Synthesis of Highly μ and δ Opioid Receptor Selective Peptides Containing a Photoaffinity Group

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A series of cyclic, conformationally constrained photolabile peptides related to the enkephalins and to somatostatin were designed and synthesized in an effort to develop highly selective and potent peptides for the δ and μ opioid receptors. The following new peptides were prepared and tested for their δ opioid receptor potency and selectivity in the guinea pig ileum assay, the mouse vas deferens assay, and the rat brain binding assay: H-Tyr-D-Pen-Gly-*p*-NH_2Phe-D-Pen-OH (**1**, [*p*-NH_2Phe^4]DPDPE) and H-Tyr-D-Pen-Gly-*p*-N_3Phe-D-Pen-OH (**2**, [*p*-N_3Phe^4]-DPDPE). The following new peptides were prepared and tested for their μ opioid receptor potency and selectivity in the same assays: H-D-Phe-Cys-*p*-NH_2Phe-D-Trp-Lys-Thr-Pen-Thr-NH_2 (**3**, [*p*-NH_2Phe^3]CTP) and D-Phe-Cys-*p*-N_3Phe-D-Trp-Lys-Thr-Pen-Thr-NH_2 (**4**, [*p*-N_3Phe^3]CTP). The δ selective photoaffinity peptide **2** displayed both high affinity (IC_{50} = 9.5 nM) and good selectivity (IC_{50} μ/IC_{50} δ = 1053) as an agonist at δ opioid receptors in bioassays, and **2** also displayed moderate affinity (33 nM) and excellent selectivity (IC_{50} μ/IC_{50} δ = 110) for rat brain δ opioid receptors. The μ selective photoaffinity peptide **4** displayed very weak affinity (8% contraction at 300 nM) at μ opioid receptors in bioassays, but good affinity (IC_{50} = 48.6 nM) and excellent selectivity (IC_{50} δ/IC_{50} μ = 412) for the rat brain μ opioid receptors. These conformationally constrained cyclic photoaffinity peptides may be useful tools to investigate the pharmacology of δ and μ opioid receptors.

The synthesis of conformationally constrained δ receptor selective opioid agonist peptides such as [D-Pen^2,D-Pen^5]enkephalin[1] (DPDPE)[2-4] and μ selective receptor opioid antogonist peptides such as D-Phe-Cys-Tyr-D-Trp-Lys-Thr-Pen-Thr-NH_2[5,6] has led to increased knowledge regarding the structural and conformational features necessary for δ and μ opioid receptor–ligand interactions. In order to investigate opioid receptors in more detail, various authors have prepared ligands designed to permit irreversible labeling of the opioid receptors.[7-9] These photoreactive ligands have been useful, but generally have had low potency and/or selectivity for opioid receptors. Thus, we have decided to examine whether our highly potent and selective δ and μ opioid peptides might be suitably modified to provide highly selective photoaffinity analogues for the opioid receptors. For this purpose the azido group was selected as the photolabile group for the following reasons: (1) its relatively small spatial requirements; (2) it can be readily synthesized (in principle) from *p*-aminophenylalanine; (3) its storage stability; and (4) it can covalently react with target molecules after photolysis.[10]

The highly δ selective agonist H-Tyr-D-Pen-Gly-Phe-D-Pen-OH (DPDPE) has been used extensively to investigate the pharmacological properties of the δ receptor. Its high δ receptor selectivity minimizes its interactions at μ and κ opioid receptors. Therefore this compound was selected as a prime candidate for photoaffinity labeling of δ receptors. The phenylalanine in the 4-position is an excellent location for substitution because it interacts closely with the active site of the δ receptor, yet modifications in the para position in δ selective peptides apparently can be tolerated without significant loss in biological activity.[11,12] Consequently the photoreactive enkephalin H-Tyr-D-Pen-Gly-*p*-N_3Phe-D-Pen-OH (**2**, [*p*-N_3Phe^4]DPDPE) was synthesized, and its pharmacological activities were investigated.

The recently synthesized μ opiod receptor antagonist[5,6] H-D-Phe-Cys-Tyr-D-Trp-Lys-Thr-Pen-Thr-NH_2 (CTP) was chosen because of its high selectivity for the μ opioid receptor. The Tyr in the 3-position lends itself to substitution by the *p*-azidophenylalanine residue. The photoreactive μ selective antagonist H-D-Phe-Cys-*p*-N_3Phe-D-Trp-Lys-Thr-Pen-Thr-NH_2 (**4**, [*p*-N_3Phe^3]CTP) was synthesized with this substitution. As part of this work, a modified synthesis of N^{α}-*t*-Boc-L-*p*-NHZ-phenylalanine[13] is presented which makes synthesis of photoaffinity peptides containing sulfur by solid phase synthesis meth-

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(1) Symbols and abbreviations are in accord with the recommendations of the IUPAC–IUB Commission of Biochemical Nomenclature (*J. Biol. Chem.* **1972**, *247*, 977). All optically active amino acids are of the L variety unless otherwise stated. Other abbreviations include the following: DCC, dicyclohexylcarbodiimide; TFA, trifluoroacetic acid; Z or Cbz, benzyloxycarbonyl; HOBt, *N*-hydroxybenzotriazole; N^{α}-Boc, N^{α}-*tert*-butyloxycarbonyl; Pen, penicillamine; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; FAB-MS, fast atom bombardment mass spectrometry; DPDPE, [D-Pen^2,D-Pen^5]enkephalin; HF, hydrogen fluoride; DCHA, dicyclohexylamine.

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Table I. Binding Potencies and Selectivities of DPDPE and CTP Photoaffinity Analogues in Competition with [^{3}H]CTOP and [^{3}H]DPDPE: Receptor Binding to Rat Brain Membranes[a]

compounds	IC_{50}, nM: binding vs [^{3}H]CTOP	IC_{50}, nM: binding vs [^{3}H]DPDPE	selectivity ratio
I. δ-selective analogues			
H-Tyr-D-Pen-Gly-Phe-D-Pen-OH (DPDPE)	385	3	117
H-Tyr-D-Pen-Gly-*p*-NH_2Phe-D-Pen-OH (1)	2326	1052	2
H-Tyr-D-Pen-Gly-p-N_3Phe-D-Pen-CO_2H (2)	3615	33	110
II. μ-selective analogues			
H-D-Phe-Cys-Tyr-D-Trp-Lys-Thr-Pen-Thr-NH_2 (CTP)	3	8400	3230
H-D-Phe-Cys-*p*-NH_2Phe-D-Trp-Lys-Thr-Pen-Thr-NH_2 (3)	500	>10000	>20
H-D-Phe-Cys-*p*-N_3Phe-D-Trp-Lys-Thr-Pen-Thr-NH_2 (4)	49	20000	412

[a] All values are the arithmetic mean from at least three separate experiments done in duplicate.

Table II. Biological Potencies of DPDPE Analogues and CTP Analogues[a]

compounds	IC_{50}, nM: MVD	IC_{50}, nM: GPI	GPI/MVD ratio
I. δ receptor selective analogues			
H-Tyr-D-Pen-Gly-Phe-D-Pen-OH (DPDPE)	4.3	16240	3810
H-Tyr-D-Pen-Gly-*p*-NH_2Phe-D-Pen-OH (1)	138	>10000	>72.5
H-Tyr-D-Pen-Gly-*p*-N_3Phe-D-Pen-OH (2)	9.5	6% inhibn at 10000 nM	≫1050
II. μ receptor selective analogues		pA_2[b]	
H-D-Phe-Cys-Tyr-D-Trp-Lys-Thr-Pen-Thr-NH_2 (CTP)	NA[c]	7.1	
H-D-Phe-Cys-*p*-NH_2Phe-D-Trp-Lys-Thr-Pen-Thr-NH_2 (3)	NA[c]	5% at 3000 nM	
H-D-Phe-Cys-*p*-N_3Phe-D-Trp-Lys-Thr-Pen-Thr-NH_2 (4)	NA[c]	8% at 300 nM	

[a] DPDPE, H-Tyr-D-Pen-Gly-Phe-D-Pen-OH; CTP, H-D-Phe-Cys-Tyr-D-Trp-Lys-Thr-Pen-Thr-NH_2; MVD, mouse vas deferens assay; GPI, guinea pig ileum assay. [b] The pA_2 is a measure of the affinity of a competitive antagonist for its receptor.[27] [c] NA = no activity at 10^{-5} M.

odology more facile.

Analysis of the pharmacological activities and the δ selectivity of Tyr-D-Pen-Gly-*p*-NH_2Phe-D-Pen-CO_2H (**1**, [*p*-NH_2Phe^4]DPDPE) and **2** and of the pharmacological activities and the μ receptor selectivity of D-Phe-Cys-*p*-NH_2-Phe-D-Trp-Lys-Thr-Pen-Thr-NH_2 (**3**, [*p*-NH_2Phe^3]-CTP) and **4** are presented.

Results and Discussion

To develop peptide analogues containing photoaffinity groups and still retain selectivity for the μ and δ receptors, we have synthesized *p*-azidophenylanine analogues of the μ receptor selective somatostatin-related compound H-D-Phe-Cys-Tyr-D-Trp-Lys-Thr-Pen-Thr-NH_2 (CTP) and the δ receptor selective enkephalin-like compound H-Tyr-D-Pen-Gly-Phe-D-Pen-OH (DPDPE). A major synthetic problem is evident when trying to synthesize these compounds. Most photoreactive peptides are made with N^{α}-*t*-Boc-*p*-nitrophenylalanine, which requires catalytic hydrogenation to form the amine, the precursor to the azido group. In the presence of cystine, cysteine, penicillamine, methionine, or other sulfur-containing compounds, this can lead to the poisoning of the catalysts and desulfation, consequently increasing reaction times and decreasing yields. The synthesis of Boc-L-*p*-NHZ-phenylalanine[13] facilitated the synthesis of these sulfur-containing peptides by solid-phase methodology. This protected derivative of phenylalanine did not require catalytic reduction and could be directly converted into *p*-azidophenylalanine when incorporated into disulfide-containing peptides.

These peptides were prepared by the solid-phase method of peptide synthesis utilizing either a *p*-methylbenzhydrylamine resin for carboxamide terminal peptides or a standard chloromethylated Merrifield resin for carboxylate terminal peptides. Preformed symmetrical anhydrides[14] of the N^{α}-protected amino acids were employed for most of the coupling reactions. Peptides were cyclized to their disulfide form under high dilution (1 mg of peptide/4 mL of solvent) with $K_3Fe(CN)_6$ at pH 8.4 as the oxidizing agent. Purification was accomplished by gel filtration followed by reverse-phase high-performance liquid chromatography (RP-HPLC) using a Vydac C_{18} reverse-phase semipreparative column. Purity was assessed by thin-layer chromatography, analytical RP-HPLC, amino acid analysis, and fast atom bombardment mass spectrometry (FAB-MS). See the Experimental Section for details.

The enkephalin analogue [*p*-NH_2Phe^4]DPDPE demonstrated a 2.2-fold selectivity for the δ receptor on the rat brain membranes. The *p*-amino group in the 4-position significantly decreased both the potency and the selectivity of DPDPE at δ opioid receptors (Table I). This was not a result of the steric requirements of the δ receptor site, since the pseudoisoster [*p*-MePhe^4]DPDPE has high potency at this receptor.[11] Rather, this effect seems to be electronic in that the amino group is basic with nonbonding electrons leading to an analogue in which the binding potency decreases over 300 times at the δ receptor and about 6-fold at the μ receptor, relative to the binding potencies of DPDPE. The [*p*-NH_2Phe^4]DPDPE analogue was a weak agonist on the mouse vas deferens (MVD) with an IC_{50} of 138 nM and appeared to be inactive in the guinea pig ileum (GPI) assay ($IC_{50} \gg 10000$ nM). The μ antagonist [*p*-NH_2Phe^3]CTP demonstrated weak binding

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Table III. Reactivity Study of [p-N_3Phe^4]DPDPE to Irradiation[a]

time, min	area, % GE sunlamp 5 cm	GE sunlamp 15 cm	mineralamp, 5 cm
0	100	100	100
2	40	100	100
5	15	74	97
10	5	50	91
20	0	25	68
30	0	15	44

[a] These values are the integrated peak areas of the [p-N_3Phe^4]-DPDPE peak remaining after irradiation of a 20-mL vial containing a 4.4×10^{-5} M solution at 5 and 15 cm with a GE 250-W sunlamp and at 5 cm with a UV Mineralamp. Aliquots of sample were removed at various time intervals. The integrated peak area of [p-N_3Phe^4]DPDPE at $t = 0$ was taken to be 100%. Chromatographic conditions are the same as those described in the Experimental Section.

potency and poor selectivity in the rat brain binding assay. This may reflect the importance of the para position of the phenyl ring in position 3 of the peptide chain. This compound demonstrated no intrinsic inhibition in the MVD or GPI bioassay preparations (Table II). The p-aminophenylalanyl peptides were the synthetic precursers to the p-azidophenylalanyl peptides and were not expected to demonstrate increased potency or greater selectivity at the μ and δ opioid receptors.

The [p-N_3Phe^3]CTP demonstrated a 412-fold selectivity for the μ receptor over the δ receptor and the [p-N_3Phe^4]DPDPE showed at 110-fold selectivity for the δ receptor over the μ receptor in the rat brain binding assays. In the mouse vas deferens (MVD) and guinea pig ileum (GPI) smooth muscle assays, [p-N_3Phe^4]DPDPE demonstrated a greater than 1000-fold selectivity for the δ receptor over the μ receptor. Further evidence for the δ receptor selectivity and reversibility was provided by the ability of the selective δ antagonist ICI 174,864 to abolish completely the inhibition of contractions by [p-N_3Phe^4]-DPDPE in the MVD assay, whereas the μ selective antagonist H-D-Phe-Cys-Tyr-D-Trp-Lys-Thr-Pen-Thr-NH_2 (CTP) had no significant effect. The μ antagonist analogues [p-NH_3Phe^3]CTP and [p-N_3Phe^3]CTP were inactive in these bioassays. The reason for its inactivity in the MVD and GPI is not understood at this time. These analogues bind well to rat brain membranes and this might suggest that they would have activity in the in vitro MVD and GPI assays, but this was not observed. One possible explanation is that there is a difference between rat brain membrane (CNS) μ receptors and guinea pig ileum (peripheral) μ receptors in their structural requirements.

Although the p-azido-substituted analogues of DPDPE and CTP are somewhat less potent than their parent analogues, they do retain high receptor selectivity, suggesting that they will be very useful in the labeling of δ and μ receptors, respectively, by photochemical methods.

The photoreactivity of the analogue [p-N_3Phe^4]DPDPE was examined by irradiating the sample and following the disappearance of the parent peak by HPLC (Table III). A GE 250-W sunlamp at a distance of 5 cm from the sample was the most effective. A Mineralamp at 5 cm was the least effective. These preliminary photoreactivity values are necessary when initiating an investigation of photoreactive analogues in vitro since the opioid receptors are very sensitive to light with a $t_{1/2} = 7.5$ min.[15] This is most likely due to the many amino acid side chains in the receptor (i.e., possibly tryptophan, histidine, cystine, etc.) that can be sensitive to irradiation. Therefore these results can be used when determining the irradiation times necessary to photolyze the azido group yet maintain the biological integrity of the receptor. The irradiation times should be long enough to photolyze the azido group but short enough not to affect the receptor.

Experimental Section

General Methods and Materials. Amino acid analyses[16] were performed on a Beckman Model 120C amino acid analyzer after acid hydrolysis of the peptides in sealed tubes with either 2 M mercaptoethanesulfonic acid (0.4% phenol) or 4 M methanesulfonic acid (0.2% 3-(2-aminoethyl)indole) at 110 °C for 22 h in vacuo. No corrections were made for the destruction of amino acid during hydrolysis. Fast atom bombardment mass spectra (FAB-MS) were obtained on a Varian 311 A spectrophotometer equipped with an Ion Tech Ltd. source with xenon as the bombarding gas. Elemental analysis was performed by MicAnal (Tucson, AZ) on a Perkin-Elmer 240 elemental analyzer. A Perkin-Elmer 552 UV–vis spectrophotometer and a Perkin-Elmer 983 infrared spectrometer were used for spectral analysis of the azido compounds. Optical rotations were obtained on an Autopol III automatic MC polarimeter at the sodium line. Ascending TLC was performed on a Merck silica gel 60 F-250 plate with the following solvent systems: (A) BuOH/HOAc/H_2O/pyridine (15:3:10:12), (B) BuOH/HOAc/pyridine/H_2O (6:1.2:6:4.8), (C) EtOAc/pyridine/HOAc/H_2O (60:20:6:11), (D) BuOH/HOAc/H_2O (5:2:3). Peptides were visualized by UV fluorescence quenching, ninhydrin, Pauly reagent, and iodine. All peptides gave a negative Ellman's test.[17] HPLC was performed with the following solvent systems on a Perkin-Elmer Series 3B solvent delivery system, using a Perkin-Elmer LC-75 UV spectrophotometer detector and laboratory computer integrator LC1-100 (Perkin-Elmer): (I) Vydac 218TP15-16C_{18}RP column (25 cm × 4.6 mm) with 0.1% aqueous trifluoroacetic acid/CH_3CN, 78:22 (v/v), at a flow rate of 2.5 mL/min, (II) Vydac 218TP15-C_{18}RP column (25 cm × 4.6 mm) with 0.1% trifluoroacetic acid/CH_3CN, 77:23 (v/v), at flow rate of 1.5 mL/min, and (III) a Vydac 218TP15-C_{18}RP column (25 cm × 4.5 mm) with 0.1% trifluoroacetic acid/CH_3CN, 77:23 (v/v), at a flow rate of 1.5 mL/min. All NMR analyses were done on a Bruker WM-250 spectrometer. The peptides DPDPE[2] and CTP[5] were prepared as previously described.

Solid Phase Peptide Synthesis of Cyclic Analogues. The peptides were prepared by standard solid-phase synthetic techniques previously used in our laboratory.[18,19] Briefly, N^{α}-*tert*-butyloxycarbonyl (Boc) protected amino acids were used throughout the synthesis and were purchased from Vega Biotechnologies (Tucson, AZ) and Bachem (Torrence, CA) or were prepared by published methods. Peptides with the COOH-terminal carboxylic acid groups were prepared by first attaching N^{α}-Boc-*S*-(*p*-methylbenzyl)-D-penicillamine (4.74 g, 13.4 nmol) to 10 g of chloromethylated copoly(styrene–1% divinylbenzene) beads (Lab Systems, 1.34 mequiv of Cl^-/g of resin) with 4.4 g (13.4 mmol) of cesium carbonate.[20] Carboxamide terminal peptides were prepared by using a *p*-methylbenzhydrylamine resin (pMBHA resin, 0.91 mmol/g of resin) synthesized according to published procedures.[21] Reactive side chains were protected as follows: Thr, *O*-benzyl ether; Lys, 2-chlorobenzyloxycarbonyl; Tyr, 2-bromobenzyloxycarbonyl; Cys and Pen, *p*-methylbenzyl; Trp was used without protection on the indole nitrogen; *p*-aminophenylalanine, *N*-benzyloxycarbonyl. Peptides were synthesized on a Vega Model 250 peptide synthesizer. A 1.5 M excess of preformed symmetrical anhydrides[14] was used for all coupling reaction when applicable, which were monitored by ninhydrin[22]

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Table IV. Chemical Characteristics of Synthetic Peptides

peptides[a]	thin-layer chromatography[b] R_f values				HPLC (K')[c]		FAB-MS[d]	
	I	II	III	IV	V	VI	$[M + H]_{obsd}$	$[M + H]_{calcd}$
[p-NH_2Phe^4]DPDPE	0.19	0.74	0.56	0.53	3.8	3.6	661.1	661.8
[p-N_3Phe^4]DPDPE	0.53	0.83	0.65	0.65	4.0	3.8	687.9	687.2
[p-NH_2Phe^3]CTP	0.24	0.56	0.61	0.61	3.2	3.0	1075.1	1074.7
[p-N_3Phe^3]CTP	0.40	0.64	0.64	0.65	3.6	3.5	1102.0	1101.6

[a] DPDPE = H-Tyr^1-Pen^2-Gly^3-Phe^4-Pen^5-OH; CTP = H-D-Phe^1-Cys^2-Tyr^3-D-Trp^4-Lys^5-Thr^6-Pen^7-Thr^8-NH_2. [b] Merck 5 cm × 20 cm silica gel 60 glass plates were used. TLC systems are as follows: (I) butanol/acetic acid/water (4:1:5) (v/v) (upper phase), (II) 2-propanol/ammonia/water (3:1:1) (v/v), (III) butanol/acetic acid/water/pyridine (6:1:5:6) (v/v), (IV) butanol/acetic acid/water/pyridine (15:3:10:12) (v/v). [c] Capacity factor for the following systems: (V) Vydac 218TP15-C_{18}RP reverse-phase (RP) column (25 cm × 4.6 mm) with 0.1% trifluoroacetic acid/CH_3CN, 78:22 (v/v), as a flow rate of 2.5 mL/min, (VI) Vydac 218TP15-C_{18}RP column (25 cm × 4.6 cm) with 0.1% trifluoroacetic acid/CH_3CN, 77:23 (v/v), at a flow rate of 1.0 mL/min. All peptides were monitored at $\lambda \approx 214$ nm. [d] FAB-MS were obtained on a Varian 311A spectrometer equipped with an Ion Tech Ltd. source with xenon as the bombarding gas. Molecular weights are in grams per mole.

and/or chloranil[23] tests and repeated as necessary. N^{α}-Boc protection was removed at each step by two treatments with 40% TFA in CH_2Cl_2 for 5 and 20 min each, except that after D-tryptophan was incorporated into the growing peptide chain, the TFA solution was modified to also contain 10% ethanedithiol and 5% carbon disulfide. Peptides were deprotected and removed from the resin with anhydrous liquid HF (10 mL/g of resin) containing 10% anisole at 4 °C for 45 min. After removal of the HF in vacuo, the free peptides were washed with either ethyl ether or ethyl acetate to remove anisole and organic soluble byproducts and then extracted with 30% acetic acid and the aqueous solution was lyophylized. After cyclization with 0.01 M $K_3Fe(CN)_6$ at pH 8.4, the analogues were purified by gel filtration depending on the analogue. The eluent was monitored by UV absorbance at 254 nm on an ISCO optical unit, Tris pump, and UA-5 chart recorder. The selected fractions were lyophilized and then purified by HPLC. Purity was assessed by thin-layer chromatography in a minimum of four solvent systems and analytical HPLC. Integration of the HPLC chromatograms (at 214 nm) indicated purity in excess of 95%. Analytical data are given in Table IV. The p-aminophenylalanine-containing peptide analogues were then placed in a 20-mL vial at 0 °C and wrapped in aluminum foil. With moderate stirring, 1 M HCl, 1 M $NaNO_2$, 1 M NaN_3, and 1 M sulfamic acid were added sequentially and then neutralized by 1 N $NaHCO_3$. The synthesized p-azido compound was then purified by HPLC, and the correct fraction was collected and lyophilized.

N^{α}-Boc-p-nitro-L-phenylalanine (MW 310.3) (A). In a 500-mL round-bottom flask, p-nitro-L-phenylalanine[24] (19 g, 0.090 mol) was reacted with di-*tert*-butyl dicarbonate (21.6 g, 0.099 mol) to yield compound A (22 g, 78% yield, 0.071 mol): mp 110–112 °C, $[\alpha]^{25}_D$ +8.0° (*c* 1, MeOH) [lit.[25] $[\alpha]^{25}_D$ +7.94° (*c* 1.55, MeOH), mp 107 °C]; TLC R_f values (A) 0.58, (B) 0.66, (C) 0.64; ^{13}C NMR (58.7 mg/0.5 mL, DMSO) 28.14 (q), 36.34 (t), 54.61 (d), 78.26 (s), 123.26 (d), 130.62 (d), 146.59 (s), 155.52 (s), 173.23 (s); ^1H NMR 1.95 (s), 3.62 (dd), 3.84 (dd), 4.85 (m), 7.88 (d), 8.49 (dd). Anal. Calcd for $C_{14}H_{18}N_2O_6$: C, 54.2; H, 5.80; N, 9.00; Found: C, 54.3; H, 5.93; N, 8.91.

N^{α}-Boc-p-amino-L-phenylalanine (MW 280.3) (B). To a 250-mL Parr hydrogenation vessel was added a solution of compound A (10 g, 0.032 mol) dissolved in 200 mL of absolute ethanol (purged with argon for 40 min). Then 1.5 g of Raney nickel (pore size 50 μm, surface area 80–100 m^2/g, Aldrich) was added and the pressure was maintained at 50 psi on a Parr hydrogenation apparatus (Parr Co., Moline, IL) for 24 h. The material was filtered through a 2-cm Celite pad under N_2 and washed three times with 30 mL of N_2-purged EtOAc. The filtrate was collected and then evaporated to 50 mL in vacuo. Then under N_2, $MgSO_4$ was added and the solution filtered through a medium-size funnel. The filtrate was reduced in vacuo to 25 mL. Then product B (7.86 g, 84% yield, 0.027 mol), under a N_2 hood, was precipitated by the addition of N_2-purged petroleum ether at 0 °C: mp 130–132 °C; $[\alpha]^{20}_D$ +23° (*c* 1, MeOH); TLC R_f values (A) 0.57, (B) 0.64, (D) 0.51; ^{13}C NMR (63 mg/0.4 mL) 28.28 (q), 35.70 (t), 55.55 (d), 77.93 (s), 113.42 (d), 115.16 (s), 126.80 (d), 130.20 (s), 155.44 (s), 173.99 (s). Anal. Calcd for $C_{14}H_{20}N_2O_4$: C, 60.0; H, 7.20; N, 10.0; Found: C, 59.7; H, 7.18; N, 10.0.

N^{α}-Boc-L-p-[N-(benzyloxycarbonyl)amino]phenylalanine (MW 414.4) (C). A portion of B (5 g, 0.017 mol) was protected at the 4′-amino group by carbobenzoxy chloride (3.2 g, 0.019 mol) in dioxane/water (1:1), pH = 9. The crude product was precipitated from EtOAc/petroleum ether to produce C (6.77 g, 93% yield, 0.016 mol): mp 165–167 °C; $[\alpha]^{25}_D$ +17° (*c* 1, MeOH); TLC R_f values (A) 0.65, (B) 0.69, (D) 0.76; ^1H NMR 1.34 (s), 2.77 (dd), 2.97 (dd), 4.06 (m), 5.16 (s), 7.09 (d), 7.24 (d), 7.39 (m); ^{13}C NMR (44.9 mg/0.5 mL, DMSO) 28.18 (q), 35.84 (t), 55.34 (d), 65.69 (t), 78.09 (s), 118.06 (d), 128.09 (d), 128.48 (d), 129.44 (d), 132.00 (s), 136.69 (s), 137.42 (s), 153.41 (s), 155.50 (s), 173.70 (s). Anal. Calcd for $C_{22}H_{26}N_2O_6$: C, 63.75; H, 6.32; N, 6.76. Found: C, 63.63; H, 6.42; N, 6.87.

[D-Pen^2,p-NH_2Phe^4,D-Pen^5]enkephalin ([p-NH_2-Phe^4]-DPDPE, MW 660.7) (1). Starting with 1.6 g of N^{α}-Boc-D-Pen(*S*-4-MeBzl)-Merrifield resin (1.0 mmol amino acid/g resin), the protected peptide resin to the title peptide was obtained after stepwise coupling of the N^{α}-Boc-protected amino acids (in order of addition): N^{α}-Boc-4′-Cbz-aminophenylalanine (1.7 g, 4.0 mmol), N^{α}-Boc-Gly (0.7 g, 4.0 mmol), N^{α}-Boc-D-Pen(*S*-4-MeBzl) (1.4 g, 4.0 mmol), and N^{α}-Boc-Tyr(2,4-Cl_2-Bzl) (1.8 g, 4.0 mmol). The product N^{α}-Boc-Tyr(2,4-Cl_2-Bzl)-D-Pen(*S*-4-MeBzl)-Gly-(4′-Cbz-NH_2)Phe-D-Pen(*S*-4-MeBzl)-Merrifield resin (2.50) was dried in vacuo. Then 2.50 g of the protected peptide was cleaved from the resin along with all the protecting groups by treatment with anhydrous HF (20 mL), anisole (2 mL), and 1,2-ethanedithiol (1 mL) at 0 °C for 45 min. After evaporation of HF, anisole, and 1,2-ethanedithiol in vacuo, the dried product was washed with diethyl ether (2 × 50 mL) and extracted successively with glacial HOAc (60 mL, stirring for 20 min), 30% HOAc (3 × 30 mL), 10% HOAc (3 × 30 mL), and distilled water (3 × 30 mL). All solvents were previously purged with nitrogen. The combined extracts were lyophilized to a white powder (929 mg). A portion (508.4 mg) was cyclized immediately by dilution with 2000 mL of distilled water (N_2-purged), the pH was adjusted to 8.4 with 20% NH_4OH, and then 100 mL (40% excess) of 0.01 N $K_3Fe(CN)_6$ was added. After 30 min at 40 °C, the reaction was terminated by the addition of 10% HOAc until a pH of 5.0 was reached. Excess ferro- and ferricyanate ion was removed by the addition of a 40-mL suspension of Bio-Rad AG3-X4A for 20 min followed by suction filtration. The solution was lyophilized to give 688 mg of crude peptide. The peptide was dissolved in 25 mL of warmed 30% HOAc and applied to a P-2 Bio-Gel (Bio-Rad) column (97 × 2.4 cm) and eluted with 25% HOAc at 18 mL/h. The three peaks (peak 1 = 28 mg, $t_{elution}$ = 9 h; peak 2 = 18 mg, $t_{elution}$ = 15 h; peak 3 = 405 mg, $t_{elution}$ = 22 h) were collected (280-nm detection) and lyophilized. Peak 3 (405 mg/10 mL of CH_3CN/0.1% TFA (50:50)) was injected in 500-μL aliquots for HPLC purification. RP-HPLC Vydac C_{18} preparative column (10 μm, 2.5 × 25 cm) with a gradient of 15–30% CH_3CN/0.1% TFA buffer system over a 15-min in-

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terval at a flow rate of 5 mL/min (λ = 214 nm). The analytical HPLC was done on a Vydac C_{18} reverse-phase column (17 μm, 0.45 × 2.5 cm). The purified powder was isolated by lyophilization to give 90.9 mg of a white powder (yield 8.6% based on starting substitution of the resin); $[\alpha]^{25}_D$ −15.0° (c 1.0, 100% EtOH). Amino acid analysis: Phe 1.0 (1.0); Gly 0.97 (1.0); Tyr 0.98 (1.0). The other analytical data are given in Table IV.

[D-Pen^2,*p*-N_3Phe^4,D-Pen^5]enkephalin ([*p*-N_3Phe^4]DPDPE, MW 686.2) (2). To a 20-mL bottle cooled to 0 °C and wrapped in foil were added the above peptide (1) (5 mg, 7.65 μmol) and 505 μL of 1 M HCl and then 22.7 μL of 1 M $NaNO_2$ (22.7 mmol, 3-fold excess) with stirring. After 10 min, the diazotization was monitored with iodine–starch paper. If negative, more $NaNO_2$ solution was added until a positive reaction was obtained. After 5 min, a 1 M solution of sulfamic acid (25.2 μL, 25.2 mmol, 3.3-fold excess) was added, followed by 15.2 μL of a 1 M solution of NaN_3 (15.2 mmol, 2-fold excess). After 5 min, the reaction mixture was neutralized by slowly adding 530 μL of 1 N $NaHCO_3$ (530 mmol, 1 mequiv) (adapted from ref 26). The sample was then kept at 0 °C and purified on a Vydac C_{18} preparative column (10 μm, 2.5 × 25 cm) using a Perkin-Elmer HPLC system. The gradient was from 20 to 40% CH_3CN/15 min (buffer A = CH_3CN, buffer B = 0.1% TFA) at a flow rate of 5 mL/min (λ = 214 nm). The yield was 2.1 mg (42%) after lyophilization in the dark; $[\alpha]^{25}_D$ −26.0° (c 1.0, 100% EtOH). Amino acid analysis: Tyr 1.0 (1.0); Gly 0.97 (1.0); IR (azido): ν = 2100 cm^{-1}; UV λ_{max} = 250 nm. The other analytical data are given in Table IV.

D-Phe-Cys-*p*-NH_2Phe-D-Trp-Lys-Thr-Pen-Thr-NH_2 ([*p*-NH_2Phe^3]CTP, MW 1073.7) (3). Starting with 0.5 g of *p*-methylbenzhydrylamine-resin (0.91 mmol of NH_2/g of resin), the following N^α-Boc-protected amino acids were added stepwise (in order of addition): N^α-Boc-Thr(*O*-Bzl), N^α-Boc-Pen(*S*-4-MeBzl), N^α-Boc-Thr(*O*-Bzl), N^α-Boc-Lys(N^ϵ-4′-ClZ), N^α-Boc-D-Trp, N^α-Boc-*p*-aminoPhe($N^{4'}$-Z), N^α-Boc-Cys(*S*-4-MeBzl), N^α-Boc-D-Phe. After coupling of the last amino acid, the protected peptide resin was dried in vacuo. Then the protected peptide resin (1.87 g) was cleaved from the resin by liquid HF (20 mL), with anisole (2 mL) and 1,2-ethanedithiol (1 mL). After evaporation of the solvents in vacuo at 0 °C, the dried product (953 mg) was washed with ethyl ether (3 × 30 mL), extracted with 30% aqueous HOAc (3 × 30 mL), and lyophilized. The peptide powder was dissolved in 3.0 L of 0.1% aqueous acetic acid under a blanket of nitrogen, and the pH was adjusted to 8.4 with aqueous ammonia. Then 0.01 M $K_3Fe(CN)_6$ was added at 40 °C until a yellow color persisted with an excess of $K_3Fe(CN)_6$ (130 mL). After 2 h at room temperature, the reaction was terminated by adjusting the pH to 4.5 with acetic acid. Excess ferro- and ferricyanate were removed by the addition of 40 mL of Bio-Rad AG3-X4A (Cl^- form) anion-exchange resin. After the mixture was stirred for 30 min, the resin was filtered off, the filtrate lyophilized, and the peptide powder (1.64 g) purified by gel filtration on a 94 × 3 cm Sephadex G-15 column using 5% acetic acid as eluent solvent. The major peptide peak was isolated and lyophilized to give a white powder. The desalted peptide was dissolved in 50% CH_3CN/0.1% TFA buffer and filtered through 4-μm filters for application to the HPLC column. The filtered peptide solution was then applied to a Vydac C_{18} semipreparative column (0.45 cm × 35 cm) in 0.1% trifluoroacetic acid/CH_3CN with a gradient of 20% CH_3CN to 45% CH_3CN over 20 min. The peak with a K' = 2.1 (58.2 mg) was identified as the correct compound; $[\alpha]^{25}_D$ +23.5° (c 1.0, 100% EtOH). Amino acid analysis: Phe 1.0 (1.0); Lys 1.0 (1.0); Thr 2.2 (2.0); *p*-NH_2-Phe 1.0 (1.0); Trp 0.90 (1.0). Other analytical data are given in Table IV.

D-Phe-Cys-*p*-N_3Phe-D-Trp-Lys-Thr-Pen-Thr-NH_2 ([*p*-N_3Phe^3]CTP, MW 1100.5) (4). To a 20-mL bottle cooled to 0 °C and wrapped in aluminum foil were added 3 (13.2 mg, 12.2 μmol) and 880 μL of 1 M HCl and then 39.6 μL of 1 M $NaNO_2$ (39.6 mmol, 3-fold excess) with stirring. After 10 min, the diazotization was monitored with starch iodine–starch paper and by HPLC. If negative, more of the $NaNO_2$ solution was added until a positive reaction was obtained. After 5 min, a 1 M solution of sulfamic acid (44.0 μL, 44 mmol, 23-fold excess) was added, followed by 26.4 μL of a 1 M solution of NaN_3 (26.4 mmol, 2-fold excess). After 5 min, the reaction mixture was neutralized by slowly adding 675 μL of 1 N $NaHCO_3$ (675 mmol, 1 equiv) (adapted from ref 26). The sample was then kept at 0 °C and purified on a Vydac C_{18} semipreparative column (10 μm, 2.5 × 25 cm) from 20–35% CH_3CN/15 min. The yield was 6.7 mg (50%) after lyophilization in the dark; $[\alpha]^{25}_D$ +35.1° (c 1.0, 100% EtOH). Amino acid analysis: Trp 0.89 (1.0); Phe 0.98 (1.0); Lys 0.96 (1.0); Thr 2.1 (2.0); IR (azido): ν = 2100 cm^{-1}; UV λ_{max} = 248 nm. The other analytical data is given in Table IV.

Rat Brain Binding Bioassay. Adult male Sprague–Dawley rats (200–250 g) were sacrificed and the brains were immediately removed and placed on ice. Whole brain minus cerebellum was homogenized with a Polytron homogenizer (Brinkman, setting no. 5, 15 s). The homogenate was then centrifuged two times at 43000*g* for 10 min before use in the radioreceptor binding assay.

[^{3}H]DPDPE (33.6 Ci/mmol, New England Nuclear, Boston, MA) and [^{3}H]CTOP (84.2 Ci/mmol, New England Nuclear, Boston, MA) binding was measured by a rapid filtration technique. A 100-μL aliquot of rat brain homogenate (0.5% final) was incubated with either 1.0 nM [^{3}H]DPDPE or 0.5 nM [^{3}H]CTOP in a total volume of 1 mL of 50 mM Na/K (pH 7.4 at 25 °C) containing 5 mM $MgCl_2$, bovine serum albumin (1 mg/mL), and phenylmethanesulfonyl fluoride (100 μM). All binding measurements were done in duplicate, and the binding displaced by 1 μM naltrexone hydrochloride was defined as specific tissue binding. Binding experiments were carried out at 25 °C for 120 min. The binding reaction was terminated by rapid filtration of samples through GF/B Whatman glass fiber filter strips pretreated with 0.1% polyethylenamine solution with a Brandel cell harvester; this was followed immediately by three rapid washes with 4-mL aliquots of ice-cold saline solution. Filters were removed and allowed to dry before assaying filter-bound radioactivity by liquid scintillation spectrophotometry.

The data were analyzed by using nonlinear least-squares regression analysis on an Apple II$^+$ computer. Programs were generously provided by SHM Research Corp., Tucson, AZ.

GPI Assay. Strips of longitudinal muscle–myenteric plexus[2] prepared from nonterminal ilea were taken from male Hartley guinea pigs weighing 150–300 g. Tissues were suspended in organ baths (20-mL capacity) containing Krebs bicarbonate buffer that was continuously bubbled with 95% O_2, 5% CO_2 and maintained at 37 °C. The tissues were attached to calibrated isometric force transducers and after a 15-min equilibration period without tension were stretched to 1.0 g of resting tension (previously determined to provide the optimal length (L_O) as measured by contractile responses to 100 nM acetylcholine). The tissues were then transmurally stimulated between platinum plate electrodes at 0.1 Hz with 0.4-ms pulses and supramaximal voltage. Twitch contractions were recorded on a Soltec multichannel recorder. All drugs were added to the baths in increments of 20–50 μL. The effect of the agonists on electrically evoked twitch tension was measured after incubation for 3 min. Antagonists were added to the bath 2 min before addition of the agonists. The changes in twitch height after drug exposure were expressed as a percentage of the twitch tension immediately before the addition of the drug. Concentration–response curves were constructed, and the IC_{50} (the concentration that reduced the response by 50%) was determined by linear regression analysis.

MVD Assay. Vas deferentia from male ICR mice (25–34 g) were removed and suspended in organ baths containing warmed (37 °C), oxygenated (95% O_2, 5% CO_2), magnesium-free Krebs bicarbonate buffer.[2] The pair of tissues from one mouse was hung as a single unit for 15 min without tension and then under 0.5 g of resting tension (L_O, as determined by measurements of contractions induced by exogenous norepinephrine). Tissues were transmurally stimulated at 0.1 Hz, with 2-ms pulses and supramaximal voltage. Drug studies were performed as described for the GPI.

The composition of Krebs solution (millimolar concentrations) was NaCl 118, KCl 4.7, CaCl 2.5, KH_2PO_4 1.19, $MgSO_4$ 1.18, $NaHCO_3$ 25, glucose 11.48 for the GPI; $MgSO_4$ was omitted from the buffer for the MVD.

Drugs. The highly selective mu agonist PL017 was a generous gift from Dr. K.-J. Chang, Burroughs-Wellcome Research Lab-

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oratories (Research Triangle Park, NC). The peptide antagonist CTP was synthesized as previously described (Pelton et al., 1986).[5] Somatostatin and DPDPE were purchases from Peninsula Laboratories (Belmont, CA). Naloxone hydrochloride and U50,488H were purchased from Sigma (St. Louis, MO) and Upjohn Diagnostics (Kalamazoo, MI), respectively. The δ receptor selective antagonist ICI 174,864 was purchased from Cambridge Research Biochemicals (Atlantic Beach, NY).

Data Analysis. All statistical calculations were carried out as described by Tallarida and Murray.[27] Tests for statistical significance included the analysis of variance, followed by a Student's *t* test for grouped data where significance was indicated.

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Registry No. 1, 118377-52-9; 2, 118377-53-0; 3, 118377-55-2; 4, 118398-12-2; A, 33305-77-0; B, 55533-24-9; C, 55533-25-0; DPDPE, 88373-73-3; CTP, 103335-28-0; *p*-NO_2Phe, 949-99-5; BOC-Gly, 4530-20-5; BOC-D-Pen(4-MeBzl), 115962-34-0; BOC-Tyr(2,4-Cl_2-Bzl), 62630-98-2; H-Tyr-D-Pen-Gly-(*p*-NH_2)Phe-D-Pen-OH, 118377-51-8; BOC-Thr(Bzl), 15260-10-3; BOC-Pen(4-MeBzl), 104323-41-3; BOC-Lys(4'-ClZ), 33640-54-9; BOC-D-Trp, 5241-64-5; BOC-Cys(4-MeBzl), 61925-77-7; BOC-D-Phe, 18942-49-9; H-D-Phe-Cys-(*p*-NH_2)Phe-D-Trp-Lys-Thr-Pen-Thr-NH_2, 118377-54-1.

Synthesis and Evaluation of Iodinated Analogues of Diacylglycerols as Potential Probes for Protein Kinase C

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Analogues of diacylglycerol containing a 3-(3-amino-2,4,6-triiodophenyl)-2-ethylpropanoyl or 3-(3-amino-2,4,6-triiodophenyl)propanoyl group in the 2-position (**1a** and **1b**, respectively) were synthesized and shown to compete with [^{3}H]phorbol dibutyrate ([^{3}H]PDBu) for binding in a crude rat brain preparation. Phorbol diesters have been shown to bind specifically to protein kinase C and the PDBu receptor has been copurified with protein kinase C activity. The four diastereomers of **1a** (**1c–f**) were synthesized from chiral starting material and studied in the same assay. The affinities for the [^{3}H]PDBu binding site of **1a**, **1b**, and two isomers of **1a** with naturally occurring L configuration were comparable to that of 1-oleoyl-2-acetyl-*rac*-glycerol (OAG), but the D isomers of **1a** were essentially inactive. The chirality of the side chain did not influence the binding affinity. Activation of protein kinase C by **1a**, **1c**, and **1e** demonstrated the same stereochemical requirements, but none were as active as OAG. For the 1,3-isomers **2**, **2a**, and **2b**, the competitive binding studies gave different results. The racemic mixture and the D isomer, **2b**, were able to compete for binding, but the L isomer, **2a**, did not compete. These studies demonstrate that diacylglycerol binding to and activation of protein kinase C is stereospecific for the glycerol backbone, but not the side chain. Furthermore, the D-1,3-isomer must exist in a conformation such that the acyl and hydroxyl oxygens assume a spatial relationship similar to that in the L-1,2-isomers.

Diacylglycerols have long been known to serve as intermediates in lipid biosynthesis, but in the last few years another role has been elucidated. They are endogenous modulators of protein kinase C (PK-C) activity.[1,2] PK-C is involved in many biological processes, including cellular signal transduction and proliferation (for reviews, see ref 3–8). Activation of many cellular receptors causes phospholipase C to cleave phosphatidylinositol 4,5-bisphosphate into inositol 1,4,5-triphosphate (IP_3) and diacylglycerol. Diacylglycerol then forms a complex at the membrane with phosphatidylserine (PS), Ca^{2+}, and PK-C, thus activating the enzyme.[9] The activated kinase phosphorylates many proteins, but the mechanism by which this produces further biological responses is not fully understood.

The acyl groups in the majority of diacylglycerols produced in cells are stearate and arachidonate in the 1- and 2-positions, respectively.[10] A series of synthetic diacylglycerols with a variety of chain lengths and degrees of unsaturation are also able to bind to and activate PK-C.[1,2,9,11,12] There is a good correlation between their binding and activation potencies.[12] Other structure–activity studies indicate that the ester linkages and the free hydroxyl in the sn-3-position are required.[12] Also, only naturally occurring sn-1,2-isomers (L or *S* enantiomers), and not sn-2,3-isomers, can induce PK-C to phosphorylate histone.[13] The ability of 1,3-substituted diacylglycerols to activate PK-C is uncertain due to conflicting reports.[2,9,14,15]

PK-C is also activated by phorbol diesters, a class of potent tumor promoters.[16] Though these molecules have

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