elution from silica gel) led to a sample of **19** (67 mg, about 8% yield) homogeneous by TLC; mass spectrum, m/e 538 $(M + 1)^+$. Anal. ($C_{27}H_{35}N_7O_5 \cdot 0.6H_2O$) C, H, N.

***N*-[4-[[(2,4-Diamino-5-methylpyrido[2,3-*d*]pyrimidin-6-yl)methyl]ethylamino]benzoyl]-L-glutamic acid (21, 5-methyl-5-deaza-10-ethylaminopterin)** was prepared by saponification of **19** as described for the conversion of **18** to **20**; yield 97% (56 mg from 60 mg (0.109 mmol) of **19**·0.6H_2O), homogeneous by HPLC. Spectral data: mass, m/e 482 $(M + 1)^+$; UV (0.1 N HCl) 228 (40.8), 313 (17.1), (pH 7) 227 (39.2), 308 (30.2), (0.1 N NaOH) 228 (36.0), 308 (29.0). Anal. ($C_{23}H_{27}N_7O_5 \cdot 2.8H_2O$) C, H, N.

***N*-[4-[[(2-Amino-5-methyl-4(3*H*)-oxopyrido[2,3-*d*]pyrimidin-6-yl)methyl]amino]benzoyl]-L-glutamic Acid (22, 5-Methyl-5-deazafolic Acid).** Hydrolysis of **12**·2.65H_2O (300 mg, 0.600 mmol) in boiling 1 N NaOH (15 mL) under N_2 during 4.25 h (as previously reported for the conversion of 5-deazaaminopterin to 5-deazafolic acid[19]) followed by acidification to pH 3.1 afforded material whose assay by HPLC revealed the presence of about 4% unchanged **12**. This sample (250 mg) was again dissolved in 1 N NaOH (10 mL), and the solution was boiled as before for 1 h. Subsequent acidification to pH 3.5 gave **22** (235 mg, 79% yield), homogeneous by HPLC. Spectral data: mass, m/e 455 $(M + 1)^+$; UV (0.1 N HCl) 220 (32.8), 280 (26.4), 295 sh (23.4), 328–340 plateau (10.5), (pH 7) 223 (39.8), 280 (25.9), 287–293 plateau (25.6), (0.1 N NaOH) 221 (34.1), 283 (25.4), 295 sh (24.8). Anal. ($C_{21}H_{22}N_6O_6 \cdot 1.25H_2O$) C, H, N.

***N*-[4-[[(2-Amino-5-methyl-4(3*H*)-oxopyrido[2,3-*d*]pyrimidin-6-yl)methyl]methylamino]benzoyl]-L-glutamic Acid (23, 5,10-Dimethyl-5-deazafolic Acid).** Treatment of **20**·3H_2O (100 mg, 0.192 mmol) with refluxing 1 N NaOH (5 mL) under N_2 for 4.25 h followed by acidification to pH 3.1 afforded **23** in 83% yield (80 mg). Assay by HPLC [acetate buffer (pH 3.6)–MeCN (85:15)] indicated 99% purity. Spectral data: mass, m/e 469 $(M + 1)^+$; UV (0.1 N HCl) 223 (32.1), 278 (21.3), 306 (24.9), (pH 7) 224 (40.8), 307 (29.9), 275 sh (17.9), (0.1 N NaOH) 226 (34.9), 309 (28.0). Anal. ($C_{22}H_{24}N_6O_6 \cdot 1.75H_2O$) C, H, N.

Acknowledgment. This investigation was supported by PHS Grants CA25236 (J.R.P.), CA18856 (F.M.S.), CA22764 (F.M.S.), and CA10914 (R.L.K.), awarded by the National Cancer Institute, DHHS. Support for the work at Memorial Sloan-Kettering Cancer Center was also provided by a grant from the Elsa U. Pardee Foundation. The in vivo studies were conducted at Southern Research Institute under PHS Contract NO1-CM-37552 awarded by the National Cancer Institute, DHHS. We thank Sheila Campbell for skillful assistance in performing the HPLC analyses.

Probes for Narcotic Receptor Mediated Phenomena. 12.[1] *cis*-(+)-3-Methylfentanyl Isothiocyanate, a Potent Site-Directed Acylating Agent for δ Opioid Receptors. Synthesis, Absolute Configuration, and Receptor Enantioselectivity

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The first enantiomeric pair of irreversible opioid ligands [(+)- and (−)-4] were synthesized in >99.6% optical purity as determined by HPLC analysis of diastereoisomeric derivatives of the intermediate 3-methyl-*N*-phenyl-4-piperidinamine (**9**) enantiomers. Single-crystal X-ray analysis of the (*R*,*R*)-L-(+)-tartaric acid salt of (−)-**9** revealed the absolute configuration to be 3*S*,4*R*. The absolute configuration of (−)-3 [*cis*-(−)-3-methylfentanyl] and (−)-4 derived from (−)-**9** is thus 3*S*,4*R* and that of (+)-3 and (+)-4 is 3*R*,4*S*. The (+) enantiomer of 4 (SUPERFIT) was shown to be highly potent and specific for acylation of δ opioid receptors (to the exclusion of μ) in rat brain membranes like its achiral prototype FIT (**2**) and was about 10 times as potent as the latter in this assay. The (+)-4 was about 5 times as potent as FIT in acylation of δ receptors in NG108-15 neuroblastoma × glioma hybrid cells and about 50 times as potent as its enantiomer. Both FIT and (+)-4 behaved as partial agonists in inhibition of δ receptor coupled adenylate cyclase in NG108-15 membranes and (+)-4 was 5–10 times more potent than FIT and about 100 times more potent than its enantiomer in this assay. Dibromination of amine **12**, catalytic exchange of bromine with tritium gas, and reaction of the labeled amine with thiophosgene afforded [^{3}H]-(+)-4 with a specific activity of 13 Ci/mmol. Previous experiments indicated (+)-4 acylates the same 58 000-dalton glycoprotein previously shown to be acylated by FIT but with less nonspecific labeling. In view of the high potency and specificity of (+)-4 and the availability of its enantiomer, it seems likely that these compounds will prove to be valuable tools for study of the opioid receptor complex.

Opioid drugs possess a wide spectrum of activity, and there has been great effort expended trying to understand the function of these agents at the molecular level. A variety of structurally diverse opioids have been used to provide evidence of a multiplicity of opioid receptors, with the three major types commonly referred to as μ, δ, and κ.[2] Presently a number of researchers are attempting to isolate and characterize individual receptor subtypes, and numerous reports of synthesis of opioid receptor irreversible ligands as tools for such studies have appeared.[3-7] We recently described the μ-specific acylating drug benzimidazole isothiocyanate (BIT) based on etonitazene, and

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[‡] National Heart, Lung and Blood Institute.
[§] National Institute of Mental Health.

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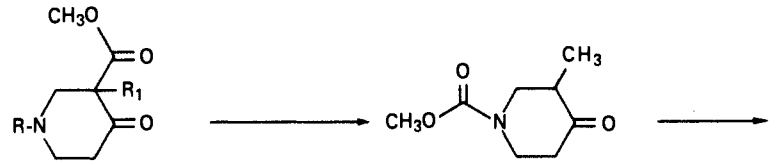
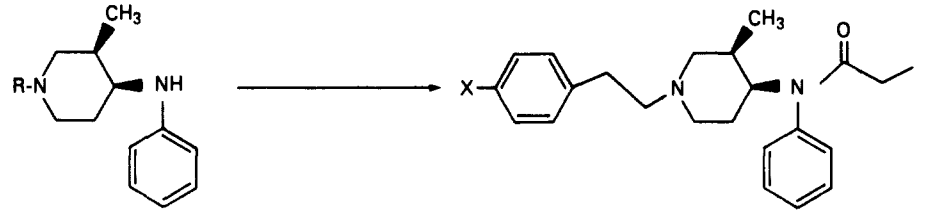
Scheme I



5: R = H, R_1 = H
6: R = CH_3OCO, R_1 = H
7: R = CH_3OCO, R_1 = CH_3

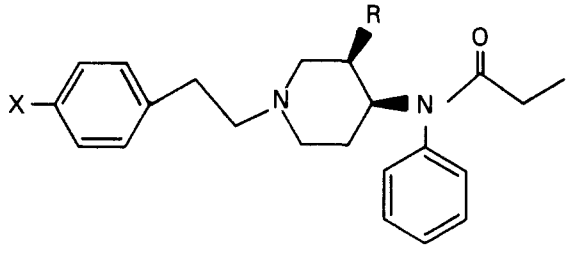
8



9: R = H
10: R = p-O_2N-$C_6H_4CH_2CH_2$

11: X = NO_2
12: X = NH_2

the δ-specific ligand fentanyl isothiocyanate (FIT, **2**),[8,9] an isothiocyanate derivative of the potent opioid agonist fentanyl (**1**). We utilized [^{3}H]FIT ([^{3}H]fentanyl isothiocyanate, **2**)[10] to demonstrate a M_r 58000 component of the δ receptor from NG108-15 neuroblastoma × glioma hybrid cells;[11] however, one difficulty encountered in this latter study was the nonspecific labeling of material not associated with the receptor.



1: X = H, R = H
2: X = NCS, R = H
3: X = H, R = CH_3
4: X = NCS, R = CH_3

We have undertaken the present study with the goals of obtaining (1) a higher affinity ligand to decrease nonspecific labeling and (2) an enantiomeric pair of opiate receptor irreversible ligands. The (+)-*cis*-3-methyl derivative of fentanyl, (+)-**3**,[12] exhibits an in vivo ED_{50} 20 times lower than that of fentanyl itself (ED_{50} = 0.011 mg/kg for fentanyl, ED_{50} = 0.000 58 mg/kg for (+)-**3** in a tail-withdrawal assay[12]) and is 100 times more potent in vivo than the enantiomeric (−)-**3** (ED_{50} = 0.068 mg/kg, tail-withdrawal assay[12]). This enhancement of potency of the more active enantiomer relative to the parent drug is also observed by the introduction of a 3-methyl substituent cis to the phenyl group in the 3-methyl-4-phenylpiperidine analgesic series.[13] These differences in in vivo potency partially reflect their binding affinities to opioid receptor preparations from rat brain. The respective IC_{50} values are 25, 21, and 1.3 nM for fentanyl, (−)-*cis*-3-methylfentanyl, and (+)-*cis*-3-methylfentanyl vs. (−)-[^{3}H]naloxone,[14] and the enantiomeric cis compounds differ by a factor of 85 in brain receptor binding vs. [^{3}H]fentanyl with the (+) enantiomer again being most potent.[15] It seemed highly probable that the analogous isothiocyanate derivative of (+)-*cis*-3-methylfentanyl [(+)-**4**] would exhibit an increased binding affinity as compared to FIT (**2**) and might show similar δ specificity. Furthermore, preparation of optical isomers of *cis*-3-methyl-FIT (**4**) could well provide enantiomers of very different affinities, which in principle would be extremely useful in receptor identification studies.

In this study, we describe the synthesis of both (+)-*cis*-3-methylfentanyl isothiocyanate [(+)-**4**] (which we refer to as SUPERFIT) and its enantiomer [(−)-**4**], the first enantiomeric pair of irreversible opioid ligands to be described. We also present comparison of their biological activities, determination of the absolute configuration of

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the antipodes by single-crystal X-ray diffractometry, and synthesis of high specific activity [^{3}H]-(+)-4. The [^{3}H]-(+)-4 has recently proven to be an essential tool for purification of the δ opioid receptor from neuroblastoma × glioma hybrid cells to apparent homogeneity.[16]

Synthesis. The routes to both (+)- and (−)-4 proceeded from a common intermediate, (±)-*cis*-3-methyl-*N*-phenyl-4-piperidinamine (9) as shown in Scheme I. Synthesis of 9 from methyl 3-methyl-4-oxo-1-piperidinecarboxylate (8) followed literature procedures.[12] The piperidone 8 was obtained in high yield from commercially available 3-carbomethoxy-4-oxopiperidine (5) in three steps. Acylation of 5 with methyl chloroformate gave methyl 3-carbomethoxy-4-oxo-1-piperidinecarboxylate (6), which was methylated (MeI/K_2CO_3 in acetone), giving methyl 3-carbomethoxy-3-methyl-4-oxo-1-piperidinecarboxylate (7). Hydrolysis and decarboxylation in refluxing aqueous HCl gave the desired piperidone (8).

Optical resolution of 9 was performed by fractional crystallization of the tartrate salts as previously reported.[12] Because a primary goal of this work was to obtain enantiomeric acylating ligands possessing a large difference in receptor affinity, it was important to have each enantiomer as optically pure as possible. This was particularly important in the case of the lower affinity (−) isomer where contamination with even traces of the higher affinity (+) isomer could have a significant effect. The resulting amines, (+)-9 (obtained by crystallization of the D-(−)-tartrate salt, $[\alpha]^{23}_D$ + 6.2°, lit.[12] + 6.1°), and (−)-9 (obtained by crystallization of the L-(+)-tartrate salt, $[\alpha]^{23}_D$ −6.9°, lit.[12] −5.95°), were examined for optical purity by conversion to their diastereoisomer 2-methyl-2-benzyl carbamates and analysis by HPLC. This derivatization procedure followed by HPLC[17] or NMR[18] analysis had previously provided a facile method of optical purity analysis in the isoquinoline series. Reaction of (+)-9 with (*S*)-(−)-α-methylbenzyl isocyanate followed by chromatography gave peaks at 12.3 and 14.6 min in a ratio of 0.30:99.70, respectively. Similarly, derivatization of (−)-9 with (*R*)-(+)-α-methylbenzyl isocyanate gave peaks at 12.3 and 14.6 min in a ratio of 0.15:99.85. Derivatization of racemic 9 with (*R*)-(+)-α-methylbenzyl isocyanate gave respective peak ratios of 1.00:1.03, indicating equal reactivity and detection for both (+)- and (−)-9. These results indicate an optical purity of 99.70% for (+)-9 and 99.85% for (−)-9. In cases such as this, where accurate measurement of very small levels of enantiomeric impurity is required, it is essential that the optical purity of the chiral derivatizing agent itself be rigorously established.[19] It had previously been shown that the chiral α-methylbenzyl isocyanates used in this procedure were free from enantiomeric contamination.[17]

Alkylation of (+)-9 and (−)-9 with 4-nitrophenethyl bromide (K_2CO_3 in 4-methyl-2-pentanone[12]) gave the corresponding *cis*-3-methyl-1-[2-(4-nitrophenyl)ethyl]-*N*-phenyl-4-piperidinamines (+)-10 and (−)-10, respectively. The white dihydrochloride salt [(+)-10·2HCl, $[\alpha]^{23}_D$ +11.2°] was unstable and dissociated to the bright yellow monohydrochloride salt [(+)-10·HCl, $[\alpha]^{23}_D$ +72.5°] when heated or brought into contact with moisture. Acylation of amines (+)-10 and (−)-10 with propionic anhydride gave

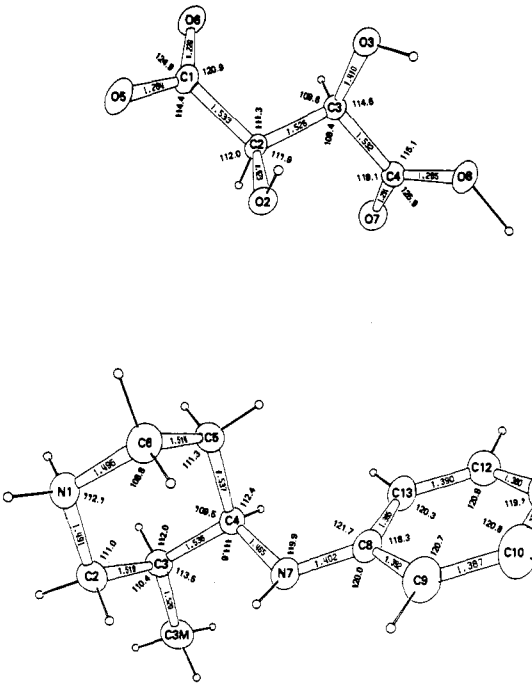

Figure 1. ORTEP[20] drawing of (−)-9 (*R*,*R*)-L-(+)-tartaric acid salt showing bond lengths and angles. Thermal ellipsoid boundaries are drawn at 36% probability and hydrogen atoms are arbitrary.

amides (+)-11 and (−)-11, respectively, which displayed almost no measurable optical rotation at the sodium D line, a feature exhibited by the remaining compounds in this series; however, rotations were larger at shorter wavelengths [(+)-11, $[\alpha]^{23}_{435.8}$ +6.2°]. Hydrogenation (Pd/C) of the enantiomeric (+)-11 and (−)-11 gave the corresponding amino derivatives (+)-12 and (−)-12, which were converted to isothiocyanates (+)-4 and (−)-4 by reaction with thiophosgene in a biphasic $CHCl_3$/aqueous $NaHCO_3$ system.

X-ray Analysis of (−)-9. An X-ray crystallographic study of the (*R*,*R*)-L-(+)-tartaric acid salt of (−)-9 established the absolute configuration as 3*S*,4*R*. The bond lengths, bond angles, and absolute configuration are as depicted in Figure 1.[20] The protonated N atom is N(1) as might be expected chemically. Two H atoms were found on this atom and the bond lengths and angles are also consistent with this finding. The establishment of the absolute configuration of (−)-9 as 3*S*,4*R* (Figure 1) indicates that the absolute configuration of the derived (−)-3 [*cis*-(−)-3-methylfentanyl] and (−)-4 is also 3*S*,4*R*. The absolute configuration of the more potent (+) enantiomer of 3 is thus 3*R*,4*S* are shown in structure 3, in contrast to a previous suggestion.[12] The same absolute configuration follows for (+)-4 (structure 4), found also to be the more potent enantiomer in the present study.

Tritiation of (+)-4. As noted below, (+)-4 can acylate δ opioid receptors selectively and with high affinity. Therefore, radiolabeled (+)-4 could be extremely useful for labeling the δ receptor. Tritiation of (+)-4 to high specific activity was accomplished in a manner similar to that used in the tritiation of FIT itself.[10] The procedure, which has also been applied to the tritiation of other irreversible ligands,[10,21] relies on the Pd/C-catalyzed exchange of aromatic bromine atoms with tritium gas. The

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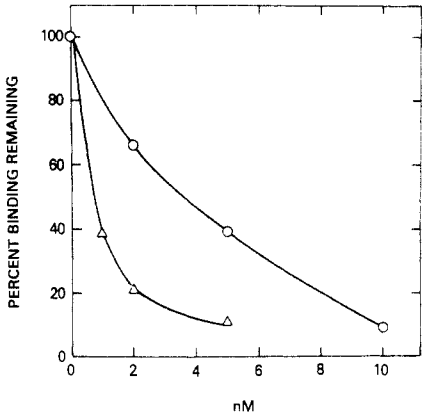

Figure 2. The number of Dalamid binding sites remaining in membranes of NG108-15 cells after incubation with FIT (2) (○) or (+)-4 (△) at the concentrations shown. Each point is the mean of duplicate determinations.

necessary brominated precursor was obtained from amine (+)-12 by reaction with Br_2 in H_2O. Catalytic exchange of bromine under an atmosphere of tritium gas gave crude [^{3}H]-(+)-12, which was purified by TLC. Reaction of [^{3}H]-(+)-12 with thiophosgene and purification of the resulting isothiocyanate by TLC gave [^{3}H]-(+)-4. The concentration of [^{3}H]-(+)-4 was determined by saturation binding to anti-FIT antibodies with use of [^{3}H]FIT-*N*-acetyl-L-lysine as a standard. From these data a specific activity of 13 Ci/mmol was calculated.

Biology. The antinociceptive activity of (+)-4 was determined in mice (sc injection) by the hot plate assay,[22] and it (or its metabolite) was found to have an ED_{50} of 0.34 (0.25–0.45) μmol/kg. Thus, it was about 9 times more potent than morphine and 15 times more potent than FIT in this assay. The 144-min duration of action observed for (+)-4 was similar to that of morphine; thus, by sc injection, (+)-4 is not a dramatically long acting agonist.

The membranes of NG108-15 neuroblastoma × glioma hybrid cells are richly endowed with opioid receptors[23] that are exclusively δ in nature.[24] When such membranes are incubated with FIT or (+)-4 for 30 min at 37 °C and washed and the remaining receptors are measured, results as shown in Figure 2 are observed. Both agents irreversibly inactivate receptors at very low concentrations, but (+)-4 does so with ca. 5 times the potency of FIT. Evidence for covalent binding of FIT to δ receptors was presented earlier[9] and similar evidence for covalent labeling by (+)-4 has been obtained. Each point in the figure is the maximum binding remaining as measured by Scatchard analysis of a complete binding isotherm with [^{3}H]Dalamid as the ligand. The number of binding sites is reduced without appreciably affecting the affinity of the remaining sites. Thus, reversibly bound FIT or (+)-4 have been removed by the washings.

Opioid agonist binding to receptors in NG108-15 membranes is accompanied by the inhibition of adenylate cyclase.[25] The data presented in Figure 3 show that both FIT and (+)-4 inhibit adenylate cyclase in these membranes and that (+)-4 is, again, more potent than FIT. The data also show that both are partial agonists because saturating concentrations of neither inhibit the activity of the enzyme to the extent that the full agonist Dalamid

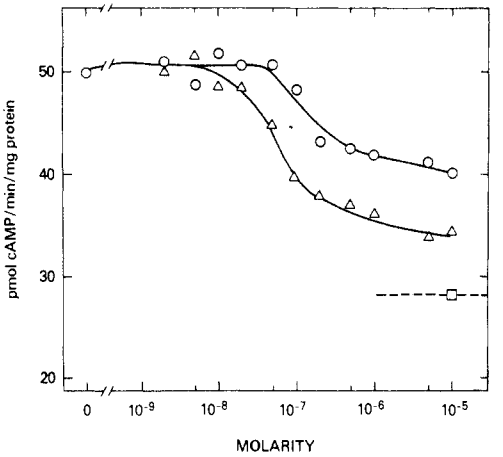

Figure 3. Inhibition of adenylate cyclase activity of membranes of NG108-15 cells by FIT (2) (○) and by (+)-4 (△) over a range of concentrations, and by Dalamid (□) at a saturating concentration (10 μM). The data shown are representative from three determinations.

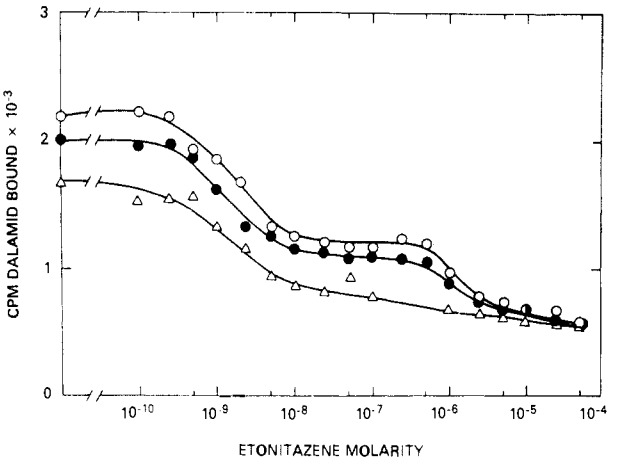

Figure 4. Displacement of 8 nM [^{3}H]Dalamid from opioid receptors of rat brain membranes by etonitazene at the concentrations shown without drug (○), with 2 nM FIT (2) (●), or with 2 nM (+)-4 (△). These are representative data from three determinations.

does. Interestingly, (+)-4 has both higher potency and higher efficacy than FIT in this test. It is not clear from this experiment whether the inhibitory effects of the ligands are due to reversible or to covalent interactions.

With rat brain membranes, we have previously demonstrated by competition experiments that etonitazene displacement of Dalamid binding is biphasic and that the two phases correspond to etonitazene binding to μ receptors (K_d near 10^{-9} M) and to δ receptors (K_d near 10^{-6} M).[3] An example of such a biphasic curve is shown in Figure 4 (upper curve) for rat brain membranes in a control experiment. After preincubation of the membrane with 2 nM FIT or (+)-4, the middle and lower curves of the figure are obtained, respectively. Neither reagent appreciably changes the number of μ receptors present and both reduce the number of δ receptors in the membrane, although to different extents. Receptor subtype numbers present are obtained simply by subtracting the appropriate plateau binding values from one another. A summary of many experiments of the type shown is provided by the data of Figure 5. In all experiments FIT and (+)-4 affect δ-receptor numbers without reducing the number of μ receptors present. The data show that (+)-4 is ca. 10 times more potent than FIT in this type of assay and that both reagents can reduce δ-receptor numbers to relatively low

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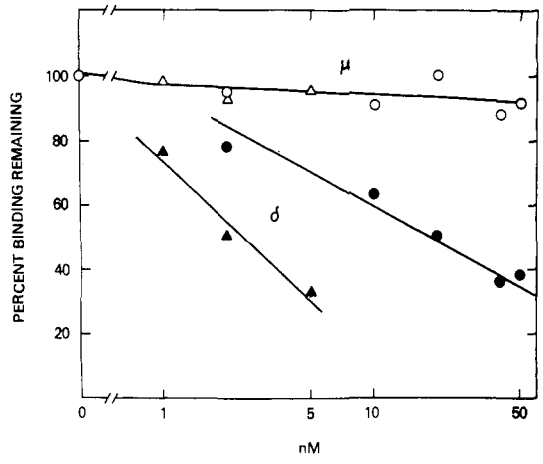

Figure 5. Number of μ and δ opioid receptors remaining in rat brain membranes after treatment with various concentrations of FIT (2) or (+)-4. The symbols represent the receptor numbers remaining as follows: μ receptors after treatment with FIT (2) (○) or (+)-4 (△); δ receptors after treatment with FIT (2) (●) or (+)-4 (▲). Each point is calculated from a complete etonitazene displacement curve as shown Figure 4 and is the average of two to three determinations.

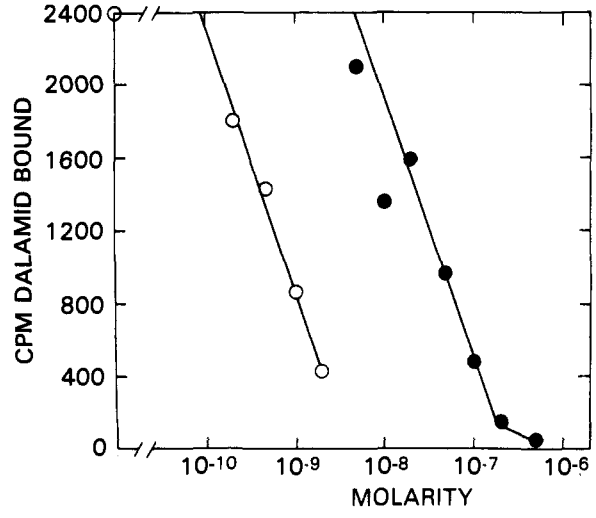

Figure 6. Comparison of the potencies of the enantiomers of 4 by measurement of residual specific Dalamid binding sites of NG108-15 membranes after preincubation with (+)-4 (○) or (−)-4 (●) at the concentrations shown. The data are representative of six determinations.

values when used at the appropriate concentrations.

As we had hoped, (+)-4 and its enantiomer (−)-4 display markedly different biological activities. Figure 6 summarizes the data of a number of experiments in which opioid binding remaining after pretreatment of NG108-15 membranes with each of the enantiomers of 4 is shown as a function of the concentration of acylating agent used. The (+)-4 is ca. 50 times were potent than its (−) enantiomer although both can inactivate essentially all of the receptors present. Similarly, (+)-4 is much more effective as an inhibitor of adenylate cyclase in these membranes than is the (−) enantiomer (Figure 7). These results are as expected since both (+)-4 and the more active (+) enantiomer of 3-methylfentanyl [(+)-3] are derived from the same amine (+)-9.

Discussion

The experiments described in this paper show that the newly prepared 3-methyl analogue of FIT, (+)-4, is, like the parent compound, an irreversible ligand for δ but not for μ opioid receptors. These experiments do not rule out

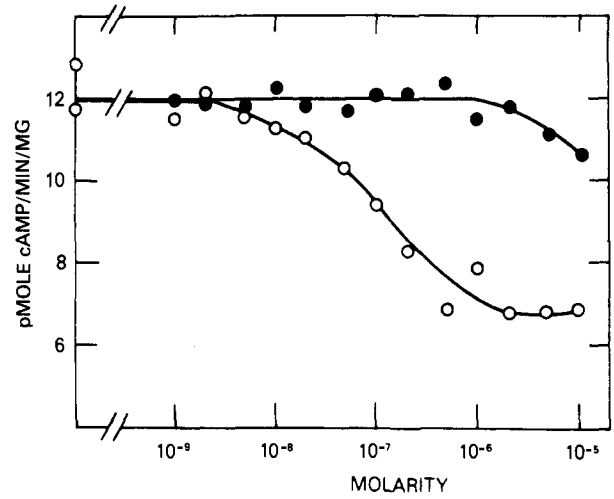

Figure 7. Inhibition of adenylate cyclase activity of NG108-15 membranes by (+)-4 (○) or (−)-4 (●) at the concentrations shown. Each point is the mean of three determinations (SE ≤ 5%).

reversible interaction of FIT and (+)-4 with μ receptors that might be expected since fentanyl is a preferential μ ligand,[26] and indeed *reversible* binding[27] of FIT to μ receptors can be demonstrated with the proper experimental design. The likely explanation[28] for the observed selectivity of FIT and (+)-4 in brain receptor acylation is that the bound drug is orientated in the μ-receptor matrix with the electrophilic isothiocyanate function located too distant from a suitably reactive nucleophile for acylation to occur, in contrast to its interaction with δ receptors where a nucleophile appears to have ready access to the electrophilic isothiocyanate moiety.

In each of the assays used, (+)-4 is more potent than FIT. Interestingly, whereas (+)-4 is only 4–5 times more potent than FIT in acylation of the δ receptors of NG108-15 membranes, it is 10 times more potent than FIT in its acylation of δ receptors of rat brain membranes. The possible significance of this small but consistently observed difference between the two types of δ receptors is unclear but may deserve further study. The high potency of (+)-4 as an irreversible δ ligand is the result of the selective interaction with the receptor. The enantiomers differ by a factor of 50 in potency, even though either will, at a sufficiently high concentration, acylate all of the δ receptors present. Although we have not yet studied the effects of (+)-4 on κ opioid receptors, we have shown that pretreatment of rat brain membranes and sections and guinea pig brain sections with FIT (4) and the μ-specific acylating agent BIT[8,9] provides membranes and sections greatly enriched with κ receptors.[29] Considering the similarity in profile of FIT and (+)-4 described here, we expect that similar results will be obtained with (+)-4.

We have found that [^{3}H]-(+)-4, reacts with the same 58 000-dalton subunit of the receptor as does FIT[11] but with considerably less nonspecific labeling of other membrane constituents.[16] This observation should facilitate study of receptors from sources less richly endowed than

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are neuroblastoma × glioma hybrid cell membranes.

Experimental Section

Melting points were determined on a Fisher-Johns apparatus and are corrected. NMR spectra were recorded with a Varian 220-MHz spectrometer. Electron-ionization mass spectra (EIMS) were obtained with a Hitachi Perkin-Elmer RMU-6E spectrometer (70 eV). 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. Column chromatography (HPLC) was performed with Gilson Model 303 pumps with Model 811 solvent mixer and Model 620 Data Master coupled to an Apple IIe computer. Optical rotations were measured with a Perkin-Elmer 241MC polarimeter using the solvents, concentrations, and wavelengths specified. Mass spectra and elemental analysis were obtainied from the Section on Analytical Services, NIADDK. All compounds gave NMR and IR spectra consistent with their assigned structure.

Methyl 3-Carbomethoxy-4-oxo-1-piperidinecarboxylate (6). Solid 3-carbomethoxy-4-oxopiperidine hydrochloride (**5**·HCl) (150.0 g, 0.78 mol) was added in portions to a 5-L flask containing a mechanically stirred mixture of $NaHCO_3$ (153.0 g, 1.94 mol) and methyl chloroformate (66 mL, 0.85 mol) in 1 L of H_2O and 1 L of ether. After 30 min the reaction was discontinued and the ether layer collected. The aqueous phase was extracted with $CHCl_3$ (200 mL), and the combined ether and $CHCl_3$ layers were allowed to remain undisturbed for 24 h during which time a red liquid separated. The red liquid was discarded and the supernatant evaporated to give **6** as a light red oil (169.0 g, 100%); high-resolution MS ($C_8H_{13}NO_5$) calcd 215.0793, found 215.0776.

Methyl 3-Methyl-3-carbomethoxy-4-oxo-1-piperidinecarboxylate (7). A mixture of **6** (107.0 g, 0.50 mol), K_2CO_3 (138.0 g, 1.0 mol), and MeI (62 mL, 1.0 mol) was stirred at reflux in acetone (600 mL). After 24 h K_2CO_3 was removed by filtration and the filtrate evaporated to an oil. Chloroform (300 mL) was added, and precipitated salts were removed by filtration. Evaporation of the filtrate gave crude **7** as a light brown oil (116.0 g). Distillation (138 °C head, 175 °C bath, 1.0 mm) gave **7** as a clear, colorless oil (97.0 g, 85%): high-resolution MS ($C_{10}H_{15}NO_5$) calcd 229.0950, found 229.0975.

Methyl 3-Methyl-4-oxo-1-piperidinecarboxylate (8). A solution of **7** (82.0 g, 0.30 mol) in MeOH (300 mL) was diluted with aqueous HCl (450 mL, 5 N) and stirred at reflux. After 20 h the mixture was cooled, diluted with H_2O (600 mL), extracted with CH_2Cl_2 (2 × 300 mL), and dried (Na_2SO_4). Evaporation gave **8** (62.0 g, 100%) as a light yellow oil: bp 86 °C (0.1 mm); high-resolution MS ($C_8H_{13}NO_3$) calcd 171.0895, found 171.0886.

(±)-*cis*-3-Methyl-*N*-phenyl-4-piperidinamine (9). The preparation of **9** was by literature methods from **8**. Product **9** was obtained as a clear coloress oil: bp 114 °C (0.2 mm) [lit.[12] bp 140–145 °C (0.4 mm)].

Optical Resolution of 9. Optical resolution of **9** was performed according to reported procedures[12] by crystallization of the D-(−)-tartaric acid and L-(+)-tartaric acid salts from MeOH/acetone to yield after conversion to the free amines (+)-*cis*-**9** and (−)-*cis*-**9**, respectively [(+)-*cis*-**9**, $[\alpha]^{23}_D$ +6.2° (*c* 3.7, MeOH) [lit.[12] $[\alpha]^{25}_D$ +6.1° (MeOH)], and (−)-*cis*-**9**, $[\alpha]^{23}_D$ −6.8° (*c* 2.6, MeOH) [lit.[12] $[\alpha]^{25}_D$ −5.9° (MeOH)]]. Six crystallizations were required to obtain 99.7% enantiomeric purity for (+)-**9**. In the case of (−)-**9**, six crystallizations were carried out and then the resolution was carried further by neutralization to the free base followed by recrystallization two more times with tartaric acid to obtain 99.85% enantiomeric purity.

Determination of Enantiomeric Purity. A sample (ca. 5 mg) of either (+)-**9** (−)-tartaric acid or (−)-**9** (+)-tartaric acid salt was partitioned between $CHCl_3$ (1 mL) and aqueous $NaHCO_3$ (1 mL). The organic phase was evaporated to dryness and then taken up in $CHCl_3$ (1 mL) and mixed with the appropriate α-methylbenzyl isocyanate (1 μL). [Amines (+)-**9** and (−)-**9** were reacted with (*R*)-(+)- and (*S*)-(−)-α-methylbenzyl isocyanates, respectively.] The reaction was complete in less than 5 min, and samples of the reaction mixture were analyzed directly by HPLC (Waters μ-Porasil column, hexane–isopropyl alcohol (88:12), 1 mL/min, absorbance 254 nm). For both mixtures, the major urea peak occurred at 14.6 min with the minor urea peak resulting from enantiomeric contamination occurring at 12.3 min. Integration of peak areas indicated enantiomeric purities of 99.7% and 99.85% for (+)-**9** and (−)-**9**, respectively. Derivatization of racemic **9** with (*R*)-(+)-isocyanate yielded HPLC peaks from (−)-**9** and (+)-**9** in a ratio of 1.03:1.00, respectively, indicating equal reactivity and detection for both enantiomers.

(3*R*,4*S*)-(+)-*cis*-3-Methyl-1-[2-(4-nitrophenyl)ethyl]-*N*-phenyl-4-piperidinamine [(+)-10]. A mixture of (+)-**9** (2.23 g, 11.7 mmol), 4-nitrophenethyl bromide (8.10 g, 58.7 mmol), and a few crystals of KI were stirred at 105 °C in 4-methyl-2-pentanone[12] for 18 h. Solvent was evaporated and residue partitioned between $CHCl_3$ (2 × 100 mL) and NaOH (1.0 N, 100 mL). Evaporation of solvent gave an oil, which was purified by silica gel flash chromatography (CH_2Cl_2 then 10% MeOH in CH_2Cl_2), yielding a brown oil. This was dissolved in MeOH, acidified with 37% HCl, evaporated to a syrup, and crystallized (MeOH/2-propanol) to yield (+)-**10** as a mixture of mono and dihydrochloride salts (3.8 g, mp 251–253 °C). A sample was dissolved in MeOH, a drop of 37% HCl was added, and then the solution was diluted with isopropyl ether to yield (+)-**10**·2HCl as a white salt: mp 254–255 °C; $[\alpha]^{23}_D$ +11.2° (*c* 0.5, 0.05 N HCl in MeOH). Anal. ($C_{20}H_{25}N_3O_2 \cdot 2H_2O$) C, H, N. Another sample was dissolved in boiling H_2O to afford a colorless solution, which deposited (+)-**10**·HCl as bright yellow crystals: mp 250–252 °C; $[\alpha]^{23}_D$ +72.5° (*c* 0.5, MeOH). Anal. ($C_{20}H_{23}N_3$) C, H, N.

(3*S*,4*R*)-(−)-*cis*-3-Methyl-1-[2-(4-nitrophenyl)ethyl]-*N*-phenyl-4-piperidinamine [(−)-10]. In a manner analogous to the preparation of (+)-**10**·2HCl, (−)-**10**·2HCl was prepared in 56% yield: mp 248–250 °C; $[\alpha]^{23}_D$ −11.1° (*c* 1.4, ~0.05 N HCl in MeOH). Anal. ($C_{20}H_{25}N_3O_2 \cdot 2HCl \cdot 2H_2O$) C, H, N.

(3*R*,4*S*)-(+)-*cis*-*N*-[3-Methyl-1-[2-(4-nitrophenyl)ethyl]-4-piperidyl]-*N*-phenylpropanamide [(+)-11·HCl]. Neutralization of (+)-**10**·2HCl gave an oil (2.1 g, 6.2 mmol), which was dissolved in toluene and refluxed 16 h with propionic anhydride (3.4 mL, 6 equiv). The solvent was evaporated and the syrup acidified with aqueous methanolic HCl, evaporated, and crystallized from 2-propanol/isopropyl ether to yield (+)-**11**·HCl as a white salt (1.69 g, 63%): mp 180 °C (partial melting), 200–202 °C (complete melting); $[\alpha]^{23}_D$ 0°, $[\alpha]^{23}_{435.8}$ +6.2° (*c* 4, MeOH). Anal. ($C_{23}H_{29}N_3O_3 \cdot HCl \cdot {}^1/_2H_2O$) C, H, N.

(3*S*,4*R*)-(−)-*cis*-*N*-[3-Methyl-1-[2-(4-nitrophenyl)ethyl]-4-piperidyl]-*N*-phenylpropanamide [(−)-11·HCl]. In a manner analogous to the preparation of (+)-**11**·HCl, (−)-**11**·HCl was obtained from (−)-**10** in 75% yield: mp 180 °C (partial melting), 202–204 °C (complete melting); $[\alpha]^{23}_{435.8}$ −5.4° (*c* 4, MeOH). Anal. ($C_{23}H_{29}N_3O_3 \cdot HCl \cdot {}^1/_2H_2O$) C, H, N.

(3*R*,4*S*)-(+)-*cis*-*N*-[1-[2-(4-Aminophenyl)ethyl]-3-methyl-4-piperidyl]-*N*-phenylpropanamide Hydrochloride [(+)-12·HCl]. A solution of (+)-**11**·HCl (2.0 g, 4.6 mmol) in MeOH (30 mL) was hydrogenated under 40 psig H_2 over 5% Pd/C (250 mg). After 3 h the mixture was filtered, evaporated to a syrup, and crystallized (2-propanol/isopropyl ether), yielding (+)-**12**·HCl as a white salt (1.89 g, 96%): mp 181–183 °C; $[\alpha]^{23}_{365}$ +18.5° (*c* 1, MeOH): high-resolution MS ($C_{23}H_{32}N_3O$) calcd 366.2545, found 366.2523 (CIMS, NH_3).

(3*S*,4*R*)-(−)-*cis*-*N*-[1-[2-(4-Aminophenyl)ethyl]-3-methyl-4-piperidyl]-*N*-phenylpropanamide Hydrochloride [(−)-12·HCl]. In a manner similar to the preparation of (+)-**12**·HCl, (−)-**12**·HCl was prepared in 96% yield from (−)-**11**·HCl: mp 181–183 °C; $[\alpha]^{23}_{365}$ −18.5° (*c* 1, MeOH); high-resolution MS ($C_{23}H_{32}N_3O$) calcd *m/e* 366.2545, found *m/e* 366.2576 (CIMS, NH_3).

(3*R*,4*S*)-(+)-*cis*-*N*-[1-[2-(4-Isothiocyanatophenyl)ethyl]-3-methyl-4-piperidyl]-*N*-phenylpropanamide Hydrochloride [(+)-4·HCl]. A mixture of (+)-**12**·HCl (300 mg, 0.75 mmol) in $CHCl_3$ (20 mL) was stirred with $NaHCO_3$ (440 mg, 5.24 mmol) in H_2O (10 mL) to which redistilled thiophosgene (68 μL, 0.90 mmol) was added. After 15 min the $CHCl_3$ was collected, the aqueous layer extracted (10 mL of $CHCl_3$), and the organic phase evaporated to yield a syrup, which was acidified with methanolic HCl. Evaporation and crystallization from 2-propanol yielded (+)-**4**·HCl as white crystals (210 mg, 67%): mp 204–211 °C; $[\alpha]^{23}_{365}$ +18.3° (*c* 1.2, MeOH). Anal. ($C_{24}H_{29}N_3SO \cdot HCl$) C, H, N.

(3*S*,4*R*)-(−)-*cis*-*N*-[1-[2-(4-Isothiocyanatophenyl)ethyl]-3-methyl-4-piperidyl]-*N*-phenylpropanamide Hy-

drochloride [(−)-4·HCl]. In a manner similar to that above, (−)-4·HCl was prepared from (+)-12·HCl in 92% yield: mp 201–209 °C; $[\alpha]^{23}_{365}$ −17.8° (c 1, MeOH). Anal. ($C_{24}H_{29}N_3SO$·HCl) C, H, N.

Tritiation of (+)-4. To a vigorously stirred solution of (+)-12·HCl (200 mg, 0.5 mmol) in H_2O (20 mL) was added a mixture of Br_2 (77 μL, 1.5 mmol) in H_2O (10 mL). The resulting suspension of yellow solid was stirred for 15 min and then made alkaline (pH >10) by addition of saturated aqueous Na_2CO_3. The suspension was then extracted (2 × 30 mL of $CHCl_3$), evaporated, and chromatographed through a short silica gel column (CH_2Cl_2–MeOH, 10:1). Evaporation of the appropriate column fractions, acidification of the resulting foam (48% HBr in MeOH), and trituration of the residue with ether gave an amorphous powder (200 mg: CIMS (NH_3) $(M + 1)^+$ 523, 524, 525 indicated dibromination). A sample of this powder (10 mg in 2 mL of MeOH with 15 mg of 10% Pd/C) was tritiated under an atmosphere of $^3H_2$ (25 Ci) for 24 h. (Tritiation was performed at New England Nuclear Corp., Boston, MA.) Labile tritium was removed in vacuo, yielding 230 mCi of remaining activity. In an identical procedure using H_2 rather than $^3H_2$, product was obtained that was identical (TLC, MS, NMR) with (+)-12. Crude [^{3}H]-(+)-12 was purified by TLC ($CHCl_3$–MeOH–NH_4OH, 9:1:0.1). Reaction with thiophosgene as previously reported[10] for the synthesis of 4 and purification by TLC on silica gel eluted with $CHCl_3$–MeOH (20:1) gave [^{3}H]-(+)-4 in 10% overall radiochemical yield with a specific activity of 13 Ci/mmol.

X-ray Analysis. Cell dimensions: a = 7.7890 (5) Å, b = 7.3626 (4) Å, c = 14.804 (1) Å, β = 92.56 (1)°; $P2_1$; Z = 2. The phase problem was solved by generating sets of 100 random phases for input to MULTAN78[30] and all heavy atoms were found in an E map. All expected H atoms were found later, and the structure was refined, using the programs of XRAY72,[31] with anisotropic thermal parameters for the heavy atoms to an R factor of 3.29%. The model atomic coordinates were chosen so that the anion had the known R,R configuration of L-(+)-tartaric acid and thus the absolute configuration of the cation follows. Tables of atomic parameters, structural factors, and comments on crystal packing and hydrogen bonding are available as supplemental material.

Determination of Residual Dalamid Binding Sites in Membranes of NG108 Neuroblastoma × Glioma Hybrid Cells after Treatment with FIT (2) or (+)-4 (Figure 2). Incubation of the membranes[25] with the isothiocyanates was at 37 °C in 10 mM potassium phosphate buffer, pH 8.0, at 1 mg of protein/mL. After 30 min, the membranes were diluted with 5 volumes of 10 mM Tris·Cl, pH 7.5, and centrifuged at 20 000 rpm for 30 min. After two more such washes, [^{3}H]Dalamid binding was measured at a series of concentrations between 1 and 20 nM. Bound radioactivity was separated from free by gel filtration as previously described,[32] and specific binding was estimated as the difference between total binding and that found in the presence of 10 μM nonradioactive Dalamid. Numbers of binding sites remaining were estimated by extrapolation of the data to infinite concentration by the method of Scatchard.[33]

Inhibition of Adenylate Cyclase Activity of Membranes of NG108-15 Cells by FIT (2), (+)-4, and Dalamid (Figure 3). Incubation with a range of concentrations of the drugs were for 5 min at 37 °C; using a previously described modification[25] of the method of Salomom.[34]

Displacement of [^{3}H]Dalamid from Opioid Receptors of Rat Brain Membranes by Etonitazene before and after Treatment with FIT (2) or (+)-4 (Figure 4). The membranes, prepared as described earlier,[35] were pretreated for 30 min at 37 °C without drug, with 2 nM FIT (2), or with 2 nM (+)-4. The membranes were washed and the binding of 8 nM [^{3}H]Dalamid was then measured as described for the generation of data shown in Figure 2.

Measurement of μ and δ Opioid Receptors Remaining after Treatment with FIT (2) and (+)-4 (Figure 5). Receptor type numbers were measured from curves as shown in Figure 4 after treatment with the concentrations of drugs indicated in the figure. Each experiment included a control of membranes incubated without drug and receptor numbers remaining are expressed as percent of that control value. The points are averages of two or three such measurements that agreed with one another within 10%.

Comparison of the Potencies of (+)- and (−)-4 by Measurement of Residual Dalamid Binding Sites of NG108-15 Membranes after Preincubation with the Enantiomers (Figure 6). Experimental conditions are as described in the experiments of Figure 2. Specific Dalamid binding is the difference between the amount of opioid bound to membranes incubated with 10 nM [^{3}H]Dalamid and that bound in the presence also of 10 μM nonradioactive Dalamid. The concentration of [^{3}H]Dalamid used is 5 times its dissociation constant.

Inhibition of Adenylate Cyclase Activity of NG108-15 Membranes by (+)- and (−)-4 (Figure 7). The activity of the enzyme was determined as described previously[25] after a 5-min incubation at 37 °C with (+)- and (−)-4 at the concentrations shown.

Registry No. 2, 85951-63-9; (+)-4, 101472-19-9; (−)-4, 101472-20-2; [^{3}H]-(+)-4, 101472-14-4; (+)-4·HCl, 101472-11-1; (−)-4·HCl, 101472-12-2; (±)-5·HCl, 101493-71-4; (±)-6, 93757-83-6; (±)-7, 101472-04-2; (±)-8, 101472-05-3; (±)-*cis*-9, 53757-54-3; (+)-*cis*-9, 53757-49-6; (−)-*cis*-9, 53757-51-0; (+)-9·(−)-tartrate, 53757-50-9; (−)-9·(+)-tartrate, 53757-52-1; (+)-10, 101472-06-4; (−)-10, 101472-07-5; (+)-10·HCl, 101472-15-5; (+)-10·2HCl, 101472-16-6; (−)-10·2HCl, 101472-17-7; (+)-11·HCl, 101472-08-6; (−)-11·HCl, 101472-09-7; (+)-12·HCl, 101472-10-0; (−)-12·HCl, 101493-72-5; [^{3}H]-(+)-12, 101472-13-3; (+)-α-methylbenzyl isocyanate, 33375-06-3; 4-nitrophenethyl bromide, 5339-26-4; propionic anhydride, 123-62-6; (−)-α-methylbenzyl isocyanate, 14649-03-7.

Supplementary Material Available: Tables of atomic parameters for heavy atoms and hydrogens, structural factors, and comments on crystals packing and hydrogen bonding (11 pages). Ordering information is given on any current masthead page.

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