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CYCLIC, DISULFIDE- AND DITHIOETHER-CONTAINING OPIOID TETRAPEPTIDES: DEVELOPMENT OF A LIGAND WITH HIGH DELTA OPIOID RECEPTOR SELECTIVITY AND AFFINITY

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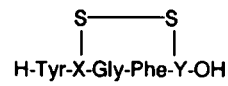
Summary

Tetrapeptides of primary sequence Tyr-X-Phe-YNH_2, where X is D-Cys or D-Pen (penicillamine) and where Y is D-Pen or L-Pen, were prepared and were cyclized via the side chain sulfurs of residues 2 and 4 to disulfide or dithioether-containing analogs. These peptides are related to previously reported penicillamine-containing pentapeptide enkephalin analogs but lack the central glycine residue of the latter and were designed to assess the effect of decreased ring size on opioid activity. Binding affinities of the tetrapeptides were determined to both μ and δ opioid receptors. Binding affinity and selectivity in the tetrapeptide series were observed to be highly dependent on primary sequence. For example, L-Pen^4 analogs displayed low affinity and were nonselective, while the corresponding D-Pen^4 diastereomers were of variable affinity and higher selectivity. Among the latter compounds were examples of potent analogs in which selectivity shifted from δ selective to μ selective as the ring size was increased. The relatively high binding affinity and δ receptor selectivity observed with one of the carboxamide terminal disulfide analogs led to the synthesis of the corresponding carboxylic acid terminal, Tyr-D-Cys-Phe-D-PenOH. This analog displayed δ receptor binding selectivity similar to that of the standard δ ligand, [D-Pen^2,D-Pen^5]enkephalin (DPDPE), and was found to have a 3.5-fold higher binding affinity than DPDPE. All the tetrapeptides were further evaluated in the isolated mouse vas deferens (mvd) assay and all displayed opioid agonist activity. In general, tetrapeptide potencies in the mouse vas deferens correlated well with binding affinities but were somewhat lower. Receptor selectivity in the mvd, assessed by examining the effect of opioid antagonists on the tetrapeptide concentration-effect curves, was similar to that determined in the binding studies.

We have previously described (1-3) a series of enkephalin analogs of the general structure:



in which X = D-Cys or D-Pen
Y = D(or L)-Cys or D(or L)-Pen

where Pen, penicillamine, is β,β-dimethylcysteine. These analogs, which are conformationally restricted because of the imposed cyclization through the side chain sulfurs and are further restricted because of the rigidizing effect of the penicillamine gem dimethyl groups (1), are notable for their high degree of selectivity

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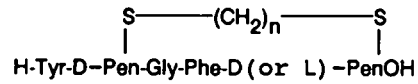
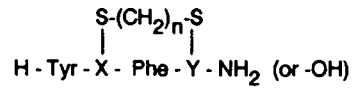
for the δ opioid receptor. The bis-penicillamine analogs within this series, [D-Pen^2, D-Pen^5]enkephalin (DPDPE) and [D-Pen^2, L-Pen^5]enkephalin (DPLPE) display the highest selectivity yet reported for the δ receptor, while those analogs with a single penicillamine residue, such as [D-Pen^2, L-Cys^5]enkephalin (DPLCE) and [D-Cys^2, D-Pen^5]enkephalin (DCDPE), are less selective but are more potent. More recently we have examined the effect of ring size on opioid activity for the bis-penicillamine analogs by means of the dithioether-containing series:



In this series δ receptor binding affinity and selectivity were observed to decrease with increasing ring size (4).

Schiller and coworkers have reported a series of smaller, cyclic des-Gly^3 enkephalin tetrapeptides in which cyclization is effected by amide bond formation via side chain functions of residues 2 and 4 (5,6). Within this series are members which display considerable μ opioid receptor selectivity resulting primarily from reduced δ receptor affinity. In view of this finding and our studies on expanded ring size in the pentapeptide series, we undertook an investigation of the effect of ring contraction on opioid receptor affinity and selectivity in a series of des-Gly^3 disulfide- and dithioether-containing tetrapeptides of general structure:



where X = D-Cys or D-Pen
Y = D-(or L-)Pen
n = 0,1,2,3

Several analogs in this series have interesting opioid receptor binding profiles. Particularly noteworthy among these is an analog which exhibits higher δ receptor affinity than the bis-penicillamine enkephalins while maintaining comparable selectivity.

Methods

Peptide synthesis. All peptides reported here were prepared by solid phase methods similar to those previously described (2,4,7). Chloromethylated polystyrene (Merrifield) resin crosslinked with 1% divinylbenzene was used for the synthesis of peptides with carboxy terminal carboxylic acid functions, while p-methylbenzhydrylamine resin was used for the synthesis of carboxamide terminal peptides. t-Butyloxycarbonyl protection of α-amino functions was used throughout, while S-p-methylbenzyl protection was used for the labile side chain sulfurs of Cys and Pen. In all cases the linear free-sulfhydryl containing peptides were generated by treatment of the peptide-resin with anhydrous HF in the presence of 5% anisole and 5% dithioethane, as has been previously described (2). Prior to cyclization, the linear, free sulfhydryl-containing peptides were purified by reverse phase-high performance liquid chromatography (RP-HPLC) on a Vydac 218TP C-18 column (2.5 x 22cm) using the solvent system 0.1% trifluoroacetic acid (TFA) in H_2O / 0.1% TFA in acetonitrile. A gradient of 10-50% organic component over a course of 40 min was employed for all purifications. Disulfide containing analogs were prepared by treating an aqueous solution (pH 8.5) of the corresponding free sulfhydryl-containing species with $K_3Fe(CN)_6$ (2), while dithioether-containing analogs were obtained by treating a dilute solution of the free sulfhydryl-containing peptide in dimethyl formamide with potassium tert-butoxide followed by addition of the appropriate alkyl dibromide (4). Disulfide and dithioether-containing peptides were purified by RP-HPLC as described above. Purity of the final product peptides was assessed by analytical HPLC monitored at 280nm and at 230nm. All peptides were >98% pure by this measure. Analytical evaluation of the final peptides also included testing with 5,5′-dithiobis-(2-

nitrobenzoic acid) to detect the presence of free sulfhydryl groups (8). Final confirmation of the putative structures was obtained by fast atom bombardment-mass spectrometry which yielded the appropriate molecular weights for all of the peptides.

Receptor binding assays. The binding assays, based on the displacement by the test compounds of radiolabelled sufentanil (μ ligand) or DPDPE (δ ligand) in cerebral membranes from rat brain, were performed as previously described (9,10). Briefly, the assay mixture, containing membrane suspension in 50mM Tris buffer (pH 7.4), 150mM NaCl, the radiolabelled ligand and the test compound, was incubated to reach binding equilibrium (40min for assays using 0.5nM [^{3}H]sufentanil; 60min for 1.5nM [^{3}H]DPDPE) at 25°C. Subsequently, the samples were rapidly filtered and the radioactivity on the filter determined by liquid scintillation counting. Inhibition of radiolabelled ligand binding by the test compound was computed from maximal specific binding, determined with an appropriate excess of unlabelled sufentanil or DPDPE. IC_{50} values were obtained by linear regression from plots relating inhibition of the specific binding in probit units to the log of five different ligand concentrations (9). In every case the correlation coefficient, r^2, of the log-probit plot was higher than 0.97.

Isolated mouse vas deferens (mvd) assay. The mvd assays were performed as previously described (11). Briefly, 1.5cm vas deferens segments from male, albino ICR mice were suspended in organ baths which contained 30mL of a modified Krebs' buffer (118mM NaCl, 4.75mM KCl, 2.54mM $CaCl_2$, 1.19mM $MgSO_4$, 1.19mM KH_2PO_4, 11mM glucose, 25mM $NaHCO_3$, 0.3mM pargyline HCl, 0.2mM tyrosine, 0.1mM ascorbic acid, and 0.03mM sodium EDTA) saturated with 95% O_2 - 5% CO_2 and kept at 37°C. The segments were attached to strain gauge transducers and suspended between two platinum electrodes. After a 30min equilibrium period, the segments were stimulated once every 10s with pairs of pulses of 2ms duration, 1ms apart and at supramaximal voltage. Test compounds were evaluated for their ability to inhibit the electrically stimulated smooth muscle contractions in this preparation. One vas deferens of each pair of vasa deferentia was studied in the presence of either 100nM naltrexone or 100nM ICI-174864 and the other served as the control. IC_{50} values were determined by probit analysis and values reported are the means of 3-9 determinations. Because there were no appreciable differences among the IC50 values for the control preparations studied with either naltrexone or ICI-174864, the control IC50 reported for each agonist is the mean of all control values determined in experiments with both antagonists.

Results

The binding affinities of eleven cyclic tetrapeptides to μ and δ opioid receptors in brain membranes as determined by the ability of the test compounds to displace the μ selective ligand [^{3}H]sufentanil and the δ selective ligand [^{3}H]DPDPE (9) are listed in Table I. Also provided are the corresponding values for the reference μ ligands sufentanil and [D-Ala^2, NMePhe^4, Gly^5-ol]enkephalin (DAGO) and the reference δ ligand DPDPE. The initial tetrapeptides chosen for synthesis were those which had a carboxamide terminal penicillamine residue, D-Cys or D-Pen as the second residue, and which were cyclized to either the disulfide or ethylene dithioether. Such a series would allow direct comparison with both the disulfide-containing pentapeptides such as DPDPE as well as the highly μ receptor selective cyclic tetrapeptide, Tyr-D-Orn-Phe-AspNH_2, reported by Schiller and coworkers (5,6) which, like the ethylene dithioethers, contains a 13-membered ring. Unlike the analogs reported here, however, Schiller's analog is cyclized via an amide bond between the ornithine δ-amino group and the aspartic acid β-carboxylate function.

The carboxamide terminal tetrapeptides 1-7 and 9 display several interesting features. Within this series, analogs 3-6, which have a L-Pen^4 residue, display rather low affinity and are essentially devoid of receptor selectivity in the binding assay. In contrast, corresponding analogs with a D-Pen^4 residue (1,2,7 and 9) exhibit considerable variation in both affinity and selectivity. For example, analogs 1 and 7 show significant δ receptor selectivity with 7 displaying considerable affinity for the δ receptor. However, analog 9, which contains the same primary sequence as 7 but which is cyclized as the ethylene dithioether rather than the disulfide, is significantly μ receptor selective and displays a 2.4-fold higher affinity for μ receptor binding sites than does the prototypical μ ligand, DAGO. The result of altering the ring size by two carbon atoms between analogs 7 and 9 is a 130-fold shift in selectivity.

The striking selectivity shift seen between 7 and 9 as a result of altering the ring size was further explored by preparing the methylene-dithioether (actually, a dithioacetal) analog, 8, and the propylene-dithioether analog, 10. Analogs 7-10 thus represent a subseries in which primary sequence is maintained

TABLE I

Opioid Receptor Binding Profiles of Cyclic Tetrapeptides

	Analog	IC_{50}(nM) [^{3}H]sufentanil	IC_{50}(nM) [^{3}H]DPDPE	$\frac{IC_{50}(\mu)}{IC_{50}(\delta)}$
1	Tyr-D-Pen-Phe-D-PenNH$_2$	1320	61	21.6
2	Tyr-D-Pen-Phe-D-PenNH$_2$	1580	215	7.35
3	Tyr-D-Pen-Phe-L-PenNH$_2$	188	103	1.83
4	Tyr-D-Pen-Phe-L-PenNH$_2$	679	632	1.07
5	Tyr-D-Cys-Phe-L-PenNH$_2$	350	373	0.94
6	Tyr-D-Cys-Phe-L-PenNH$_2$	294	222	1.32
7	Tyr-D-Cys-Phe-D-PenNH$_2$	320	17.2	18.6
8	Tyr-D-Cys-Phe-D-PenNH$_2$	76	238	0.32
9	Tyr-D-Cys-Phe-D-PenNH$_2$	5.6	39.6	0.14
10	Tyr-D-Cys-Phe-D-PenNH$_2$	22.4	193	0.12
11	Tyr-D-Cys-Phe-D-PenOH	1210	1.90	637
	DPDPE	7720	6.44	1200
	DAGO	13.2	690	0.02
	Sufentanil	1.3	45	0.03

Binding assays were performed on rat brain membrane preparations as described in Methods. Reported IC_{50} values represent the mean of 1-6 experiments run in duplicate with 5 different concentrations of each compound. The average range ($n < 4$) or standard deviation ($n \geq 4$) for the μ and δ selective assays was $\pm$ 6.5% and $\pm$ 10%, respectively (9).

while ring size is varied from an 11-membered ring to a 14-membered ring. Increasing the ring size from the 11-membered ring in 7 to the 12-membered ring of 8 increases μ receptor affinity 4-fold while decreasing δ receptor affinity by a factor of 14 (Table I). As a result, analog 8 exhibits a slight binding preference for μ opioid receptors. Increasing the ring size by an additional carbon results in a slight improvement in μ receptor selectivity for analog 9 as compared with analog 8. Accompaning this improved selectivity are impressive 6-fold and 13-fold enhancements in δ and μ receptor binding affinities, respectively. Further expanding the ring size to the propylene-dithioether, 10, has little effect on selectivity but results in a 4-fold reduction in binding affinity.

The results obtained with analogs 2,4,6,and 9, which contain 13-membered ring systems, are quite different from those observed for Schiller's analogous tetrapeptide. In the current series analogs 4 and 6, which like Schiller's contain an L-amino acid in position 4, show very weak binding affinity and are essentially nonselective. Only analog 9 among those with a 13-membered ring displays μ receptor binding selectivity and this selectivity is modest. The high μ affinity of this analog is nonetheless indicative of a favorable conformation for μ receptor binding. That the analogs presented here differ in binding profile from those reported by Schiller is not surprising given the different steric and electronic characteristics expected for these analogs.

The significant δ receptor selectivity of analogs 1 and 7 and the relatively high δ receptor affinity of the latter are reminiscent of earlier results in the pentapeptide penicillamine-containing enkephalin series in which carboxamide terminal analogs were observed to display δ receptor selectivity (12). Since carboxylic acid terminal enkephalin analogs are generally observed to exhibit considerably enhanced δ selectivity, the results obtained for analogs 1 and 7 suggested that the corresponding carboxylic acid terminal analogs, particularly that of analog 7, might lead to greatly improved δ selectivity and/or binding affinity. As shown in Table 1 this is in fact the case; analog 11 shows greatly improved δ receptor selectivity and affinity compared to analog 7. Indeed, analog 11 is almost as selective as DPDPE, the current standard for δ receptor selectivity, and has 3.5-fold higher affinity for δ receptors.

The opioid tetrapeptides were further evaluated on the isolated, electrically stimulated mouse vas deferens preparation and were compared to DAGO and DPDPE (Table II). Both DAGO and DPDPE were full agonists in this assay with DPDPE exhibiting approximately 15-fold higher potency than DAGO. All but three of the tetrapeptides were full agonists which produced complete inhibition of the twitch. Analogs 2, 4, and 10 were partial agonists which produced maximal inhibitions of 85.1 $\pm$ 2.1%, 65.8 $\pm$ 3.8%, and 91.9 $\pm$ 2.0%, respectively. The most potent in this series in inhibiting the twitch was analog 11 which was essentially equipotent with DPDPE. Analog 9 had about one-half the potency and analog 8, about one-eighth the potency of DPDPE. All other analogs were less potent than DAGO as agonists in this preparation, and the partial agonists, analogs 2 and 4 were the least potent of the series. Because of the much lower potency of analog 2 on the vas deferens than in the binding assay, this peptide was evaluated as a possible antagonist, however no such activity was observed.

In order to determine the types of opioid receptors which mediate the agonist activity of the tetrapeptide analogs, complete concentration-effect relationships were determined in the presence and absence of two opioid antagonists, naltrexone, which is somewhat more selective for μ receptors than for δ receptors in the mouse vas deferens (11), and ICI-174864, which is a highly selective antagonist at δ opioid receptors and is virtually devoid of activity at μ receptors (13). Naltrexone (100nM) caused a 27.4-fold shift to the right in the DAGO concentration-effect curve which is typical of those shifts seen with highly selective μ receptor agonists (14). In the presence of 100nM ICI-174864, there was no shift in the DAGO concentration-effect curve. In contrast, naltrexone produced a smaller shift in the DPDPE concentration-effect curve (7.1-fold), and ICI-174864 significantly shifted the DPDPE concentration-effect curve to the right (2.8-fold).

Three of the tetrapeptide analogs appeared to be even more selective than DPDPE for δ receptors in the mouse vas deferens. The shifts produced by 100nM naltrexone and 100nM ICI-174864 were 5.7- and 4.5-fold for analog 11, 3.2 and 4.8-fold for analog 1, and 3.9- and 4.0-fold for analog 7, respectively. These results are in qualitative agreement with the displacement studies which found these analogs to exhibit the highest δ binding selectivity in the series. Two of the tetrapeptide analogs appeared to act only on μ receptors in the vas deferens. The shifts produced by 100nM naltrexone and 100nM ICI-174864 were 5.2- and 1.0-fold for analog 10 and 41.3- and 1.2-fold for analog 9. Again these results are in agreement with the displacement results which established analogs 9 and 10 as having the highest μ binding selectivity in this

TABLE II

Tetrapeptide Potencies in the Mouse Vas Deferens Preparation in the Presence and Absence of Opioid Antagonists

	Analog	Control	IC_{50}(nM) (± s.e.m.) + Naltrexone (100nM)	+ ICI-174864 (100nM)
1	Tyr-D-Pen-Phe-D-PenNH_2	409 ± 140	1290 ± 430	1950 ± 968
2	Tyr-D-Pen-Phe-D-PenNH_2	4600 ±1050	7830 ± 130	7030 ± 20
3	Tyr-D-Pen-Phe-L-PenNH_2	524 ± 120	6370 ±2410	2910 ±1620
4	Tyr-D-Pen-Phe-L-PenNH_2	1630 ± 230	*	*
5	Tyr-D-Cys-Phe-L-PenNH_2	919 ± 276	1650 ± 107	2910 ± 780
6	Tyr-D-Cys-Phe-L-PenNH_2	371 ± 96	947 ± 41	452 ± 108
7	Tyr-D-Cys-Phe-D-PenNH_2	120 ± 24	469 ± 130	477 ± 143
8	Tyr-D-Cys-Phe-D-PenNH_2	46 ± 6	405 ± 285	95 ± 11
9	Tyr-D-Cys-Phe-D-PenNH_2	11 ± 3	435 ± 136	12 ± 4
10	Tyr-D-Cys-Phe-D-PenNH_2	119 ± 24	622 ± 211	119 ± 30
11	Tyr-D-Cys-Phe-D-PenOH	4.6 ± 0.5	26 ± 6	21 ± 5
	DPDPE	5.5 ± 3	39 ± 16	15 ± 3
	DAGO	81 ± 12	2230 ± 446	73 ± 17

* Due to low potency and large shift by both antagonists, accurate IC_{50} values could not be determined. Each reported IC_{50} value represents the mean of 3-9 determinations.

series. Both antagonists shifted responses to analog 4 to such a degree that IC_{50} values could not be reliably determined, while neither antagonist produced an appreciable shift in the concentration-effect curve of analog 2. Both of these analogs were partial agonists of extremely low potency. The interactions with the two antagonists suggested that the remaining tetrapeptides had significant activity at both μ and δ receptors, which, again, is consistent with the results of the binding assays.

Discussion

As noted above analogs 3-6, which have a carboxy terminal L-Pen residue, have low opioid binding affinity and are essentially nonselective. In contrast, the corresponding D-Pen[4] analogs display significant receptor selectivity and, except for 2, have higher binding affinities. This is particularly evident in analog 7, which has μ receptor binding affinity like that of analog 5 but which has δ receptor binding affinity 22-fold higher than 5. Similarly, analog 9 has 5.6 and 53-fold higher affinity than analog 6 for δ and μ receptors, respectively. Potencies in the mvd assay parallel the binding results, with analog 7 exhibiting 7.7-fold higher potency than 5 and analog 9 displaying 35-fold higher potency than 6. Further, while analogs 5 and 6 are relatively nonselective based on antagonist shifts in the mvd, analog 7 appears to be selective for δ and analog 9, selective for μ receptors in this preparation. These findings suggest a requirement for a carboxy-terminal D-amino acid for optimal binding to both δ and μ receptors in this series, a more stringent condition than is found in the penicillamine-containing pentapeptide series (1-3). Such a divergence of effect due to altering the configuration of the carboxy terminal residue may be due to the increased rigidity resulting from the elimination of the glycine residue in the tetrapeptide series. In the pentapeptide series this flexible glycine residue has been suggested to serve as a means of achieving a similar topography for the various analogs in the series (15). The lack of such a flexible hinge in the tetrapeptide series eliminates much of this conformational compensation.

The changes in receptor binding selectivity observed among analogs 7-10, in which the ring size is varied from 11- to 14-membered without altering the primary sequence, also reflect the importance of conformation on pharmacological activity. As was seen in a series of dithioether-containing pentapeptides derived from DPDPE and DPLPE, increasing the ring size in the tetrapeptide series diminishes δ receptor selectivity (4). In the pentapeptide series, however, this resulted from a large decrease in δ receptor affinity with little effect observed on μ receptor affinity. By contrast, in analogs 7-10 increasing ring size leads to greatly enhanced μ receptor binding affinity (and consequent μ receptor selectivity), particularly in 9, while δ receptor affinity is more variable. Nonetheless, the general trend previously observed, namely that more compact conformations tend to favor δ receptor binding selectivity, is supported by the data presented here. The observed changes in binding selectivities in analogs 7-10 are in agreement with the behavior of these analogs in the mvd preparation. As seen from the antagonist shifts presented in Table II, analog 7 displays high δ selectivity in the mvd while 9 and 10 act primarily on μ receptors in this assay.

We have previously proposed that the high δ receptor selectivity of DPDPE is due in part to adverse steric interactions between the β,β-dimethyl substituents of the Pen[2] residue and the receptor binding site which decrease binding affinity to δ and particularly to μ receptors (4,15). Further evidence is seen in the current series from a comparison of binding affinities of analog 2 with analog 9. In these ethylene-dithioether analogs, analog 2, which has a D-Pen residue in position 2, has 5.4-fold and 280-fold lower affinity at δ and μ receptors, respectively than does analog 9. This result, which is similar to that observed with the pentapeptides, DPDPE and DCDPE, reflects adverse steric interactions of the D-Pen[2] β,β-dimethyl groups particularly at μ receptor binding sites. It should be noted that the disulfide-containing analogs 1 and 7 show a much less impressive steric effect due to the side chain of residue 2. In this case the β,β-dimethyls of residue 2 in analog 1 result in approximately 4-fold reductions in binding affinity to both δ and μ receptors. In both analogs, μ receptor binding affinities are low suggesting that these more compact structures may lead to adverse steric (or other) interactions with the μ receptor which are not observed in more extended structures such as 9.

The design of analog 11 was, as indicated above, straightforward given the observed δ receptor selectivity and potency of the carboxamide terminal analog 7. The resulting high selectivity (similar to that of DPDPE) and high δ affinity (3.5 fold higher than that of DPDPE) displayed by analog 11 are impressive. The improved affinity may prove to be useful for in vivo experiments. Perhaps of greater significance, analog 11 provides an important tool for elucidating the optimal ligand conformation for δ opioid receptor binding. Since both 11 and DPDPE must assume similar conformations at the δ binding site, comparisons of possible

conformations for these two compounds should allow the elimination of unlikely binding conformations and greatly facilitate the determination of the optimal ligand conformation at the δ opioid receptor.

Acknowledgements

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