

Synthesis and Biological Activities of Cyclic Lanthionine Enkephalin Analogues: δ -Opioid Receptor Selective Ligands

Yosup Rew,[†] Shelle Malkmus,[‡] Camilla Svensson,[‡] Tony L. Yaksh,[‡] Nga N. Chung,[§] Peter W. Schiller,[§] Joel A. Cassel,^{||} Robert N. DeHaven,^{||} and Murray Goodman^{*,†}

Department of Chemistry and Biochemistry, University of California, San Diego, 9500 Gilman Drive, La Jolla, California 92093-0343, Department of Anesthesiology, University of California, San Diego, 9500 Gilman Drive, La Jolla, California 92093-0818, Clinical Research Institute of Montreal, 110 Pine Avenue West, Montreal, Quebec, H2W 1R7, Canada, and Adolor Corporation, 371 Phoenixville Pike, Malvern, Pennsylvania 19355

Received March 7, 2002

The synthesis and biological test results of a series of enkephalin analogues incorporating the lanthionine modification are presented. The syntheses of four monosulfide-bridged analogues of enkephalins, Tyr-c[D-Ala_L-Gly-Phe-D-Ala_L]-OH (**1a**), Tyr-c[D-Val_L-Gly-Phe-D-Ala_L]-OH (**1b**), Tyr-c[D-Ala_L-Gly-Phe-Ala_L]-OH (**1c**), and Tyr-c[D-Val_L-Gly-Phe-Ala_L]-OH (**1d**), where Ala_L and Val_L denote the lanthionine amino acid ends linked by a monosulfide bridge to form the lanthionine structure, were successfully carried out via preparation of the linear peptide on solid support and cyclization in solution. In vitro binding assays against μ -, δ -, and κ -opioid receptors and in vitro tests using GPI and MVD assays revealed that the dimethyl lanthionine analogues **1b** and **1d**, denoted as D-Val_L in position 2, show substantial selectivity toward the δ -opioid receptor, while the unsubstituted analogues **1a** and **1c**, denoted as D-Ala_L in position 2, bind to both μ - and δ -opioid receptors. The in vivo thermal escape assay by intrathecal administration showed that the analogues **1b** and **1d** are among the most potent ligands at producing antinociception through the δ -opioid receptor. The picomolar potencies of analogues **1a** and **1c** in the intrathecal (it.) assay strongly indicate that μ - and δ -opioid receptors interact synergistically to modulate the antinociceptive responses.

Introduction

The pharmacological effects of opioids are due to specific interactions of the drugs with opioid receptors. To date, three major opioid receptors (μ , δ , and κ) have been cloned and characterized.¹ Morphine and the other alkaloid opioids, currently in use for the relief of pain, are μ -opioid receptor selective agonists. Many of the serious side effects associated with morphine administration such as respiratory depression, physiological dependence, tolerance, and a reduction in gut motility are mediated by the μ -opioid receptor,² while κ -agonists show some promise as nonaddictive analgesics.³ The κ -agonists, however, are generally less efficacious as analgesics^{4,5} and have been shown to cause dysphoric side effects.⁶ The δ -opioid receptor is an attractive target that may possess potential clinical benefits including sufficient analgesic efficacy, negligible cross tolerance to morphine, reduced respiratory depression, reduced inhibition of gut motility, and minimal physical dependence.⁷ No δ -opioid receptor selective agonist that exhibits potent systemic in vivo activity has been found to date.⁸ Therefore, our goal was to develop opioid ligands with a high degree of selectivity and potency for the δ -receptor subtype.

Methionine and leucine enkephalins, identified from mammalian tissues in 1975, have been considered as endogenous peptide ligands toward the δ -opioid receptor.⁹ As with other bioactive endogenous peptides, there are several major considerations that limit the clinical applications of naturally occurring enkephalins including rapid degradation under physiological conditions, poor selectivity for receptor subtypes, modest absorption, and relatively poor central bioavailability.¹⁰ Since their discovery, exhaustive synthetic modifications of enkephalins have been conducted in an attempt to improve their biological profiles. Studies to obtain structure–activity relationships of enkephalins have revealed that two aromatic groups of Tyr and Phe and the protonated nitrogen at the N terminus act as pharmacophores when enkephalins interact with the receptors.¹¹ Changes in the amino acid composition of these endogenous peptides can lead to compounds with high potency and selectivity for the δ -receptor, such as DADLE ([D-Ala², D-Leu⁵]-enkephalin),¹² BUBU [Tyr-D-Ser(OtBu)-Gly-Phe-Leu-Thr(OtBu)],¹³ and the cyclic peptide DPDPE ([D-Pen², D-Pen⁵]-enkephalin).¹⁴ These and other δ -receptor selective peptides have been used for in vitro studies, but their metabolic instability and/or their relatively poor distribution properties and low in vivo potency have limited their usefulness.

Lanthionines are monosulfide analogues of cystine and are key constituents of bioactive peptide antibiotics. They can act as food preservatives, antibacterials, immunostimulants, and antitumor agents.^{15–18} For

* To whom correspondence should be addressed. Phone: (858) 534-4466. Fax: (858) 534-0202. E-mail: mgoodman@ucsd.edu.

[†] Department of Chemistry and Biochemistry, University of California, San Diego.

[‡] Department of Anesthesiology, University of California, San Diego.

[§] Clinical Research Institute of Montreal.

^{||} Adolor Corporation.

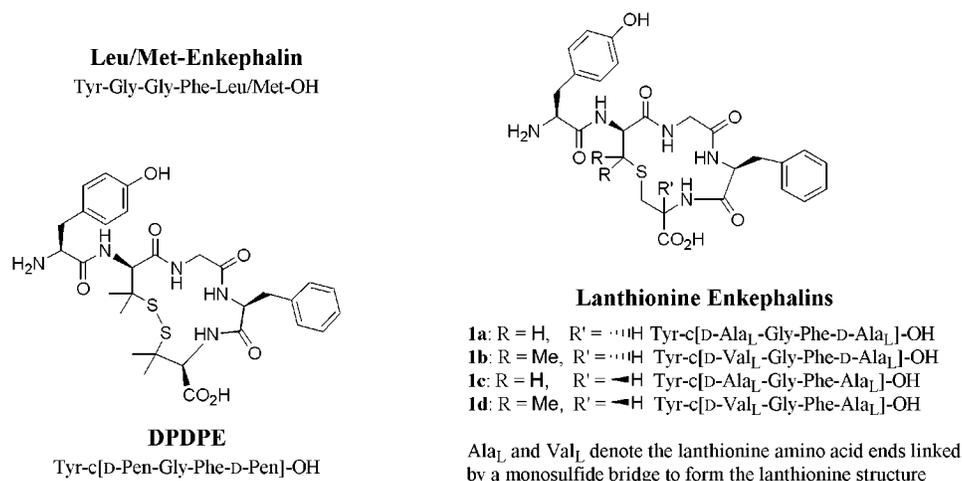
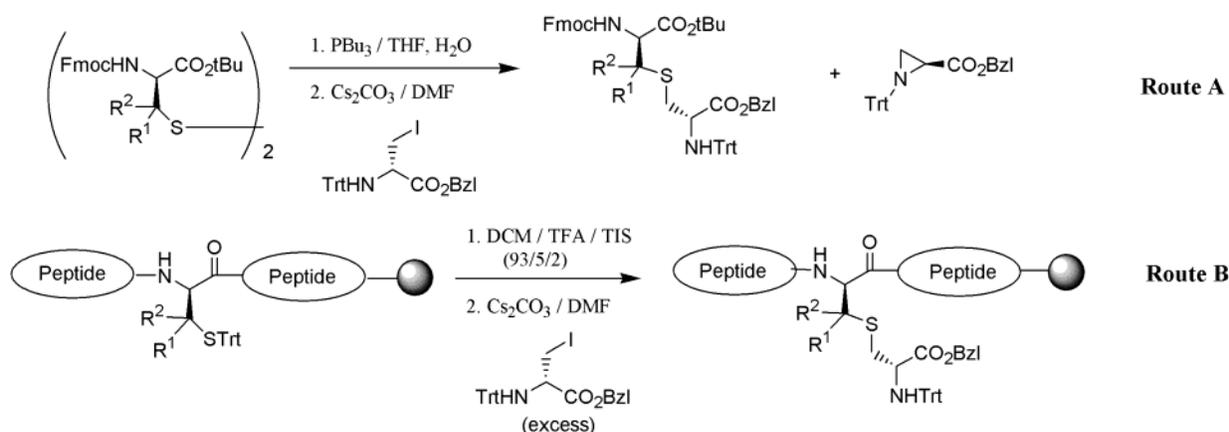


Figure 1. Enkephalins, DPDPE, and lanthionine enkephalin analogues.

Scheme 1. Synthesis of Lanthionine Building Blocks



some time, our group has pursued the introduction of the lanthionine structure as novel peptidomimetics into various drug families, i.e., sandostatin,¹⁹ cell adhesion modulator,²⁰ and somatostatin.^{21–23} The monosulfide bridge of lanthionine provides more constrained peptide structures with greater stability toward enzymatic degradation compared to the labile disulfide bridge of cystine in natural or unnatural cyclic peptide sequences.

Molecular modeling and X-ray diffraction studies of DPDPE indicated that the δ -selectivity of DPDPE is due to the presence of the *gem*-dimethyl group in the 2 position, which causes more severe steric interference at the μ -receptor than at the δ -receptor.^{24,25} On the basis of this finding, we designed and synthesized a series of enkephalin analogues incorporating the lanthionine modification (**1a–d**, Figure 1).^{26–28}

In this paper we present novel routes for the synthesis of lanthionine enkephalin analogues and the results of biological assays (in vitro opioid binding assays, in vitro GPI and MVD assays, and in vivo thermal escape assay by intrathecal (it.) administration).

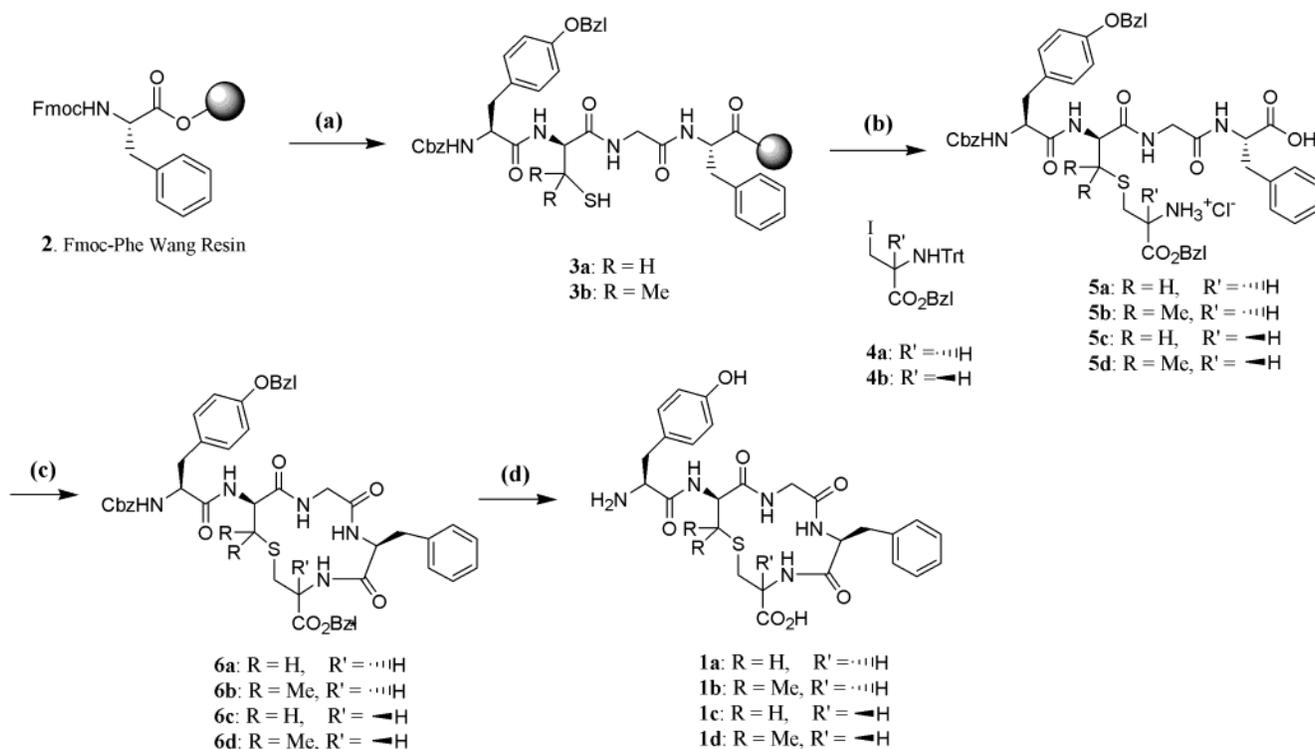
Results and Discussion

Design and Synthesis of Target Peptides. To compare the biological activities and stabilities of the lanthionine analogues with DPDPE, we synthesized lanthionine enkephalin analogues with a minimal change of the spatial arrangement of the pharmacophoric groups (Figure 1). We believe that the replacement of a

disulfide with a thioether side chain linkage and the consequent reduced ring size will lead to more stable and selective opioids.

Previously, we reported the synthesis of similar lanthionine enkephalin analogues by several methods that have been developed to prepare synthetic lanthionines or natural lantibiotics including sulfur extrusion and biomimetic Michael addition of cysteine to dehydroalanine.^{29,30} Unfortunately, inherent problems such as lack of regioselectivity and epimerization made these methods impractical for the development of a generalized synthetic route for the target lanthionine analogues. To overcome the lack of stereoselectivity, we developed a facile synthesis of orthogonally protected lanthionine using the β -lactone³¹ developed by Vederas et al.³² Because this route did not provide a building block in a suitably protected form for Fmoc solid-phase peptide synthesis,³³ the target lanthionine enkephalins were synthesized in solution.³⁴ Although a preliminary report of the synthesis of lanthionine opioids has appeared, the method of synthesis in solution led to serious problems of yield and optical purity of the target structures.

Recently, the synthesis of Fmoc-protected lanthionines for solid-phase peptide synthesis was reported by Ménez (Scheme 1, route A).³⁵ The *N*-trityl-3-iodoalanine derivatives were used to avoid epimerization at the α -carbon or β -elimination. However, when the reaction to form the lanthionine was carried out in solution, a

Scheme 2. Synthesis of Lanthionine Enkephalin Analogues^a

^a (a) (i) 20% piperidine in NMP, (ii) Trt-Gly-OH, PyBOP, HOBT, DIEA, DMF, (iii) DCM/TFA/TIS (97/2/1), (iv) preactivated Fmoc-D-Cys(Trt)-OH (for **3a**) or Fmoc-D-Pen(Trt)-OH (for **3b**) with DIC, DIEA, DMF/DCM (1/1), (v) 20% piperidine in NMP, (vi) Cbz-Tyr(Bzl)-OH, PyBOP, HOBT, DIEA, DMF, (vii) DCM/TFA/TIS (94/3/3); (b) (i) **4a** or **4b**, Cs₂CO₃, DMF, (ii) TFA/H₂O/TIS (93/5/2), (iii) 4 N HCl/dioxane; (c) DPPA, NaHCO₃, DMF, 0 °C, 72 h; (d) (i) HBr/AcOH, room temp, 4 h, (ii) purification by RP-HPLC.

significant amount of undesired aziridine was obtained (up to 35%). As a result, we applied this method to solid-phase reactions in which any aziridine formed was removed by washing the solid support (Scheme 1, route B). The target lanthionine enkephalin analogues (**1a–d**) were successfully synthesized via preparation of the linear peptide on the solid support and cyclization in solution (Scheme 2).^{26–28} Synthesis of analogue **1a** commenced with commercially available Fmoc-Phe Wang resin **2**. The tetrapeptide chain **3** was initially assembled on the Wang resin. The Fmoc-Phe Wang resin **2** was allowed to react with Trt-Gly-OH using PyBOP/HOBT, after deprotection of the Fmoc group with 20% piperidine in NMP. The Trt-Gly-Phe Wang resin was then deprotected with CH₂Cl₂/TFA/TIS (97/2/1, v/v/v) and coupled with Fmoc-D-Cys(Trt)-OH, which was preactivated with DIC and HOBT under neutral conditions to prevent diketopiperazine formation and epimerization.^{36,37} The Fmoc protecting group of the tripeptide resin was removed by 20% piperidine in DMF, and Cbz-Tyr(Bzl)-OH was then coupled to the resulting peptide resin using PyBOP/HOBT. After removal of the trityl protecting group of cysteine by CH₂Cl₂/TFA/TIS (94/3/3, v/v/v), the lanthionine linkage was introduced by reaction of *N*-trityl-D-3-iodoalanine benzyl ester **4a** with the tetrapeptide resin **3a** in the presence of Cs₂CO₃ in DMF. The cleavage of the pentapeptide **5a** from the resin and the removal of the Trt protecting group were achieved simultaneously by TFA/H₂O/TIS (93/5/3, v/v/v). The resulting crude peptide was cyclized in dilute DMF solution in the presence of DPPA and NaHCO₃. Purification of the less polar desired cyclic peptide from polar impurities was effected by silica gel column

chromatography, followed by removal of benzyloxycarbonyl, benzyl ether, and benzyl ester protecting groups by 30% HBr/AcOH. Final purification by RP-HPLC provided the target peptide **1a**. The other target peptides (**1b–d**) were synthesized by this synthetic route.

Biological Results. The synthesized lanthionine enkephalin analogues were evaluated for their in vitro binding affinities at μ -, δ -, and κ -opioid receptors by measuring the inhibition of binding of [³H]-diprenorphine to cloned human opioid receptors expressed in CHO cell membranes (Table 1). Morphine and DPDPE were used as reference compounds. As shown in Table 1, analogues **1b** and **1d**, which have a *gem*-dimethyl group in the β -position of D-Ala₁² at the second residue, are δ -opioid receptor selective peptides, while analogues **1a** and **1c**, lacking the *gem*-dimethyl group in position 2, bind with high affinity to both μ - and δ -receptors. The lanthionine enkephalin analogues bind to the δ -receptor with affinities ranging from 0.63 to 2.0 nM and thus are slightly more potent than DPDPE. The binding affinity for the μ -receptor of analogue **1b** is reduced 320-fold relative to that of the parent compound **1a**, whereas analogue **1d** has a 60-fold-reduced μ -affinity compared with analogue **1c**. Analogue **1b** is the most δ -selective ligand among these analogues.

The functional in vitro activities of the analogues were determined by measuring the inhibition of electrically evoked contractions of isolated muscle preparations. The guinea pig ileum (GPI) contains μ - and κ -opioid receptors, of which 70% are μ -receptors, and the mouse vas deferens (MVD) contains μ - and δ -opioid receptors, of which 80% are δ -receptors. The resulting IC₅₀ values determined in the GPI and MVD assays delineate the

Table 1. In Vitro Binding Affinities of Lanthionine Enkephalin Analogues at Cloned Human μ -, δ -, and κ -Opioid Receptors in CHO Cell Membranes^a

analogues	K_i (nM)			K_i ratio μ/δ
	μ	δ	κ	
1a Tyr-c[D-Ala _L -Gly-Phe-D-Ala _L]-OH	2.0 (1.8–2.2) <i>n</i> = 11	2.0 (1.7–2.3) <i>n</i> = 5	1600 (1400–1900) <i>n</i> = 7	1
1b Tyr-c[D-Val _L -Gly-Phe-D-Ala _L]-OH	630 (580–680) <i>n</i> = 3	0.93 (0.35–2.5) <i>n</i> = 3	>10 000	677
1c Tyr-c[D-Ala _L -Gly-Phe-Ala _L]-OH	2.3 (1.3–3.7) <i>n</i> = 5	0.63 (0.62–0.65) <i>n</i> = 4	6400 (4700–8500) <i>n</i> = 4	3.7
1d Tyr-c[D-Val _L -Gly-Phe-Ala _L]-OH	130 (110–150) <i>n</i> = 4	0.79 (0.66–0.95) <i>n</i> = 4	>1000	165
DPDPE	>10 000	2.2 (1.0–3.9) <i>n</i> = 5	>10 000	>4550
morphine	17 (6.8–43) <i>n</i> = 4	150 (63–360) <i>n</i> = 4	260 (100–690) <i>n</i> = 3	0.11

^a The K_i values are presented with 95% confidence intervals and the number (*n*) of determinations. Reference ligand is [³H]-diprenorphine.

Table 2. In Vitro and In Vivo Bioactivities of Lanthionine Enkephalin Analogues

analogues	IC ₅₀ ^a [nM]		IC ₅₀ ratio GPI/MVD	ED ₅₀ ^d [nM]
	GPI ^b	MVD ^c		
1a Tyr-c[D-Ala _L -Gly-Phe-D-Ala _L]-OH	0.56 ± 0.037 <i>n</i> = 3	1.58 ± 0.13 <i>n</i> = 3	0.35	0.0015 (0.0010–0.0024) <i>n</i> = 24
1b Tyr-c[D-Val _L -Gly-Phe-D-Ala _L]-OH	730 ± 136 <i>n</i> = 3	2.33 ± 0.02 <i>n</i> = 3	313	0.26 (0.17–0.41) <i>n</i> = 24
1c Tyr-c[D-Ala _L -Gly-Phe-Ala _L]-OH	1.06 ± 0.15 <i>n</i> = 5	0.35 ± 0.04 <i>n</i> = 5	3.02	0.0018 (0.0010–0.0034) <i>n</i> = 20
1d Tyr-c[D-Val _L -Gly-Phe-Ala _L]-OH	82.0 ± 11.5 <i>n</i> = 5	0.26 ± 0.03 <i>n</i> = 5	315	0.12 (0.04–0.33) <i>n</i> = 13
DPDPE	7300 ± 1700 <i>n</i> = 3	4.1 ± 0.5 <i>n</i> = 3	1780	130 (98–163) <i>n</i> = 24
morphine	58.8^e	644^e	0.09^e	15 (10–24) <i>n</i> = 30

^a IC₅₀ values are given ±SEM with number (*n*) of determinations. ^{b,c} GPI for μ receptor and MVD for δ receptor. ^d ED₅₀ values are presented with 95% confidence intervals and the number (*n*) of determinations. ^e Data taken from reference 60.

opioid activities of the synthesized analogues at the μ - and δ -receptors, respectively. The ratio of the IC₅₀ values in the GPI versus the MVD therefore provides a functional index of the μ - or δ -selectivity (Table 2). The compounds displayed a wide range of potencies in the GPI assay, from IC₅₀ values of 0.56 and 1.06 nM for analogues **1a** and **1c**, respectively, to IC₅₀ values of 730 and 82 nM for analogues **1b** and **1d**, respectively. In contrast, the IC₅₀ values of these lanthionine analogues displayed a narrow range of potencies in the MVD assay from 0.26 to 2.33 nM. These data indicated that analogues **1a** and **1c** had low nanomolar potencies at both μ - and δ -receptors. On the other hand, the dimethylated analogues **1b** and **1d** maintained the high potency of analogues **1a** and **1c** toward the δ -receptor while showing much decreased μ -activities. These results are consistent with the binding assay data. Analogue **1b**, which has a D residue at the fifth position,

showed reduced μ -potency with the same δ -potency (greater selectivity for the δ -receptor) compared to analogue **1d**, which possesses an L residue at the fifth position.

Previous work demonstrated the presence of μ - and δ -receptors in the spinal dorsal horn,³⁸ and the activation of each results in antinociception.³⁹ The thermal escape latency assay with it. drug administration measures the magnitude of the spinal antinociceptive effect. The results are reported as the dosage necessary to produce a half-maximum effect (ED₅₀) (Table 2). All agents examined produced a dose-dependent increase in the thermal escape latency with a maximum elevation produced by the highest doses of all drugs. At doses where a maximum thermal escape latency was observed, there was no concomitant change in motor function or general behavior. All of the lanthionine analogues had much lower ED₅₀ values than DPDPE

or morphine. The ED₅₀ values for **1a** and **1c** were 0.0015 and 0.0018 nM. Dimethylated analogues were less potent, and the ED₅₀ values were 0.26 nM for **1b** and 0.12 nM for **1d**. The ED₅₀ values of analogues **1a** and **1c** indicate that these compounds were almost 10⁶ times more potent than DPDPE and 10⁴ times more potent than morphine. The dimethylated lanthionine analogues **1b** and **1d**, which are δ -selective agonists, exhibited ED₅₀ values that reflect potencies 60–120 times greater than morphine as well as nearly 10³ times greater than DPDPE.

The enzymatic degradation studies of **1b** and DPDPE have been carried out by incubating the peptides in the rat brain homogenates solution for 24 h.⁴⁰ The results indicate that both **1b** and DPDPE are too stable to establish a quantitative difference between them. The extraordinary resistances is presumably caused by the presence of a β,β -dimethyl group in the position 2 or position 5, which stabilizes the compound toward enzymatic degradation. A previous study of enzymatic degradation of a similar lanthionine enkephalin amide, which does not have a β,β -dimethyl group in the position 2, has shown that the lanthionine enkephalinamide Tyr-c[D-Ala_L-Gly-Phe-Ala_L]-NH₂ is approximately 5 times more stable than the corresponding disulfide analogue Tyr-c[D-Cys-Gly-Phe-Cys]-OH toward degradation by rat brain homogenates.⁴¹ The enhanced potencies of the δ -selective lanthionine analogues **1b** and **1d** over DPDPE can be explained in part by improved biostability of the lanthionine structure compared with the disulfide bond.

It is important to stress that spinal δ -receptors can mediate significant antinociception. Thus, spinal delivery of agents with high δ -selectivity as defined by binding and in vitro bioassays (e.g., analogues **1b** and **1d**) can yield potent analgesia. To determine the role of the δ -opioid receptor in mediating the spinal action of the analogue **1b**, we examined the effects of the δ -receptor-preferring antagonist naltrindole, given spinally (30 μ g/10 μ L) on the antinociception produced by a just maximally effective dose of intrathecal **1b** (30 μ g). Alone, this agonist produced a maximum possible effect (MPE) of 92 \pm 9%. Following pretreatment with naltrindole, the MPE was significantly reduced to 23 \pm 4%; $P < 0.05$; $n = 5$. In contrast, this antagonist treatment had no effect on the antinociception produced by intrathecal morphine (data not shown).⁴⁰

Studies in recombinant mice, where expression of the μ -opioid receptor was disrupted, demonstrated that δ -opioid receptor selective agonists do not require functional μ -receptors to mediate antinociception.⁴² While spinal δ -agonists are clearly distinct in their pharmacology from μ -opioid agonists,⁴³ the role played by the δ -opioid receptor is clearly complicated. For instance, while the effects of δ -selective agonists are antagonized by naltrindole and those of μ -agonists are not, CTAP, a μ -selective antagonist, antagonizes the effects of intrathecally administered μ -agonists and DPDPE, a δ -selective agonist, with the same potency.⁴⁴ It has also been reported that δ -receptor-mediated analgesia is partially decreased in μ -opioid receptor deficient mice.⁴⁵

These observations are consistent with synergistic interaction between μ - and δ -opioid receptors and could

account for the potency of analogues **1a** and **1c** relative to μ -agonists such as morphine and DAMGO. Because of its potency for the three δ -preferring ligands (**1b**, **1d**, and DPDPE) in this study and other μ/δ -peptidomimetics previously studied covary with the in vitro affinity and the activity for both the μ - and δ -receptors, we cannot exclude the possibility that some of their apparent potency also reflects a minimal μ -contribution to their apparent antinociceptive potency.

Conclusion

Synthesis of the target lanthionine analogues (**1a**–**1d**) was accomplished by the reaction of thiol with *N*-trityl-D- or *N*-trityl-L-3-iodoalanine benzyl ester on a solid support. As shown by the data for in vitro binding affinity, the β,β -dimethylation at the second residue led to a dramatic decrease in binding affinity at the μ -receptor for both **1b** and **1d** analogues without altering δ binding affinity. Furthermore, the K_i value at the μ -receptor for **1b**, which has a D-Ala_L⁵ substitution, is 5-fold higher at the μ -receptor and decreases 5 times more than that of **1d**, which has an Ala_L⁵ substitution. The functional assay results from GPI and MVD assays parallel the results from the binding assay. All the lanthionine analogues had much lower antinociceptive ED₅₀ values than DPDPE and were also more potent than morphine after spinal delivery. The subnanomolar analgesic potencies of δ -selective lanthionine analogues may be due to their potent δ -activity and improved stability. The picomolar potencies of nonselective lanthionine analogues is consistent with the existence of synergistic interactions between spinal μ - and δ -receptors. The previous work systemically examining spinal μ - and δ -interactions have indeed demonstrated such synergy. Thus, the lanthionine-bridged enkephalins represent a new family of interesting δ -receptor selective opioid agonists.

Further biological studies (ip administration, agonist and antagonist tests) and acid stabilities of these lanthionine enkephalin analogues for systemic availability will be reported elsewhere soon,⁴⁰ and the conformational analyses of these lanthionine analogues by NMR are underway.

Experimental Section

General Procedures and Notes. NMR spectra were obtained on Varian HG-400 (400 MHz) and Bruker AMX 500 (500 MHz) spectrometers. Chemical shifts (δ) are reported in parts per million (ppm) relative to residual undeuterated solvent as an internal reference. The following abbreviations were used to explain the multiplicities: s, single; d, doublet; t, triplet; q, quartet; dd, doublet of doublets; dt, doublet of triplets; m, multiplet; b, broad. Optical rotations were recorded on a Perkin-Elmer 241 polarimeter. IR spectra were recorded on a Nicolet Magna-IR 550 series II spectrometer. Mass spectroscopic analyses were carried out by the facility at The Scripps Research Institute, La Jolla, CA. The final products were purified and analyzed by RP-HPLC using Vydac protein peptide C₁₈ columns. Column dimensions were 4.5 mm \times 250 mm (90 Å silica, 5 μ m) for analytical HPLC and were 22 mm \times 250 mm (90 Å silica, 10 μ m) for preparative HPLC. UV absorbance was monitored at 220 nm. A binary system of water and acetonitrile, both containing 0.1% TFA, was used throughout. Two analytical HPLC profiles were obtained on a Waters Millennium PDA system using both a linear gradient of 10–50% acetonitrile over 30 min (condition A, t_R^a) and an isocratic elution of 16% acetonitrile at 1 mL/min flow rate

(condition B, t_R^b). Preparative HPLC was carried out at 10 mL/min flow rate using condition A, and the materials so obtained were further purified by condition B in the aforementioned system.

All reagents were purchased from Aldrich Chemical unless otherwise indicated. DMF was purchased from Fisher Scientific and treated with sodium aluminosilicate molecular sieves (4 Å nominal pore diameter) obtained from Sigma and with Amberlite IR 120(plus) cation-exchange resin. CH_2Cl_2 was distilled from calcium hydride. NMP was purchased from Applied Biosystems and used without further purification. Protected amino acids, Fmoc-Phe Wang resin, and PyBOP were purchased from Novabiochem. Fmoc-D/L-Pen(Trt)-OH were purchased from BACHEM Americas. Trt-D/L-Ser-OBzl were prepared according to the method of Nakajima et al.⁴⁶ The reactions were monitored by thin-layer chromatography carried out on 0.25 mm E. Merck silica gel plates (60F-254) using UV light as the visualizing agent and 7% ethanolic phosphomolybdic acid and heat as the developing agent. The Kaiser test⁴⁷ was used as the qualitative test for the presence or absence of free amino groups, and the Ellman test^{48,49} was employed for the detection of a free thiol group during reactions on solid support.

N-Trt-D/L-3-iodoalanine Benzyl Ester (4a/4b). To a solution of Trt-D-Ser-OBzl (8.16 g, 18.7 mmol), Ph_3P (6.87 g, 26.2 mmol), and imidazole (1.79 g, 26.2 mmol) in acetonitrile/ether (2/1, v/v, 90 mL) was added I_2 (6.63 g, 52.5 mmol) slowly at 0 °C. After the reaction was stirred for 1 h (monitored by TLC) at room temperature, the reaction was suspended in water (300 mL) and extracted with EtOAc (3 × 200 mL). The combined organic layers were rinsed with saturated aqueous $\text{Na}_2\text{S}_2\text{O}_3$ solution (300 mL), saturated aqueous CuSO_4 solution (300 mL), and brine (300 mL), dried over MgSO_4 , and concentrated under reduced pressure. The resulting reaction mixture was triturated with *n*-hexane/ether (2/1, v/v) and filtered to remove the triphenylphosphine oxide. The filtrate was then concentrated under reduced pressure. Flash chromatography (*n*-hexane/EtOAc, 15/1 to 9/1, v/v) afforded **4a** as a pale-yellow paste (9.52 g, 17.4 mmol, 93%): $R_f = 0.70$ (*n*-hexane/EtOAc, 4/1, v/v); $[\alpha]_D^{25} - 14^\circ$ (*c* 1.06, CHCl_3); $^1\text{H NMR}$ (CDCl_3) δ 7.50 (bd, $J = 7.2$ Hz, 6H), 7.40–7.17 (m, 14 H), 4.79 (d, $J = 12.0$ Hz, 1H), 4.60 (d, $J = 12.0$ Hz, 1H), 3.54 (m, 1H), 3.32 (dd, $J = 9.6$ and 3.2 Hz, 1H), 3.30 (dd, $J = 9.6$ and 7.2 Hz, 1H), 2.91 (d, $J = 10.0$ Hz, 1H); $^{13}\text{C NMR}$ (CDCl_3) δ 171.8, 145.4, 134.9, 128.5–126.4 (complex), 71.1, 67.2, 56.2, 9.9; MS (ESI) 570 $[\text{M} + \text{Na}]^+$; HRMS(MALDI) $[\text{M} - \text{I}]^+$ calcd for $\text{C}_{29}\text{H}_{26}\text{NO}_2$ 420.1963, found 420.1962; IR (cm^{-1}) 3318, 2059, 3032, 1733, 1490, 1455, 1210, 1167, 746, 698. **4b** was prepared as described above for **4a** but starting with Trt-Ser-OBzl to provide **4b** (95% yield) as a pale-yellow paste: $[\alpha]_D^{25} + 14.7^\circ$ (*c* 0.37, chloroform); $[\alpha]_D^{25}$ from the reference is $+5^\circ$.⁵⁰

Tyr-c[D-Ala₁-Gly-Phe-D-Ala₁]-OH (1a). The Fmoc-Phe Wang resin **2** (1.0 g, 1.0 mmol/g) was treated with 20% piperidine in NMP (10 mL) for 10 min and was washed sequentially with CH_2Cl_2 , MeOH, CH_2Cl_2 , and DMF. The resin in DMF (10 mL) was allowed to react with Trt-Gly-OH (2.5 equiv), PyBOP (2.5 equiv), HOBT (1.0 equiv), and DIEA (4 equiv) for 12 h. After being washed with DMF, MeOH, and CH_2Cl_2 , the resin product was treated twice with $\text{CH}_2\text{Cl}_2/\text{TFA}/\text{TIS}$ (97/2/1, v/v/v, 10 mL) for 5 min and then was washed again with CH_2Cl_2 , MeOH, CH_2Cl_2 , and DMF. The washed resin was suspended in DMF/ CH_2Cl_2 (1/1, v/v, 15 mL) and was treated with a solution of Fmoc-D-Cys(Trt)-OH (2.5 equiv), which was preactivated with DIC (2.5 equiv) and HOBT (2.5 equiv) in CH_2Cl_2 (5 mL) and 2,6-lutidine (1 equiv) for 12 h. After being washed with DMF, MeOH, CH_2Cl_2 , and NMP, the resin was treated with 20% piperidine in NMP (10 mL) for 20 min and was washed (CH_2Cl_2 , MeOH, CH_2Cl_2 , and DMF). The washed resin was suspended once more in DMF (10 mL), treated with Cbz-Tyr(Bzl)-OH (2.5 equiv), PyBOP (2.5 equiv), HOBT (1.0 equiv), and DIEA (4.5 equiv) for 12 h, and then washed successively with DMF, MeOH, and CH_2Cl_2 . The resin was treated twice with $\text{CH}_2\text{Cl}_2/\text{TFA}/\text{TIS}$ (94/3/3, v/v/v, 10 mL) for 5 min and then was washed with CH_2Cl_2 , MeOH, CH_2Cl_2 , and

DMF to yield the tetrapeptide resin, which contained a free thiol group **3a**.

To an argon-agitated suspension of resin **3a** and CsCO_3 (4 equiv) in DMF was added *N*-Trt-D-3-iodoalanine benzyl ester **4a** (3 equiv), and this was agitated for 8 h. After successive washings with DMF, H_2O , MeOH, benzene, and CH_2Cl_2 , the resin was treated with TFA/ $\text{H}_2\text{O}/\text{TIS}$ (93/5/2, v/v/v, 15 mL) for 2 h. The filtrate from the cleavage reaction was collected and combined with TFA washes (2 × 10 mL) of the cleaved peptide resin. After addition of 4 N HCl in dioxane (1 mL), concentration of the combined filtrates under reduced pressure, precipitation in *n*-hexane/IPE (1/1, v/v, 10 mL), and centrifugation yielded a crude linear intermediate **5a** (310 mg, 0.33 mmol) $[\text{MS}(\text{ESI}) 890 [\text{M} + \text{H}]^+$, 912 $[\text{M} + \text{Na}]^+$] as a yellow solid, which was used without further purification in the next step.

Crude intermediate **5a** (310 mg, 0.33 mmol) was dissolved in DMF (130 mL), and NaHCO_3 (280 mg, 3.3 mmol) was added. The solution was cooled to -20 °C under Ar, and DPPA (0.280 mL, 1.3 mmol) was added. The reaction mixture was then slowly allowed to reach 0 °C, and stirring was continued for 72 h. The solvent was removed under reduced pressure, and flash chromatography ($\text{CH}_2\text{Cl}_2/\text{MeOH}$, 100/1 to 60/1, v/v) afforded a crude cyclic intermediate (88 mg) $[\text{MS}(\text{ESI}) 894 [\text{M} + \text{Na}]^+$, 906 $[\text{M} - \text{Cl}]^-]$ as an oily solid, which was used without further purification in the next step.

To the protected cyclic peptide **6a** (88 mg) was added 30% HBr/AcOH (5 mL) at 0 °C under Ar. After the reaction mixture was stirred for 2 h at room temperature, precipitation in *n*-hexane/IPE (1/1, v/v, 10 mL) and centrifugation yielded the target compound **1a** (45 mg, 0.07 mmol; overall yield based on the Fmoc Wang resin is 7%). Compound **1a** was further purified by RP-HPLC as described in General Procedures and Notes: $^1\text{H NMR}$ ($\text{DMSO}-d_6$) δ 9.33 (s, 1H, OH), 9.00 (dd, $J = 7.2$ and 4.0 Hz, 1H, Gly³NH), 8.84 (d, $J = 7.6$ Hz, 1H, D-Ala²NH), 8.30 (d, $J = 8.4$ Hz, 1H, Phe⁴NH), 8.06 (bs, 3H, Tyr¹NH₃), 7.35 (d, $J = 5.2$ Hz, 1H, D-Ala⁵NH), 7.25–7.18 (m, 5H, Phe⁴H_{Ar}), 7.01 (d, $J = 8.4$ Hz, 2H, Tyr¹H_{2,6}), 6.71 (d, $J = 8.4$ Hz, 2H, Tyr¹H_{3,5}), 4.61 (m, 1H, D-Ala²H_α), 4.39 (m, 1H, Phe⁴H_α), 4.00 (m, 2H, Gly³H_α, Tyr¹H_α), 3.46–3.20 (m, 4H, D-Ala⁵H_α, D-Ala⁵H_{β2}, Gly³H_α), 3.12 (dd, $J = 14.0$ and 5.2 Hz, 1H, Phe⁴H_β), 2.93 (dd, $J = 14.0$ and 6.0 Hz, 1H, Tyr¹H_β), 2.85–2.71 (m, 4H, Phe⁴H_β, D-Ala²H_{β2}, Tyr¹H_β); MS (ESI) 558 $[\text{M} + \text{H}]^+$, 580 $[\text{M} + \text{Na}]^+$, 556 $[\text{M} - \text{H}]^-$; HRMS (MALDI) $[\text{M} + \text{H}]^+$ calcd for $\text{C}_{26}\text{H}_{31}\text{N}_5\text{O}_7\text{S}$ 558.2017, found 558.2015; RP-HPLC $t_R^a = 14.39$, $t_R^b = 9.34$.

Tyr-c[D-Val₁-Gly-Phe-D-Ala₁]-OH (1b). The target compound (**1b**) was obtained using the described procedure for the synthesis of compound **1a** with Fmoc-D-Pen(Trt)-OH and **4a**. During the synthesis, intermediates **5b** and **6b** were confirmed by mass spectroscopy and used without further purification in the next steps as described above. **5b**: MS (ESI) 918 $[\text{M} + \text{H}]^+$, 916 $[\text{M} - \text{H}]^-$. **6b**: $R_f = 0.25$ ($\text{CH}_2\text{Cl}_2/\text{MeOH}$, 15/1, v/v); MS (ESI) 900 $[\text{M} + \text{H}]^+$, 922 $[\text{M} + \text{Na}]^+$, 934 $[\text{M} + \text{Cl}]^-$, 1012 $[\text{M} + \text{TFA}]^-$.

The analytical results for compound **1b** are as follows: $^1\text{H NMR}$ ($\text{DMSO}-d_6$) δ 9.32 (s, 1H, OH), 8.84 (d, $J = 7.2$ Hz, 1H, Phe⁴NH), 8.81 (dd, $J = 8.0$ and 2.4 Hz, 1H, Gly³NH), 8.71 (d, $J = 8.8$ Hz, 1H, D-Val²NH), 8.00 (b, 3H, Tyr¹NH₃), 7.43 (d, $J = 7.2$ Hz, 1H, D-Ala⁵NH), 7.29–7.17 (m, 5H, Phe⁴H_{Ar}), 7.09 (d, $J = 8.0$ Hz, 2H, Tyr¹H_{2,6}), 6.70 (d, $J = 8.4$ Hz, 2H, Tyr¹H_{3,5}), 4.71 (d, $J = 9.2$ Hz, 1H, D-Ala²H_α), 4.31–4.13 (m, 3H, D-Ala⁵H_α, Phe⁴H_α, Gly³H_α), 3.25–3.06 (m, 4H, D-Ala⁵H_{β2}, Gly³H_α, Phe⁴H_β), 2.94 (dd, $J = 14.0$ and 6.0 Hz, 1H, Tyr¹H_β), 2.84 (dd, $J = 14.0$ and 11.6 Hz, 1H, Phe⁴H_β), 2.78 (dd, $J = 13.6$ and 8.4 Hz, 1H, Tyr¹H_β), 1.16 (s, 3H, D-Val²H₃), 1.07 (s, 3H, D-Val²H₃); MS (ESI) 586 $[\text{M} + \text{H}]^+$, 608 $[\text{M} + \text{Na}]^+$, 630 $[\text{M} + 2\text{Na}]^+$, 584 $[\text{M} - \text{H}]^-$; HRMS (MALDI) $[\text{M} + \text{Na}]^+$ calcd for $\text{C}_{28}\text{H}_{35}\text{N}_5\text{O}_7\text{S}$ 608.2149, found 608.2141; RP-HPLC $t_R^a = 15.05$, $t_R^b = 10.36$.

Tyr-c[D-Ala₁-Gly-Phe-Ala₁]-OH (1c). The target compound was obtained using the described procedure for the synthesis of compound **1a** with Fmoc-D-Cys(Trt)-OH and **4b**. During the synthesis, intermediates **5c** and **6c** were confirmed by mass spectroscopy and used without further purification

in the next steps as described above. **5c**: MS(ESI) 890 [M + H]⁺, 912 [M + Na]⁺. **6c**: $R_f = 0.21$ (CH₂Cl₂/MeOH, 15/1, v/v); MS(ESI) 894 [M + Na]⁺, 906 [M + Cl]⁻.

The analytical results for compound **1c** are as follows: ¹H NMR (DMSO-*d*₆) δ 9.37 (s, 1H, OH), 8.90 (dd, $J = 7.6$ and 3.6 Hz, 1H, Gly³NH), 8.72 (d, $J = 8.4$ Hz, 1H, D-Ala²NH), 8.40 (d, $J = 9.2$ Hz, 1H, Phe⁴NH), 8.09 (bs, 3H, Tyr¹NH₃), 7.39 (d, $J = 8.0$ Hz, 1H, Ala⁵NH), 7.26–7.15 (m, 5H, Phe⁴H_{Ar}), 7.00 (d, $J = 8.8$ Hz, 2H, Tyr¹H_{2,6}), 6.71 (d, $J = 8.4$ Hz, 2H, Tyr¹H_{3,5}), 4.50 (m, 2H, d-Ala²H_α, Phe⁴H_α), 4.35 (m, 1H, Ala⁵H_α), 4.02 (dd, $J = 14.0$ and 8.0 Hz, 1H, Gly³H_α), 3.93 (m, 1H, Tyr¹H_α), 3.24 (dd, $J = 13.6$ and 3.6 Hz, 1H, Gly³H_β), 3.14 (dd, $J = 14.0$ and 4.8 Hz, 1H, Phe⁴H_β), 3.02 (dd, $J = 12.0$ and 9.2 Hz, 1H, Ala⁵H_β), 2.92 (dd, $J = 12.0$ and 3.6 Hz, 1H, Ala⁵H_β), 2.88–2.77 (m, 3H, Tyr¹H_β, Phe⁴H_β), 2.65 (dd, $J = 12.8$ and 4.4 Hz, 1H, D-Ala²H_β), 2.35 (dd, $J = 12.8$ and 10.0 Hz, 1H, D-Ala²H_β); MS(ESI) 580 [M + Na]⁺, 556 [M – H]⁻; HRMS (MALDI) [M + Na]⁺ calcd for C₂₆H₃₁N₅O₇S 580.1842, found 580.1846; RP-HPLC $t_R^a = 13.81$, $t_R^b = 8.38$.

Tyr-c[D-Val¹-Gly-Phe-Ala¹]-OH (1d). The target compound was obtained using the described procedure for the synthesis of compound **1a** with Fmoc-D-Pen(Trt)-OH and **4b**. During the synthesis, intermediates **5d** and **6d** were confirmed by mass spectroscopy and used without further purification in the next steps as described above. **5d**: MS(ESI) 918 [M + H]⁺, 916 [M – H]⁻. **6d**: $R_f = 0.28$ (CH₂Cl₂/MeOH, 15/1, v/v); MS(ESI) 900 [M + H]⁺, 922 [M + Na]⁺, 898 [M – H]⁻, 934 [M + Cl]⁻.

The analytical results for compound **1d** are as follows: ¹H NMR (DMSO-*d*₆) δ 9.34 (s, 1H, OH), 8.75 (d, $J = 9.2$ Hz, 1H, D-Val²NH), 8.69 (dd, $J = 8.8$ and 1.6 Hz, 1H, Gly³NH), 8.29 (d, $J = 8.0$ Hz, 1H, Phe⁴NH), 8.05 (s, 3H, Tyr¹NH₃), 7.39 (d, $J = 9.2$ Hz, 1H, Ala⁵NH), 7.26–7.15 (m, 5H, Phe⁴H_{Ar}), 7.09 (d, $J = 8.4$ Hz, 2H, Tyr¹H_{2,6}), 6.69 (d, $J = 8.4$ Hz, 2H, Tyr¹H_{3,5}), 4.59 (d, $J = 9.6$ Hz, 1H, D-Val²H_α), 4.42–4.30 (m, 2H, Phe⁴H_α, D-Ala⁵H_α), 4.22 (dd, $J = 13.6$ and 9.6 Hz, 1H, d-Ala⁵H_β), 4.12 (dd, $J = 6.8$ and 6.0 Hz, 1H, Tyr¹H_α), 3.15–3.06 (m, 3H, Gly³H_α, Phe⁴H_β, Ala⁵H_β), 2.98–2.83 (m, 3H, Ala⁵H_β, Tyr¹H_β, Phe⁴H_β), 2.76 (dd, $J = 13.6$ and 8.8 Hz, 1H, Tyr¹H_β), 1.16 (s, 3H, D-Val²H_γ), 0.97 (s, 3H, D-Val²H_γ); MS(ESI) 586 [M + H]⁺, 584 [M – H]⁻; HRMS (MALDI) [M + Na]⁺ calcd for C₂₈H₃₅N₅O₇S 608.2155, found 608.2138; RP-HPLC $t_R^a = 17.66$, $t_R^b = 10.73$.

In Vitro Radioligand Binding Assay. 1. Preparation of Cell Membranes Expressing Opiate Receptors. This method is a modification of the method of Raynor et al.⁵¹ The cloned human μ-, δ-, and κ-receptors were expressed in CHO cells, and these CHO cells were harvested from the culture flasks. The cells were centrifuged at 1000*g* for 10 min, resuspended in assay buffer (50 mM tris(hydroxymethyl)aminomethane HCl, pH 7.8, 1.0 mM ethylene glycol bis(β-aminoethyl ether) *N,N,N,N*-tetraacetic acid (EGTA free acid), 5.0 mM MgCl₂, 10 mg/L leupeptin, 10 mg/L pepstatin A, 200 mg/L bacitracin, 0.5 mg/L aprotinin), and centrifuged again. The resulting pellet was resuspended in assay buffer homogenized with a Polytron homogenizer (Brinkmann, Westbury, NY) for 30 s at a setting of 1. The homogenate was centrifuged at 48000*g* for 10 min at 4 °C, and the pellet was resuspended at 1 mg of protein/mL of assay buffer and stored at –80 °C until use.

2. [³H]-Diprenorphine Binding to μ-, δ-, and κ-Opioid Receptors. After dilution in assay buffer and homogenization as before, membrane proteins (50–100 μg) in 250 μL of assay buffer were added to mixtures containing test compound and [³H]-diprenorphine (final concentration of 0.4 nM, 30 000 dpm) in 250 μL of assay buffer in 96-well deep-well polystyrene titer plates (Beckman) and were incubated at room temperature for 60 min. Reactions were terminated by vacuum filtration with a Brandel MPXR-96T harvester through GF/B filters in filter bottom 96-well plates that had been pretreated with a solution of 0.5% polyethylenimine and 0.1% bovine serum albumin for at least 1 h. The filters were washed four times with 1.0 mL of ice-cold 50 mM Tris-HCl, pH 7.8, 30 μL of Microscint-20 (Packard Instrument Co., Meriden, CT) was

added to each filter, and radioactivity on the filters was determined by scintillation spectrometry in a Packard Top-Count.

[³H]-Diprenorphine was purchased from Amersham Life Science, Inc. (Arlington Heights, IL) and had a specific activity of 66 Ci/mmol. Preliminary experiments were performed to show that no specific binding was lost during the wash of the filters, that binding achieved equilibrium within the incubation time and remained at equilibrium for at least an additional 60 min, and that binding was linear with regard to protein concentration. Nonspecific binding, determined in the presence of 10 μM unlabeled naloxone, was less than 10% of total binding.

The data from competition experiments were fit by nonlinear regression analysis by Prism (GraphPad Software, Inc., San Diego, CA) using the four-parameter equation for one-site competition and subsequently calculating the K_i from the EC₅₀ value by the Cheng–Prusoff equation.

In Vitro Biological Assay. The in vitro bioactivities of the compounds were tested in the GPI⁵² and MVD⁵³ assays as reported elsewhere.^{54,55} A log dose–response curve was determined with [Leu⁵]-enkephalin as the standard for each ileum and vas preparation, and the IC₅₀ values of the compounds were normalized according to a published procedure.⁵⁶

In Vivo Thermal Escape Assay. 1. Rat Preparation. Animal surgeries and tests were approved by the institutional animal care committee of the University of California, San Diego. Male Sprague–Dawley rats (Harlan Industries, Indianapolis, IN), weighing 250–350 g, were housed in separate plastic cages and maintained on a 12 h cycle (on, 6:00 a.m.; off, 6:00 p.m.) with food and water given ad libitum.

Chronic intrathecal (it.) catheters were implanted under 2–3% isoflurane (50% O₂/air) anesthesia.⁵⁷ Briefly, for intrathecal injection, an 8.5 cm polyethylene catheter (PE-10) was inserted through a slit in the cisternal atlanto-occipital membrane and was passed to the rostral edge of the lumbar (L2) subarachnoid space. The external portion was tunneled subcutaneously to exit at the top of the skull. Prior to insertion, the catheter was flushed with saline and, after insertion, plugged with stainless steel wire to prevent leakage of cerebrospinal fluid.

2. Assay of Thermal Nociception. This test was described previously in detail.⁵⁸ Briefly, the animal was placed on a glass surface that was maintained at 30 °C by a feedback-controlled, under-glass, forced-air heating system.⁵⁹ A projection bulb under the glass was focused on the foot pad of the animal, which induced an abrupt withdrawal. A cutoff time was set at 20 s to prevent tissue damage. The test was started approximately 1 h after the animal was placed in the chamber for acclimation. After the baseline latency was measured, the drugs were injected intrathecally and the test was performed.

3. Drugs and Injections. All agonists were administered it. in a total volume of 10 μL followed by 10 μL of saline to flush the catheter. Lanthionine enkephalin analogues (**1a–d**) and DPDPE ([D-Pen²,D-Pen⁵]-enkephalin; RBI, MA) were dissolved in 20% 2-hydroxypropyl-β-cyclodextrin (Research Biochemicals, Inc., Natick, MA). Morphine sulfate (Merck, Sharp and Dohme, West Point, PA) was dissolved in physiological saline (0.9% NaCl w/v).

4. Data Analysis. Response latency data from the thermal escape test are presented as the mean ± SE at 0, 15, 30, 60, 90, and 120 min after drug injection and were converted to percent maximum possible effect (% MPE) according to the formula

$$\% \text{ MPE} = \frac{\text{postdrug latency} - \text{predrug latency}}{\text{cutoff latency}^* - \text{predrug latency}} \times 100\%$$

where the cutoff latency was 20 s in this study.

Abbreviations. All optically active amino acids are of the L variety unless otherwise stated. Other abbreviations are the following: Bzl, benzyl; Cbz, benzyloxycarbonyl; CDCl₃, deuterated chloroform; CHO, Chinese hamster ovary; DIC, *N,N*-diisopropylcarbodiimide; DIEA, diisopropylethylamine; DMF, dimethylformamide; DMSO-*d*₆, fully deuterated dimethyl sul-

foxide; DPDPE, Tyr- ϵ [D-Pen-Gly-Phe-D-Pen]-OH; DPPA, di-phenylphosphoryl azide; Fmoc, fluorenylmethoxycarbonyl; GPI, guinea pig ileum; HOBt, 1-hydroxybenzotriazole monohydrate; ip, intraperitoneal; IPE, diisopropyl ether; it, intrathecal; MVD, mouse vas deferens; *n*-hex, *n*-hexane; NMP, *N*-methylpyrrolidinone; Pen, penicillamine; PyBOP, benzotriazole-1-ylxytrispyrrolidinophosphonium hexafluorophosphate; RP-HPLC, reverse-phase high-performance liquid chromatography; TFA, trifluoroacetic acid; TIS, triisopropylsilane; Trt, triphenylmethyl.

Acknowledgment. This work was supported by NIH Grant R01-DA 05539 (M.G.). We thank Dr. Li Zhang for the interpretation of NMR spectra and Sandra Blaj Moore for her helpful assistance in the preparation of this manuscript.

Supporting Information Available: HRMS spectra and HPLC profiles of the final products **1a–d**. This material is available free of charge via the Internet at <http://pubs.acs.org>.

References

- Reisine, T.; Bell, G. I. Molecular Biology of Opioid Receptors. *Trends Neurosci.* **1993**, *16*, 506–510.
- Reisine, T.; Pasternak, G. Opioid Analgesics and Antagonists. In *Goodman & Gilman's the pharmacological basis of therapeutics*, 9th ed.; Hardman, J. G., Limbird, L. E., Eds.; McGraw-Hill: New York, 1996; pp 521–555.
- Millan, M. J. κ -Opioid Receptors and Analgesia. *Trends Pharmacol. Sci.* **1990**, *11*, 70–76.
- Stevens, C. W.; Yaksh, T. L. Dynorphin A and Related Peptides Administered Intrathecally in the Rat: A Search for Putative κ Opiate Receptor Activity. *J. Pharmacol. Exp. Ther.* **1986**, *238*, 833–838.
- Nakazawa, T.; Ikeda, M.; Kaneko, T.; Yamatsu, K. Analgesic Effects of Dynorphin A and Morphine in Mice. *Peptides* **1985**, *6*, 75–78.
- Caudle, R. M.; Mannes, A. J. Dynorphin: Friend or Foe? *Pain* **2000**, *87*, 235–239.
- Quock, R. M.; Burkey, T. H.; Varga, E.; Hosohata, Y.; Hosohata, K.; Cowell, S. M.; Slate, C. A.; Ehlert, F. J.; Roeske, W. R.; Yamamura, H. I. The δ -Opioid Receptor: Molecular Pharmacology, Signal Transduction, and the Determination of Drug Efficacy. *Pharmacol. Rev.* **1999**, *51*, 503–532.
- Przewlocki, R.; Przewlocka, B. Opioids in Chronic Pain. *Eur. J. Pharmacol.* **2001**, *429*, 79–91.
- Hughes, J.; Smith, T. W.; Kosterlitz, H. W.; Fothergill, L. A.; Morgan, B. A.; Morris, H. R. Identification of Two Related Pentapeptides from the Brain with Potent Opiate Agonist Activity. *Nature (London)* **1975**, *258*, 577–579.
- Dupont, A.; Cusan, L.; Garon, M.; Alvarado-Urbina, G.; Labrie, F. Extremely Rapid Degradation of [3 H]-Methionine-enkephalin by Various Rat Tissues In Vivo and In Vitro. *Life Sci.* **1977**, *21*, 907–914.
- Hruby, V.; Gehrig, C. A. Recent Developments in the Design of Receptor-Specific Opioid Peptides. *Med. Res. Rev.* **1989**, *9*, 343–401.
- James, I. F.; Goldstein, A. Site-Directed Alkylation of Multiple Opioid Receptors. I. Binding Selectivity. *Mol. Pharmacol.* **1984**, *25*, 337–342.
- Gacel, G.; Dauge, V.; Breuze, P.; Delay-Goyet, P.; Roques, B. P. Development of Conformationally Constrained Linear Peptides Exhibiting a High Affinity and Pronounced Selectivity for δ Opioid Receptors. *J. Med. Chem.* **1988**, *31*, 1891–1897.
- Mosberg, H. I.; Hurst, R.; Hruby, V. J.; Gee, K.; Yamamura, H. I.; Galligan, J. J.; Burks, T. F. Bis-penicillamine Enkephalins Possess Highly Improved Specificity toward δ Opioid Receptors. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 5871–5874.
- Jack, R. W.; Jung, G. Lantibiotics and Microcins. Polypeptides with Unusual Chemical Diversity. *Curr. Opin. Chem. Biol.* **2000**, *4*, 310–317.
- Bierbaum, G. Antibiotic Peptides—lantibiotics. *Chemother. J.* **1999**, *8*, 204–209.
- Kaiser, D.; Jack, R. W.; Jung, G. Lantibiotics and Microcins: novel Posttranslational Modifications of Polypeptides. *Pure Appl. Chem.* **1998**, *70*, 97–104.
- Jack, R.; Gotz, F.; Jung, G. Lantibiotics. In *Biotechnology*, 2nd ed.; Kleinkauf, H., Von Doehren, H., Eds.; VCH: Weinheim, Germany, 1997; pp 323–368.
- Li, H.; Jiang, X.; Howell, S. B.; Goodman, M. Synthesis, Conformational Analysis and Bioactivity of Lan-7, a Lanthionine Analog of TT-232. *J. Pept. Sci.* **2000**, *6*, 26–35.
- Li, H.; Jiang, X.; Goodman, M. Synthesis, Conformational Analysis and Biological Activities of Lanthionine Analogs of a Cell Adhesion Modulator. *J. Pept. Sci.* **2001**, *7*, 82–91.
- Osapay, G.; Prokai, L.; Kim, H.-S.; Medzihradsky, K. F.; Coy, D. H.; Liapakis, G.; Reisine, T.; Melacini, G.; Zhu, Q.; Wang, S. H. H.; Mattern, R.-H.; Goodman, M. Lanthionine-Somatostatin Analogs: Synthesis, Characterization, Biological Activity, and Enzymic Stability Studies. *J. Med. Chem.* **1997**, *40*, 2241–2251.
- Melacini, G.; Zhu, Q.; Osapay, G.; Goodman, M. A Refined Model for the Somatostatin Pharmacophore: Conformational Analysis of Lanthionine-Sandostatin Analogs. *J. Med. Chem.* **1997**, *40*, 2252–2258.
- Zheng, H.; Fink, D.; Li, H.; Jiang, X.; Aebi, S.; Law, P.; Goodman, M.; Howell, S. B. In Vitro Antineoplastic Activity of a Novel Lanthionine-Containing Peptide. *Clin. Cancer Res.* **1997**, *3*, 1323–1330.
- Mosberg, H. I.; Omnaas, J. R.; Goldstein, A. Structural Requirements for δ Opioid Receptor Binding. *Mol. Pharmacol.* **1987**, *31*, 599–602.
- Flippen-Anderson, J. L.; Hruby, V. J.; Collins, N.; George, C.; Cudney, B. X-ray Structure of [D-Pen 2 , D-Pen 5]Enkephalin, a Highly Potent, δ Opioid Receptor-Selective Compound: Comparisons with Proposed Solution Conformations. *J. Am. Chem. Soc.* **1994**, *116*, 7523–7531.
- Baker, T. J.; Rew, Y.; Goodman, M. Novel Reagents and Reactions for Drug Design. *Pure Appl. Chem.* **2000**, *72*, 347–354.
- Goodman, M.; Rew, Y.; Malkmus, S.; Svensson, C.; Yaksh, T. L.; Chung, N. N.; Schiller, P. W.; Daubert, J. D.; Cassel, J. A.; DeHaven, R. Design and Synthesis of Cyclic Lanthionine Enkephalin Analogs: δ Opioid Receptor Selective Ligands. Presented at the 221st National Meeting of the American Chemical Society, San Diego, CA, April 1–5, 2001; Paper ORGN-109.
- Goodman, M.; Zapf, C.; Rew, Y. New Reagents, Reactions and Peptidomimetics for Drug Design. *Biopolymers* **2001**, *60*, 229–245.
- Osapay, G.; Wang, S.; Comer, D. D.; Toy-Palmer, A.; Zhu, Q.; Goodman, M. Superactive Lanthionine-enkephalins. In *Peptides: Chemistry, Structure and Biology*, Proceedings of the 13th American Peptide Symposium; Hodges, R. S., Smith, J. A., Eds.; ESCOM: Leiden, The Netherlands, 1994; pp 435–437.
- Polinsky, A.; Cooney, M. G.; Toy-Palmer, A.; Osapay, G.; Goodman, M. Synthesis and Conformational Properties of the Lanthionine-Bridged Opioid Peptide [D-Ala 2 , Ala 5]Enkephalin As Determined by NMR and Computer Simulations. *J. Med. Chem.* **1992**, *35*, 4185–4194.
- Shao, H.; Wang, S. H. H.; Lee, C.-W.; Osapay, G.; Goodman, M. A Facile Synthesis of Orthogonally Protected Stereoisomeric Lanthionines by Regioselective Ring Opening of Serine β -Lactone Derivatives. *J. Org. Chem.* **1995**, *60*, 2956–2957.
- Pu, Y.; Martin, F. M.; Vederas, J. C. Synthesis and Acylation of Salts of L-Threonine β -Lactone: A Route to β -Lactone Antibiotics. *J. Org. Chem.* **1991**, *56*, 1280–1283.
- Probert, J. M.; Rennex, D.; Bradley, M. Lanthionines for Solid Phase Synthesis. *Tetrahedron Lett.* **1996**, *37*, 1101–1104.
- Lee, C. W.; Zhu, Q.; Shao, H.; Wang, S. H. H.; Osapay, G.; Goodman, M. Design and Synthesis of Substituted Lanthionine Enkephalin Opioids. In *Peptides 1994*, Proceedings of the 23rd European Peptide Symposium; Maia, H. L. S., Ed.; ESCOM: Leiden, The Netherlands, 1995; pp 627–628.
- Dugave, C.; Menez, A. Synthesis of Natural and non Natural Orthogonally Protected Lanthionines from *N*-Tritylserine and *allo*-Threonine Derivatives. *Tetrahedron: Asymmetry* **1997**, *8*, 1453–1465.
- Jensen, K. J.; Alsina, J.; Songster, M. F.; Vagner, J.; Albericio, F.; Barany, G. Backbone Amide Linker Strategy for Solid-Phase Synthesis of C-Terminal-Modified and Cyclic Peptides. *J. Am. Chem. Soc.* **1998**, *120*, 5441–5452.
- Han, Y.; Albericio, F.; Barany, G. Occurrence and Minimization of Cysteine Racemization during Stepwise Solid-Phase Peptide Synthesis. *J. Org. Chem.* **1997**, *62*, 4307–4312.
- Besse, D.; Lombard, M. C.; Zajac, J. M.; Roques, B. P.; Besson, J. M. Pre- and Postsynaptic Location of μ , δ and κ Opioid Receptors in the Superficial Layers of the Dorsal Horn of the Rat Spinal Cord. *Prog. Clin. Biol. Res.* **1990**, *328*, 183–186.
- Yaksh, T. L. Pharmacology and Mechanisms of Opioid Analgesic Activity. *Acta Anaesthesiol. Scand.* **1997**, *41*, 94–111.
- Svensson, C.; Rew, Y.; Malkmus, S.; Schiller, P. W.; Goodman, M.; Yaksh, T. Y. Systemic and Spinal Analgesic Activity of a δ Opioid Selective Lanthionine Enkephalin Analogue. Unpublished results.
- Wang, S. H. H.; Bahmanyar, S.; Taulane, J. P.; Goodman, M. Enzymic Stability of Disulfide-Bridged Peptides and Their Lanthionine Analogs. In *Peptides: Chemistry, Structure and Biology*, Proceedings of the 14th American Peptide Symposium; Kaumaya, P. T. P., Hodges, R. S., Eds.; Mayflower Scientific: Kingswinford, 1996; pp 715–716.

- (42) Loh, H. H.; Liu, H.-C.; Cavalli, A.; Yang, W.; Chen, Y.-F.; Wei, L.-N. μ Opioid Receptor Knockout in Mice: Effects on Ligand-Induced Analgesia and Morphine Lethality. *Mol. Brain Res.* **1998**, *54*, 321–326.
- (43) Yaksh, T. L.; Malmberg, A. B.; Ro, S.; Schiller, P.; Goodman, M. Characterization of the Spinal Antinociceptive Activity of Constrained Peptidomimetic Opioids. *J. Pharmacol. Exp. Ther.* **1995**, *275*, 63–72.
- (44) He, L.; Lee, N. M. δ Opioid Receptor Enhancement of μ Opioid Receptor-Induced Antinociception in Spinal Cord. *J. Pharmacol. Exp. Ther.* **1998**, *285*, 1181–1186.
- (45) Matthes, H. W. D.; Smadja, C.; Valverde, O.; Vonesch, J. L.; Foutz, A. S.; Boudinot, E.; Denavit-Saubie, M.; Severini, C.; Negri, L.; Roques, B. P.; Maldonado, R.; Kieffer, B. L. Activity of the δ -Opioid Receptor Is Partially Reduced, Whereas Activity of the κ -receptor Is Maintained in Mice Lacking the μ -Receptor. *J. Neurosci.* **1998**, *18*, 7285–7295.
- (46) Nakajima, K.; Takai, F.; Tanaka, T.; Okawa, K. Studies on Aziridine-2-carboxylic Acid. I. Synthesis of the Optically Active L-Aziridine-2-carboxylic Acid and Its Derivatives. *Bull. Chem. Soc. Jpn.* **1978**, *51*, 1577–1578.
- (47) Kaiser, E.; Colescott, R. L.; Bossinger, C. D.; Cook, P. I. Color Test for Detection of Free Terminal Amino Groups in the Solid-Phase Synthesis of Peptides. *Anal. Biochem.* **1970**, *34*, 595–598.
- (48) Ellman, G. L. Tissue Sulfhydryl Groups. *Arch. Biochem. Biophys.* **1959**, *82*, 70–77.
- (49) Ellman, G. L.; Courtney, K. D.; Andres, V., Jr.; Feather-Stone, R. M. A New and Rapid Colorimetric Determination of Acetylcholinesterase Activity. *Biochem. Pharmacol.* **1961**, *7*, 88–95.
- (50) Dugave, C.; Menez, A. Stereoconservative Synthesis of Orthogonally Protected γ -Functionalized Amino Acids Using *N*-Tritylserine Derivatives. *J. Org. Chem.* **1996**, *61*, 6067–6070.
- (51) Raynor, K.; Kong, H.; Chen, Y.; Yasuda, K.; Lu, L.; Bell, G. I.; Reisine, T. Pharmacological Characterization of the Cloned κ -, δ -, and μ -opioid receptors. *Mol. Pharmacol.* **1994**, *45*, 330–334.
- (52) Paton, W. D. M. Action of Morphine and Related Substances on Contraction and on Acetylcholine Output of Coaxially Stimulated Guinea Pig Ileum. *Br. J. Pharmacol.* **1957**, *12*, 119–127.
- (53) Henderson, G.; Hughes, J.; Kosterlitz, H. W. New Example of a Morphine-Sensitive Neuro-effector Junction. Adrenergic Transmission in the Mouse Vas Deferens. *Br. J. Pharmacol.* **1972**, *46*, 764–766.
- (54) DiMaio, J.; Nguyen, T. M. D.; Lemieux, C.; Schiller, P. W. Synthesis and Pharmacological Characterization In Vitro of Cyclic Enkephalin Analogs: Effect of Conformational Constraints on Opiate Receptor Selectivity. *J. Med. Chem.* **1982**, *25*, 1432–1438.
- (55) Schiller, P. W.; Lipton, A.; Horrobin, D. F.; Bodanszky, M. Unsulfated C-Terminal 7-Peptide of Cholecystokinin: A New Ligand of the Opiate Receptor. *Biochem. Biophys. Res. Commun.* **1978**, *85*, 1332–1338.
- (56) Waterfield, A. A.; Leslie, F. M.; Lord, J. A. H.; Ling, N.; Kosterlitz, H. W. Opioid Activities of Fragments of β -Endorphin and of Its Leucine⁶⁵-Analog. Comparison of the Binding Properties of Methionine- and Leucine-enkephalin. *Eur. J. Pharmacol.* **1979**, *58*, 11–18.
- (57) Yaksh, T. L.; Rudy, T. A. Chronic Catheterization of the Spinal Subarachnoid Space. *Physiol. Behav.* **1976**, *17*, 1031–1036.
- (58) Dirig, D. M.; Yaksh, T. L. Differential Right Shifts in the Dose-Response Curve for Intrathecal Morphine and Sufentanil as a Function of Stimulus Intensity. *Pain* **1995**, *62*, 321–328.
- (59) Hargreaves, K.; Dubner, R.; Brown, F.; Flores, C.; Joris, J. A. A New and Sensitive Method for Measuring Thermal Nociception in Cutaneous Hyperalgesia. *Pain* **1988**, *32*, 77–88.
- (60) Salvadori, S.; Balboni, G.; Marastoni, M.; Sarto, G.; Tomatis, R. Synthesis and Opioid Activity of [Sar⁴]Dermorphin-tetrapeptide Analogs. *Hoppe-Seyler's Z. Physiol. Chem.* **1984**, *365*, 1199–1206.

JM020108K