[D-Pen^2,D-Pen^5]enkephalin Analogues with Increased Affinity and Selectivity for δ Opioid Receptors[1]

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The conformationally restricted, cyclic disulfide-containing enkephalin analogue [D-Pen^2,D-Pen^5]enkephalin (DPDPE) was modified by halogenation (F, Cl, Br, I) of the phenylalanine-4 residue in the para position. The potency and selectivity of these analogues for the δ opioid receptor was greater than that of the parent peptide. The analogues possessed greater potency and affinity for the δ receptors than DPDPE in the mouse vas deferens assay and in radioreceptor assays (against [^{3}H]DPDPE), respectively. [*p*-ClPhe^4]DPDPE was the most selective in the radioligand binding assays ($IC_{50}(\mu)/IC_{50}(\delta)$ = 574), being about 5-fold more δ opioid receptor selective than DPDPE in this assay, whereas [*p*-IPhe^4]DPDPE was the most selective in the classical bioassay systems using the mouse vas deferens and guinea pig ileum assays (IC_{50}(GPI)/IC_{50}(MVD) = 17 374), making it nearly 9-fold more selective than DPDPE in direct comparisons using the same assay conditions.

It is now well accepted that the endogenous opioid peptides such as methionine enkephalin, leucine enkephalin, and dynorphin interact with several subtypes of opioid receptors (μ, δ, κ, and possibly other receptors) that may mediate different biological responses.[2-4] The search for opioid receptor ligands with a high degree of selectivity for one type of opioid receptor, therefore, has become essential for elucidation of the function(s) of opioid receptor types and potential subtypes.

One approach for the design of very selective ligands involves the incorporation of conformational restrictions.[5] In our laboratory this approach has led to the development of selective ligands for μ and δ opioid receptors.[5-10] The most selective analogue for δ opioid receptors was the cyclic analogue [D-Pen^2,D-Pen^5]enkephalin (DPDPE, 1), a peptide with a highly constrained 14-membered ring. The selectivity of this analogue for δ receptors was greater than that of any other synthetic enkephalin developed thus far.[6,11] Pharmacological determinations using the mouse vas deferens and the guinea pig ileum showed DPDPE to be over a 1000 times more selective for the δ receptor than the μ, while rat brain radioreceptor binding assays showed a 100-fold selectivity difference for the same receptors. Besides the high selectivity for opioid receptors, DPDPE possesses high potency (nmolar range). However, somewhat less selective δ opioid ligands such as DSLET and DTLET have higher potency at δ receptors than DPDPE.[12]

Recently we have prepared several new DPDPE analogues with modifications of the parent peptide in the Phe^4 residue. The rationale for this modification was our examination of the conformation of DPDPE,[13] which indicated that the Tyr^1 and Phe^4 aromatic side chain groups were generally in close proximity, and we suggested that this relationship probably was important for receptor recognition and transduction. Examination of the conformational model,[13] indicated that substitution of the Phe^4 residue in the para position would be possible without disruption of the conformation.[7,13] The effect of para substitution of an aromatic ring in the Phe^4 residue of metkephamide, a linear methionine enkephalin analogue, was investigated by Gesellchen et al.[14] The high potency of the analogues in this series implied that the aromatic nucleus of Phe^4 residue in metkephamide interacts with a region of opioid receptor which is capable of accepting a range of molecular volumes and electronegativities. Unfortunately, these analogues generally lacked high opioid receptor selectivity, making it difficult to draw conclusions regarding the requirements at the μ vs δ receptor.

To investigate the effect of para substitution of the Phe^4 residue in DPDPE for δ opioid receptor potency and selectivity, para-halogenated Phe^4-substituted DPDPE analogues were synthesized and evaluated for their bioactivity. The opioid receptor affinities and selectivities were evaluated in the MVD and GPI bioassays, and in rat brain membrane binding assays in comparison with the parent peptide, DPDPE.

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(1) Symbols and abbreviations are in accord with the recommendations of the IUPAC-IUB Commission on Nomenclature (*J. Biol. Chem.* **1972**, *247*, 977). All optically active amino acids are of the L variety unless otherwise noted. Other abbreviations used are: DPDPE, [D-Pen^2,D-Pen^5]enkephalin; Pen, penicillamine; CTAP, D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2; CTOP, D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2; metkephamide, Tyr-D-Ala-Gly-Phe-*N*-MeMet-NH_2; ICI 174,864, *N,N*-diallyl-Tyr-Aib-Aib-Phe-Leu; DSLET, Tyr-D-Ser-Gly-Phe-Leu-Thr; DTLET, Tyr-D-Thr-Gly-Phe-Leu-Thr; DSTBULET, Tyr-D-Ser(O^{t}Bu)-Gly-Phe-Leu-Thr; BUBU, Tyr-D-Ser(O^{t}Bu)-Gly-Phe-Leu-Thr(O^{t}Bu); FAB-MS, fast atom bombardment mass spectrometry; pMB, *p*-methylbenzyl.
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Table I. Inhibitory Potency and Selectivity of DPDPE Analogues in GPI and MVD Bioassays

peptide	IC_{50} (nM) ± S.E.E.[a] MVD	GPI	ratio IC_{50}(GPI)/IC_{50}(MVD)
1, Tyr-D-Pen-Gly-Phe-D-Pen	5.81 ± 1.63	11,600 ± 2990	2,000
2, Tyr-D-Pen-Gly-*p*-FPhe-D-Pen	0.84 ± 0.11	5,032 ± 595	5,990
3, Tyr-D-Pen-Gly-*p*-ClPhe-D-Pen	0.89 ± 0.11	4,830 ± 600	5,400
4, Tyr-D-Pen-Gly-*p*-BrPhe-D-Pen	1.50 ± 0.36	13,400 ± 3420	8,960
5, Tyr-D-Pen-Gly-*p*-IPhe-D-Pen	2.65 ± 0.68	46,067 ± 10,720	17,374

[a] S.E.E. = standard error of the estimate.

Table II. Binding Affinities and Selectivities of DPDPE Analogues in Competition with [^{3}H]CTOP and [^{3}H]DPDPE Receptor Binding

peptide	IC_{50} (nM) ± S.E.E. binding vs [^{3}H]DPDPE	[^{3}H]CTOP	ratio IC_{50}(CTOP)/IC_{50}(DPDPE)
1, Tyr-D-Pen-Gly-Phe-D-Pen	5.25 ± 0.89	609 ± 278	116
2, Tyr-D-Pen-Gly-*p*-FPhe-D-Pen	2.50 ± 0.30	623 ± 35	249
3, Tyr-D-Pen-Gly-*p*-ClPhe-D-Pen	1.57 ± 0.20	901 ± 161	574
4, Tyr-D-Pen-Gly-*p*-BrPhe-D-Pen	1.73 ± 0.39	418 ± 79	242
5, Tyr-D-Pen-Gly-*p*-IPhe-D-Pen	4.74 ± 0.17	964 ± 139	203

Results and Discussion

All of the new analogues of DPDPE reported here were prepared by the solid-phase method of peptide synthesis using methods for synthesis and purification previously developed for DPDPE and related analogues[6] (see the Experimental Section for details).

The potencies of the synthesized DPDPE analogues to inhibit electrically evoked contractions of the myenteric plexus longitudinal muscle preparation of guinea pig ileum and mouse vas deferens are summarized in Table I.

All of the new analogues were more potent in the MVD system, which contains δ, μ, and κ opioid receptors, but where the functional receptor is believed to be predominantly the δ receptor,[15] than in the GPI system which contains μ and κ opioid receptors, but in which the μ receptor mediates the effect of DPDPE. The effects of all of the compounds in the MVD were antagonized by ICI 174,864, a δ opioid receptor selective antagonist, but not by CTAP, a highly μ selective antagonist[10] (data not shown), and hence all the analogues were found to be δ selective. Also, the agonist actions of all the analogues in the GPI were completely antagonized by the highly μ opioid receptor antagonist CTAP,[10,27] indicating that μ but not κ agonist activity is obtained. Thus any possible κ agonist activity is at best extremely weak. This data clearly indicated that the para halogen substitution on Phe^4 in DPDPE is an effective modification for the δ receptor, since all of the *p*-XPhe^4 analogues reported here were more potent in the MVD than the parent DPDPE molecule. The fluoro and chloro analogues were especially potent, being approximately 7 times more potent than the DPDPE in the MVD assay. The ratios of the IC_{50} values in the two assays, which can be considered an index of selectivity for μ vs δ receptors, showed very high δ opioid receptor selectivity. The [*p*-IPhe^4]DPDPE was the most selective, being almost 9 times more selective than DPDPE.

Similar trends were observed in the radioreceptor assays using rat brain membrane preparations as shown in Table II. All of the DPDPE analogues inhibited the binding of [^{3}H]DPDPE (a δ-selective ligand) and [^{3}H]CTOP (a highly μ opioid selective ligand),[10,28] but the IC_{50} values against [^{3}H]DPDPE were always much lower than the IC_{50} values against [^{3}H]CTOP, showing the high δ opioid receptor selectivity of these analogues. [*p*-ClPhe^4]DPDPE appears to have very low affinity for the κ opioid receptor since the K_i value for U-69,543 (a high-affinity κ ligand) inhibition of [^{3}H][*p*-ClPhe^4]DPDPE is 3420 nM.[16] In the competitive-binding experiments against [^{3}H]DPDPE, all four analogues were more potent than the parent DPDPE (Table II). The chloro and bromo analogues **3** and **4** were the most potent in the δ binding assay, which is in reasonable agreement with the results from the bioassays. These results are consistent with data of Gesselchen et al.[14] and Anwer et al.[17] in that the halogenated Phe^4 of the linear enkephalin analogues reported by these investigators also displayed increased binding potencies. However, the substantially higher selectivities of the DPDPE analogues reported here for δ opioid receptors were an added bonus.

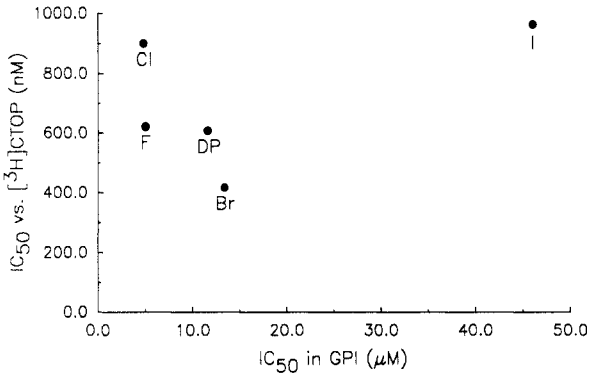

Figure 1. Lack of correlation between the potency in the peripheral guinea pig ileum (GPI) assay (IC_{50}) and in the radioreceptor binding assay vs [^{3}H]CTOP in rat brain homogenates (IC_{50}) of DPDPE (DP) and the DPDPE analogues [*p*-FPhe^4]DPDPE (F), [*p*-ClPhe^4]DPDPE (Cl), [*p*-BrPhe^4]DPDPE (Br), and [*p*-IPhe^4]DPDPE (I).

Correlations between peripheral activity (IC_{50} on GPI and MVD assays) and inhibitory potency on [^{3}H]CTOP (IC_{50}) and [^{3}H]DPDPE (IC_{50}) binding in rat brain homogenate are shown in Figures 1 and 2, respectively. There is no apparent correlation between IC_{50} (GPI) and IC_{50}

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Table III. Analytical Properties of Synthetic Peptide Analogues

peptide	TLC[a] R_f values I	II	III	IV	HPLC[b] V	VI	FAB-MS calcd	found
1, Tyr-D-Pen-Gly-Phe-D-Pen	0.43	0.66	0.86	0.81	1.85	1.95	-	-
2, Tyr-D-Pen-Gly-*p*-FPhe-D-Pen	0.45	0.66	0.91	0.83	3.11	3.10	664	664
3, Tyr-D-Pen-Gly-*p*-ClPhe-D-Pen	0.48	0.67	0.92	0.84	3.69	3.45	680	680
4, Tyr-D-Pen-Gly-*p*-BrPhe-D-Pen	0.54	0.57	-	0.78	4.94	4.15	725	724, 726
5, Tyr-D-Pen-Gly-*p*-IPhe-D-Pen	0.49	0.68	0.93	0.85	6.88	5.51	772	772

[a] Merck DC-Fertigplatten Kieselgel $60F_{254}$ 0.25-mm analytical silica gel plates were used. Solvent systems are as follows: I, butanol/acetic acid/water (4:1:1); II, butanol/acetic acid/pyridine/water (13:3:12:10); III, 2-propanol/ammonia/water (3:1:1); IV, butanol/acetic acid/ethyl acetate/water (1:1:1:1). [b] Capacity factor (k') for the following systems: Vydac 218TP104 C_{18} reversed phase (RP) column (25 × 0.46 cm) with 0.1% trifluoroacetic acid/CH_3CN (75/25 v/v) and at a flow rate 1.5 mL/min (V) and with 0.1% trifluoroacetic acid/CH_3CN (55:45) at a flow rate of 1.5 mL/min (VI). All peptides were monitored at λ = 280 nm.

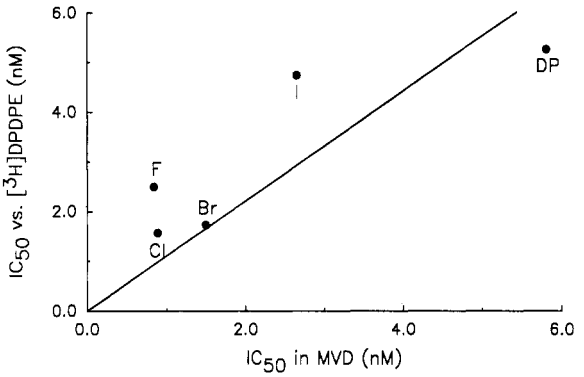

Figure 2. Correlation between the potency in the peripheral mouse vas deferens (MVD) assay (IC_{50}) and in the radioreceptor binding assay vs [^{3}H]DPDPE in rat brain homogenates (IC_{50}) of DPDPE and its analogues; same notation as in Figure 1 (r = 0.86).

(CTOP) of the various analogues (Figure 1). However, the correlation between IC_{50} (MVD) and IC_{50} (DPDPE) is strong (Figure 2), with a correlation coefficient of 0.86. Similar correlations were presented for linear enkephalin analogues by Gacel et al.,[12] and their findings were similar to those obtained here. However, these correlations should be interpreted with some caution, as the range in potencies found in either δ binding or bioassay spans less than 1 order of magnitude. Our results suggest the peripheral δ (mouse vas deference) and brain δ opioid receptors have similar recognition characteristics, while peripheral μ (guinea pig intestine) and brain μ receptor recognition characteristic may be different, as previously proposed by Chang et al.,[18] Liebman et al.,[19] and Sakaguchi et al.[20] Other explanations include κ agonist activity or lack of intrinsic μ agonist efficacy.

The δ opioid receptor potencies of the para halogenated Phe^4 analogues correlate very well with the increasing electronegativities (H = I = 2.0; Br = 2.8; Cl = 3.0; F = 4.0) of the substituents. On the other hand, as the lipophilicity of the halogenated analogues increases (Table III), the potencies decrease, although it is possible that the higher lipophilicity of all of these analogues relative to DPDPE (as can be seen from the RP-HPLC capacity factors in Table III) increase their interaction by these analogues with the μ opioid receptor, further demonstrating that these receptor subtypes are distinct. The increased molecular volumes at the Phe^4 para position of these analogues apparently does not lead to significant steric hindrance on interaction with the δ receptor, since all of the halogenated analogues are more potent than DPDPE in both the receptor binding assay and MVD assay.

Recently Delay-Goyet et al.[21] synthesized two linear leucine enkephalin analogues with bulky *tert*-butyl groups on the hydroxyl groups of D-Ser^2 (H-Tyr-D-Ser(O^{t}Bu)-Gly-Phe-Leu-Thr-OH, DSTBULET), or on D-Ser^2 and Thr^6 residues (H-Tyr-D-Ser(O^{t}Bu)-Gly-Phe-Leu-Thr-(O^{t}Bu)-OH, BUBU) to give analogues with slightly higher δ opioid receptor selectivities than DPDPE. Although direct comparison of these linear analogues and our cyclic para-halogenated Phe^4 DPDPE analogues is not available under the same assay conditions, the [*p*-ClPhe^4]DPDPE analogue reported here appears to be approximately equipotent and relatively more δ opioid receptor selective than these two linear analogues.

The results from our studies clearly demonstrate the importance of the Phe^4 residue of DPDPE in its high δ opioid receptor potency and selectivity and suggest that perhaps even higher δ selectivities may be possible. In view of the very high potency of the *p*-ClPhe^4 analogue **3** for δ receptors, it may prove to be the ligand of choice for examining the properties of δ opioid receptors.

Experimental Section

General Method for Synthesis of DPDPE Analogues. All of the analogues were synthesized by the solid-phase method of peptide synthesis by methods similar to those previously reported[6] for DPDPE. Chloromethylated (1.4 mmol/g of resin) polystyrene resin 1% cross-linked with divinylbenzene (Lab. Systems, San Mateo, CA) was used as a solid support. N^α-*tert*-Butyloxycarbonyl (Boc) protected amino acids were used throughout. The amino acids were obtained either from Bachem (San Mateo, CA) or Chemical Dynamics Corp. (South Plainfield, NJ) and were converted to the N^α-*tert*-butyloxycarbonyl-amino acids by using literature procedures.[22] The N^α-(*tert*-butyloxycarbonyl)-*S*-(*p*-methylbenzyl)-D-penicillamine was attached to the resin by using Gisin's method.[23] Dicyclohexylcarbodiimide and hydroxybenzyltriazole were used in the coupling reactions, which were monitored by the ninhydrin test.[24] Cleavage of the peptides from the resin and removal of the protecting groups was effected by treatment of the protected peptide resin with anhydrous HF (10 mL/g of resin) in the presence of anisole (2 mL/g of resin) for 60 min at 0 °C. The extraction of peptides from the resin and the cyclization of linear peptides was carried out via a manner

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similar to those previously published.[6] The analogues were purified by gel filtration on Sephadex G-10 using 15% acetic acid and partition chromatography (butanol/toluene/pyridine/acetic acid/water 6:3:0.135:0.135:8.55, upper phase) on Sephadex G-25 block polymerizate, or by HPLC (Perkin Elmer, Vydac 218TP1010 C18 reversed phase column (25 cm × 1 cm)) using a linear gradient of 20%–40% CH_3CN in 0.1% aqueous trifluoroacetic acid, 1%/min at a flow rate of 4 mL/min with UV detection at 214 nm or 280 nm.

Purity was assessed by TLC in four solvent systems on silica gel and by analytical HPLC. The amino acid analyses were performed on a Beckman 120C amino acid analyzer (Pen could not be determined). The $(M + 1)^+$ molecular ions and fragmentation patterns obtained by FAB-MS were in agreement with the amino acid sequence and composition of each analogue. ^1H NMR spectra were obtained for each analogue and were found to be consistent with the sequence and structure of the peptides.

[D-Pen^2,D-Pen^5]enkephalin (DPDPE, 1) was prepared by the methods outlined above and was found to be identical with the compound previously prepared.[6]

[D-Pen^2,*p*-FPhe^4,D-Pen^5]enkephalin (2). The title compound was prepared by the solid-phase method as outlined above, starting with 1.17 g of N^α-Boc-D-Pen(*S*-pMB)-*O*-resin (substitution = 0.86 mmol/g of resin, 1.0 mmol), and the following protected amino acids were added stepwise to the growing peptide chain: N^α-Boc-*p*-FPhe, N^α-Boc-Gly, N^α-Boc-D-Pen(*S*-pMB), N^α-Boc-Tyr. This led to 1.57 g of N^α-Boc-Tyr-D-Pen(*S*-pMB)-Gly-*p*-FPhe-D-Pen(*S*-pMB)-*O*-resin, which was treated with 15 mL of HF containing 1.5 mL of anisole for 60 min at 0 °C. The HF was rapidly removed by vacuum aspiration at 20 °C. The mixture was washed with 3 × 60 mL of ether, and the peptide was extracted with 40 mL of glacial acetic acid and washed with 3 × 30 mL of 20% aqueous acetic acid. The peptide solution was lyophilized and the residue dissolved in 1500 mL of deaerated 0.1% aqueous acetic acid. The pH was adjusted to 8.4 with 3 N ammonium hydroxide and 150 mL of 0.01 N $K_3Fe(CN)_6$ was added. After 120 min the pH was decreased to 4.5 with glacial acetic acid, and the ferro- and excess ferricyanide were removed by the anion exchange resin Amberlite IRA-45 (Cl^- form). After the mixture was stirred for 20 min, the resin was filtered off and the resin washed with 3 × 30 mL of 30% aqueous acetic acid. The solution was evaporated down to 150 mL on a rotary evaporator in vacuo at 25 °C and lyophilized. The residue was dissolved in 5 mL of 15% aqueous acetic acid and gel filtered on a 50 × 3.2 cm column containing Sephadex G-10. The major peak was isolated and lyophilized. The powder obtained was dissolved in 2 mL of 20% acetonitrile in 0.1% trifluoroacetic acid and purified on a Vydac 218TP1010 C18 RP-HPLC column (25 cm × 1 cm). Conditions for HPLC were as follows: linear gradient elution starting with 20% CH_3CN in 0.1% TFA, 1%/min for 20 min, at a flow rate of 4 mL/min. The more lypophilic impurities were washed from the column with 80% CH_3CN in 0.1% TFA for 5 min, and after equilibration (5 min 20% CH_3CN) the column was ready to use. The major peak was isolated as a white powder (the yield was 70 mg). Amino acid analysis: Gly 1.00 (1.00); Tyr, 0.93 (1.00); *p*-FPhe 1.01 (1.0). The analytical data from the purified product **2** are given in Table III.

[D-Pen^2,*p*-ClPhe^4,D-Pen^5]enkephalin (3). The title compound was prepared from 1 mmol of N^α-Boc-D-Pen(*S*-pMB)-*O*-resin as for **2** above except that N^α-Boc-*p*-ClPhe was added to the growing peptide chain instead of N^α-Boc-*p*-FPhe. This led to 1.65 g of N^α-Boc-Tyr-D-Pen(*S*-pMB)-Gly-*p*-ClPhe-D-Pen(*S*-pMB)-*O*-resin. The peptide resin was treated with HF as before, and the peptide was isolated, oxidized to the cyclic structure, and purified as for **2** above. This yielded 65 mg of the title compound **3** as a white powder. Amino acid analysis: Gly 1.00 (1.00); Tyr, 0.94 (1.00); *p*-ClPhe, 1.09 (1.00). The analytical data for the purified product **3** are given in Table III.

[D-Pen^2,*p*-BrPhe^4,D-Pen^5]enkephalin (4). The title compound was prepared from 1 mmol of N^α-Boc-D-Pen(*S*-pMB)-*O*-resin as for **2** above except that N^α-Boc-*p*-BrPhe was used in place of N^α-Boc-*p*-FPhe in synthesizing the growing peptide chain. This yielded 1.5 g of N^α-Boc-Tyr-D-Pen(*S*-pMB)-Gly-*p*-BrPhe-D-Pen(*S*-pMB)-*O*-resin. The peptide resin was treated with HF as before, and the peptide was isolated and oxidized to the cyclic structure and then purified as for **2** above to give 53 mg of the title compound **4** as a white powder. Amino acid analysis: Gly, 1.00 (1.00); Tyr, 0.89 (1.00); *p*-BrPhe, 0.95 (1.00). The analytical data for the product **4** are given in Table III.

[D-Pen^2,*p*-IPhe^4,D-Pen^5]enkephalin (5). The title compound was prepared from 1 mmol of N^α-Boc-D-Pen as for **2** above except that N^α-Boc-*p*-IPhe was used in place of N^α-Boc-*p*-FPhe in synthesizing the growing peptide chain. There was obtained 1.87 g of N^α-Boc-Tyr-D-Pen(*S*-pMB)-Gly-*p*-IPhe-D-Pen(*S*-pMB)-*O*-resin. The protected peptide resin was treated with HF as before. The peptide was isolated and oxidized to the cyclic structure as above and then purified as for **2** above to give 55 mg of the title compound **5** as a white powder. Amino acid analysis: Gly, 1.00 (1.00); Tyr, 0.91 (1.00); *p*-IPhe, 0.88 (1.00). The analytical data for **5** are given in Table III.

GPI and MVD Bioassays. Electrically induced smooth muscle contractions of mouse vas deferens and strips of guinea pig ileum longitudinal muscle–myenteric plexus were used as a bioassay.[25] Tissues came from male ICR mice weighing 25–30 g and from male Hartley guinea pigs weighing 150–400 g. The tissues were tied to gold chains with suture silk, suspended in 20-mL baths containing 37 °C oxygenated (95% O_2, 5% CO_2) Krebs bicarbonate solution (magnesium-free for the MVD) and allowed to equilibrate for 15 min. The tissues were then stretched to optimal length previously determined to be 1 g tension (0.5 g for MVD) and allowed to equilibrate for 15 min. The tissues were stimulated transmurally between platinum plate electrodes at 0.1 Hz, 0.4-ms pulses (2.0-ms pulses for MVD), and supramaximal voltage. Drugs were added to the baths in 20–60-μL volumes. The agonists remained in contact with the tissue for 3 min, and the baths were then rinsed several times with fresh Krebs solution. Tissues were given 8 min to reequilibrate and regain predrug contraction height. Antagonists were added to the bath 2 min prior to the addition of the agonist. Percent inhibition was calculated by using the average contraction height for 1 min preceding the addition of the agonist divided by the contraction height 3 min after exposure to the agonist. IC_{50} values represent the mean of not less than four tissues. IC_{50} estimates, relative potency estimates, and their associated standard errors were determined by fitting the mean data to the Hill equation by using a computerized nonlinear least-squares method.[26] The standard errors of the IC_{50} values for μ activity appear large in some cases, as the weak μ agonist actions of these analogues did not permit completion of dose–response curves in the GPI.

Radioreceptor Assay. Adult male Sprague–Dawley rats (200–250 g) were sacrificed and brains immediately removed and placed on ice. Whole brain minus cerebellum was homogenized with a Polytron homogenizer (Brinkman, setting 5, 15 s). The homogenate was preincubated at 25 °C for 30 min to remove endogenous opioids and centrifuged two times at 43000*g* for 10 min before use in the radioreceptor assay.

Binding affinities [^3H]DPDPE (43.0 Ci/mmol, 1.59 TBq/mmol, New England Nuclear, Boston, MA) and [^3H]CTOP (84.2 Ci/mmol, 3.12 TBq/mmol, New England Nuclear, Boston, MA) were measured by a rapid filtration technique. A 100-μL aliquot of rat brain homogenate (0.5% final) was incubated with either 1.0 nM [^3H]DPDPE or 0.5 nM [^3H]CTOP in a total volume of 1 mL of 50 mM Tris-HCl pH 7.4 at ~5 °C containing 5 mM $MgCl_2$, bovine serum albumin (1 mg/mL), and phenylmethanesulfonyl fluoride (100 μL). Steady-state binding experiments were carried out at 25 °C for 120 min. All binding measurements were done in duplicate, and the radioligand displaced by 1 μM naltrexone hydrochloride was defined as specific tissue binding. The binding

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reaction was terminated by rapid filtration of samples through GF/B Whatman glass fiber filter strips pretreated with 0.1% polyethylenimine 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 (43% efficiency).

The data were analyzed by using nonlinear least-squares regression analysis on the Apple II plus computer. Programs were generously written by Susan Yamamura.

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Registry No. 1, 88373-73-3; 2, 122622-48-4; 3, 122507-47-5; 4, 122622-49-5; 5, 122622-50-8; BOC-D-Pen(*p*MB)-OH, 115962-34-0; BOC-*p*-F-Phe-OH, 41153-30-4; BOC-Gly-OH, 4530-20-5; BOC-Tyr-OH, 3978-80-1; H-Tyr-D-Pen-Gly-*p*-F-Phe-D-Pen-OH, 122593-60-6; BOC-*p*-Cl-Phe-OH, 68090-88-0; H-Tyr-D-Pen-Gly-*p*-Cl-Phe-D-Pen-OH, 122593-57-1; BOC-*p*-Br-Phe-OH, 62129-39-9; H-Tyr-D-Pen-Gly-*p*-Br-Phe-D-Pen-OH, 122593-59-3; BOC-*p*-I-Phe-OH, 62129-44-6; H-Tyr-D-Pen-Gly-*p*-I-Phe-D-Pen-OH, 122593-58-2.

Alkylation of DNA by C-10 of 2,7-Diaminomitosene

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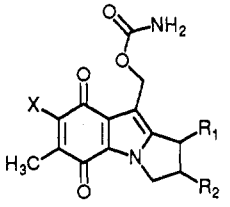
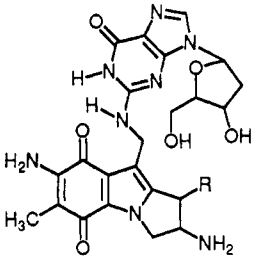
Mitomycin C and certain analogues alkylate DNA with their C-1 position and cross-link it by a second alkylation involving C-10. We now show that monoalkylation by C-10 (carbamate group) can occur for mitosene analogues that have no reactive C-1 functionality. Sodium dithionite reduction of 2,7-diaminomitosene or 2,7-diamino-1-hydroxymitosene in the presence of calf thymus DNA resulted in alkylation of the DNA to the extent of one molecule per 14 and 11 bases, respectively, although no covalent binding was observed on catalytic reduction. Reduction of each of these mitosenes by sodium dithionite in the presence of 2'-deoxyguanosine gave monoalkylation on the 2-amino group of this nucleotide. The 2,7-diaminomitosenes inhibited L-1210 leukemia cell colony formation in vitro at concentrations 3–4-fold greater (less potent) than mitomycin C. DNA single-strand breaks were also produced by each mitosene, but these lesions did not correlate with cytotoxicity and were less prominent than breaks produced by another monofunctional alkylating agent, methyl methanesulfonate. Mitosene-induced DNA strand breaks are probably due to excission–repair endonuclease activity and not from oxygen free radicals produced by redox cycling of the quinone moiety. There was no evidence of DNA–DNA cross-links by either 2,7-diaminomitosene.

The alkylation of DNA by mitomycin C has been studied by a number of investigators.[1-10] Their results have led to the definition of a process in which there is an initial alkylation on the 2-amino group of a guanine residue by C-1 of mitomycin C, following bioreductive activation and opening of the aziridine ring (Scheme I). A second alkylation by C-10 of mitomycin C, with loss of the carbamoyloxy group, is possible and results in cross-linking, providing that a second guanine residue is in an appropriate location, below the complimentary cytidine residue. If a second guanine is not in proximity, the carbamoyloxy group is simply replaced by a hydroxyl group.[10] Thus, cross-linking occurs by mitomycin C to a lesser extent than monoalkylation. Enzymatic hydrolysis of DNA bound covalently by mitomycin C has produced fragments in which the resulting mitomycin species was bound monocovalently by C-1 to the 2-amino group of 2'-deoxyguanosine or by C-1 and C-10 to the 2-amino groups of two 2'-deoxyguanosines. This confirms the chemical activation process described above.[5,6]

Products derived from monoalkylation of DNA by C-10 of mitomycin C have never been isolated. This may be explained by the observation that the aziridine ring of mitomycin C is more reactive than the carbamate group.[11] Notwithstanding this, previous biological studies on mitosenes show that compounds lacking an aziridine ring or other alkylating functionality have potent cytotoxic activity, expecially toward bacteria.[12] Thus, 1-hydroxy-7-methoxymitosene (1) which has a relatively poor leaving group at C-1, was as potent as mitomycin C against Gram-positive bacteria in a disk-plate assay.[12] It also was





1: X = CH_3O, R_1 = OH, R_2 = H
2: X = CH_3O, R_1 = R_2 = H
3: X = H_2N, R_1 = uridylate, R_2 = NH_2
4: X = H_2N, R_1 = H, R_2 = NH_2
5: X = H_2N, R_1 = OH, R_2 = NH_2

6: R = H
7: R = OH

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