acid salt, 120143-76-2; (−)-*trans*-**8**, 120143-74-0; (−)-*trans*-**8**·(−)-tartrate, 120199-49-7; (−)-*trans*-**8**·α-methylbenzylcarbamate, 120143-80-8; (±)-*trans*-**8**·oxalate, 120059-83-8; (3*S*,4*S*)-*trans*-**9**, 120059-80-5; (3*R*,4*R*)-*trans*-**9**, 120059-84-9; (3*R*,4*R*)-*trans*-**10**, 120059-76-9; (3*R*,4*R*)-*trans*-**10**·HCl, 120059-85-0; (3*S*,4*S*)-*trans*-**10**, 120059-81-6; (3*S*,4*S*)-*trans*-**10**·HCl, 120085-47-4; (3*R*,4*R*)-*trans*-**11**·HCl, 120059-86-1; (3*S*,4*S*)-*trans*-**11**·HCl, 120059-77-0; adenylate cyclase, 9012-42-4; aniline, 62-53-3; (*R*)-(+)-α-methylbenzylisocyanate, 33375-06-3; 4-nitrophenethyl bromide, 5339-26-4.

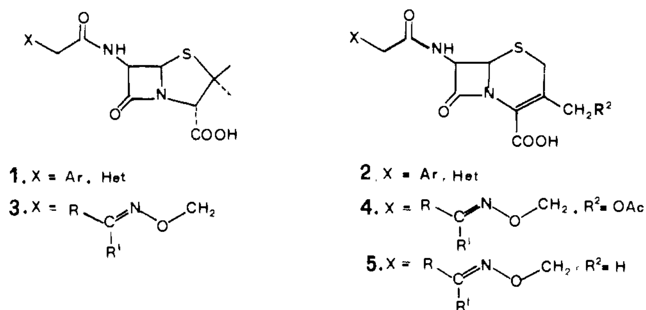
Synthesis and Antimicrobial Properties of Substituted β-Aminoxypropionyl Penicillins and Cephalosporins[1]

A. Balsamo,*,† G. Broccali,‡,§ A. Lapucci,† B. Macchia,† F. Macchia,*,⊥ E. Orlandini,† and A. Rossello†

Istituto di Chimica Farmaceutica e Istituto di Chimica Organica, Facoltà di Farmacia, Università di Pisa, 56100 Pisa, Italy, and ISF-Laboratori per la Ricerca Biomedica, 20094 Trezzano s/N, Milano, Italy. *Received May 26, 1988*

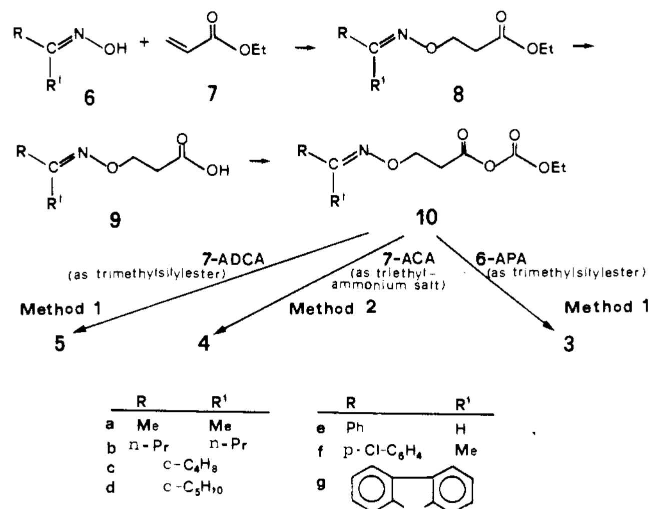
Some β-aminoxypropionyl penicillins (**3**) and cephalosporins (**4** and **5**), planned on the basis of the hypothesis that the (methyleneaminoxy)methyl group (>C=NOCH$_2$) could be a "bioisoster" of either aryls or other aromatic groups, were synthesized and assayed for their antimicrobial properties. Compounds **3**–**5**, tested on Gram-positive and Gram-negative bacteria, both sensitive to enzyme inactivation and otherwise, exhibited an activity trend that was not substantially different from that of the corresponding phenylacetamido derivatives taken as terms of comparison.

It is usually recognized that the changes on the side chain linked to the β-lactam nucleus of β-lactam antibiotics exert an influence that is sometimes decisive on the potency and range of the antimicrobial activity spectrum, stability to acids, and resistance to enzyme inactivation.[2] The numerous studies that have been carried out on the structure–activity relationships of these antibiotics have not yet made it possible to establish a precise correlation between the nature of these side chains and antimicrobial activity.[3] However, it may be pointed out that, in general, in the field of the more classic β-lactam antibiotics such as penicillins and cephalosporins, such compounds widely used in therapeutic practice such as penicillin G, ampicillin, cefalexin, and cefamandole present side chains of an acetamido type, substituted by an aryl or by an aromatic heterocycle, as shown in **1** and **2**, linked to the β-lactam nucleus.



The results of both experimental and theoretical studies[4] carried out on the mechanism of drug–receptor interactions in the field of adrenergic drugs indicated that a suitable nonaromatic moiety, such as the (methyleneaminoxy)methyl group (>C=NOCH$_2$) could be a "bioisoster"[5] of

Scheme I



† Istituto di Chimica Farmaceutica.
‡ ISF-Laboratori per la Ricerca Biomedica.
§ Present address: Lab. BT, 21047 Saronno, Varese, Italy.
⊥ Istituto di Chimica Organica.

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(2) (a) Price, K. E. *Structure Activity Relationships Among the Semisynthetic Antibiotics*; Academic Press: New York, 1977; pp 1, 61. (b) Sassiver, M. L.; Lewis, A. ref 2a; p 87. (c) Webber, J. A.; Wheeler, W. J. *Chemistry and Biology of β-Lactam Antibiotics*; Academic Press: New York, 1982; p 371. (d) Boyd, D. B. ref 2c; p 437.
(3) See for example: (a) Balsamo, A.; Macchia, B.; Macchia, F.; Rossello, A.; Giani, R.; Pinza, M,; Broccali, G. *J. Med. Chem.* **1983**, *26*, 1648 and references therein cited. (b) Cimarusti, C. M. *J. Med. Chem.* **1984**, *27*, 247.
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