Brain-Targeted Delivery of a Leucine-enkephalin Analogue by Retrometabolic Design†

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A brain-targeted chemical delivery system (CDS) based on retrometabolic drug design was applied to a Leu-enkephalin analogue, Tyr-D-Ala-Gly-Phe-D-Leu (DADLE). The molecular architecture of the peptide CDS disguises its peptide nature from neuropeptide-degrading enzymes and provides lipophilic, bioreversible functions for the penetration through the blood−brain barrier. These functions were provided by a targetor, a 1,4-dihydrotrigonellyl group, on the N-terminus and a bulky, lipophilic ester group on the C-terminus. A spacer amino acid residue was also inserted between the targetor and the parent peptide to assure the release of DADLE by specific enzymes. Four CDSs were synthesized by segment-coupling method that proved to be superior to sequential elongation in obtaining this type of peptide conjugates. Intravenous injection of the compounds produced a significant and long-lasting response in rats monitored by the tail-flick latency measurements. CDSs having the bulkier cholesteryl group showed a better efficacy than those having the smaller 1-adamantaneethyl ester. The spacer was the most important factor to manipulate the rate of DADLE release and, thus, the pharmacological activity; proline as a spacer produced more potent analgesia than alanine. The antinociceptive effect of the CDSs was naloxone-reversible and methylnaloxonium-irreversible, confirming that central opiate receptors were solely responsible for mediating analgesia induced by the peptide CDS that delivered, retained, and then released the peptide in the brain.

Introduction

Enkephalins are naturally occurring linear pentapeptides that bind to opioid receptors and have the sequence Tyr-Gly-Gly-Phe-Leu/Met ([Leu]enkephalin or [Met]-enkephalin).[1] The action of these endogenous peptides on pain perception, addictive states, and psychiatric disorders have been thoroughly investigated.[2] However, analgesia is their best-known central effect.[3,4] The metabolic stability of enkephalins is poor. Studies suggest that at least four different types of enkephalin-metabolizing enzymes contribute to their rapid deactivation. The enkephalins are predominantly cleaved by aminopeptidases[5] at the N-terminal tyrosine. Enkephalinase and angiotensin-converting enzymes[6] cleave the Gly^3-Phe^4 bond, and a dipeptidyl aminopeptidase[7] cleaves the Gly^2-Gly^3 bond. There have been numerous attempts to modify the original structure to improve metabolic stability and receptor binding (affinity, selectivity).[8] One approach to stabilize the peptide backbone against proteases incorporates unnatural D-amino acids to the peptide chain, such as replacements of Gly^2 with D-Ala and Leu with D-Leu.[9] Metabolic stability alone does not, however, warrant access of the peptide analogues to the central nervous system (CNS) upon systemic administration.

Many peptides also are potential neuropharmaceuticals. Most of them are water soluble and cannot penetrate through the lipoidal bilayer of the blood−brain barrier (BBB). The BBB, which acts as a selective partition between the CNS and peripheral nervous system, is the major obstacle for delivery of centrally active peptides to the brain.[10] Peptides may also be recognized by neuropeptide-degrading enzymes expressed in the BBB, even when their lipophilicity allows their transport through the BBB.[11] Recently, an enkephalin analogue[12,13] and a centrally active thyrotropin-releasing hormone[14] have been successfully transported into the CNS by a brain-targeting chemical delivery based on retrometabolic drug design approach.[15,16] A brain-targeting peptide chemical delivery system is defined as a biologically inert molecule which requires several steps in its conversion to the biologically active peptide and ensures drug differential delivery to the CNS, the site of action. In this approach, a redox "targetor" (1,4-dihydrotrigonellyl) moiety is attached to the N-terminal amino acid of the peptide chain via a spacer amino acid, and a bulky, lipophilic moiety is anchored to the C-terminus via an ester bond. The dihydropyridine-type targetor has definite roles. This group enhances BBB penetration and, most importantly, can be converted by enzymatic oxidation to a water-soluble, lipid-insoluble pyridinium salt that cannot leave the brain but can easily be eliminated from the peripheral circulation. A spacer amino acid residue separates the targetor from the biologically active peptide to enhance its release by specific enzymes from the inactive conjugate. This cleavage should be favored over proteolytic degradations induced by other peptidases. Attachment of the 1,4-dihydrotrigonellyl to the N-terminus alone would not give significant increase in lipid solubility to a peptide and would only protect against aminopeptidases such as aminopeptidase N (EC 3.4.11.2). Therefore, a bulky ester on the C-terminus is applied, which not only protects this part of the

† Abbreviations: DCC, 1,3-dicyclohexylcarbodiimide; DMAP, 4-(dimethylamino)pyridine; HOBt, 1-hydroxybenzotriazole; PyBOP, benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate; DCM, dichloromethane; DMF, *N*,*N*-dimethylformamide; Fmoc, fluorenylmethyloxycarbonyl; Boc, *tert*-butyloxycarbonyl; EtOAc, ethyl acetate; MeOH, methanol; TFA, trifluoroacetic acid; NMP, *N*-methylpyrrolidinone; DIEA, *N*,*N*-diisopropylethylamine; TEA, triethylamine; Nic, nicotinoyl; ᵗBu, *tert*-butyl; Su, *N*-hydroxysuccinyl.

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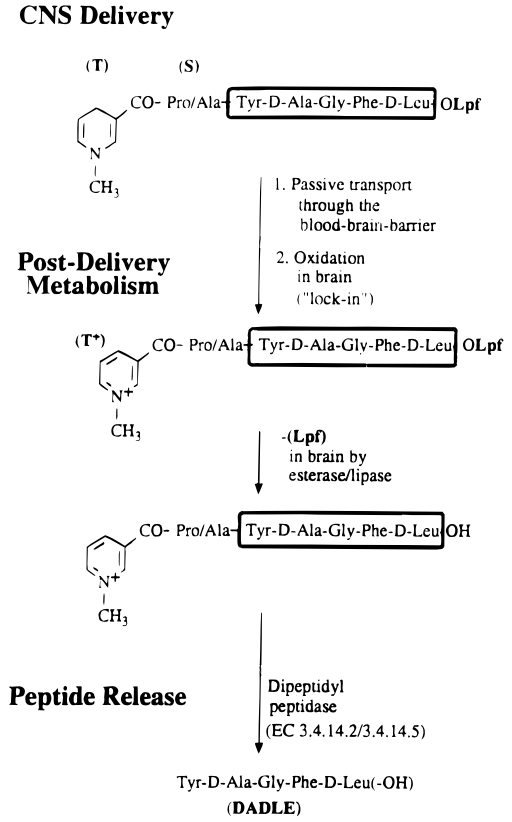
Scheme 1. Brain Targeting of DADLE by Retrometabolic Design



peptide from peptidases but also ensures the necessary lipophilicity for passive transport of the peptide conjugate through the BBB into the CNS. Simple alkyl esters do not provide these functions.[12] The ester bond is stable chemically but labile enzymatically. Sequential metabolism (involving oxidoreductases, lipases/esterases, dipeptidyl peptidases) in the brain affords the retention ("lock-in") and the subsequent release of the biologically active peptide.

In an apparent effort to improve and optimize this strategy, the objectives of our study were to develop an effective synthesis strategy for obtaining peptide chemical delivery systems (CDSs) and to determine the potential contribution of the spacer and the C-terminal ester, the two functions that may be adjusted to achieve optimal brain targeting of Tyr-D-Ala-Gly-Phe-D-Leu (DADLE), to the efficacy of CDSs to induce CNS-mediated analgesia.

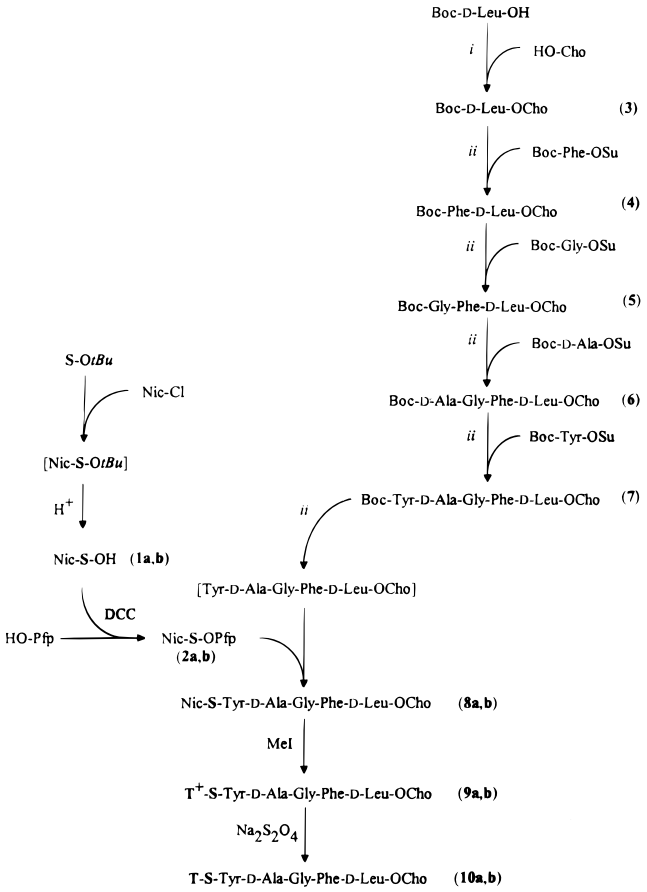
Results

Design. A chemical delivery system consistent with the activation mode of retrometabolic concept was developed for brain-targeting delivery of enkephalin analogues.[15,16] The CDS should be sufficiently lipophilic for allowing brain uptake and should undergo an enzymatic conversion to promote retention within the CNS. In this design, shown in Scheme 1, a synthetic leucine-enkephalin analogue, DADLE, a well-known opioid agonist,[17] is surrounded by functional units that protect the peptide from being recognized by peptide-degrading enzymes and provide biolabile and lipophilic functions for the penetration through the BBB by passive transport. A bulky, lipophilic group (Lpf), cholesteryl (Cho) or 1-adamantaneethyl (Ada), anchored to the COOH-terminus via an ester bond provides protection of this part of the molecule. A 1,4-dihydrotrigonellyl targetor (T) on the N-terminus enhances BBB transport and, most importantly, after reaching the CNS keeps the peptide conjugate in the brain due to the enzymatic oxidation to a lipid-insoluble ionic salt (T^+, trigonellyl). The mechanism of this oxidation has been extensively studied, and it has been suggested to be analogous to the oxidation of NAD(P)H, a coenzyme associated with numerous oxidoreductases and cellular respiration.[18] Attachment of the targetor directly to the amino-terminal residue may not afford the release of the target peptide at a desired rate because of the low amidase activity of the brain tissue.[19] Therefore, a spacer (S) function is also introduced into the peptide chain for separation of the enkephalin moiety from the targetor to promote the cleavage that yields DADLE. The selection of alanine and proline as a spacer is based on the suggested involvement of alanyl and prolyl aminodipeptidyl peptidases in the enkephalinergic transmission in the brain.[20,21] These enzymes have specific cleaving site in the peptide sequence. We assumed that trigonelline (synthetic precursor and oxidative metabolite of T) represents an amino acid residue for these dipeptidyl peptidases because it is attached to the spacer by a peptide-like bond and has a permanent positive charge like an N-terminal amino acid. Furthermore, pyridinium salt-type amino acids can replace natural ones in various peptides such as LHRH.[22]

After passive transport through the BBB the targetor undergoes an enzymatically mediated oxidation in the CNS to a lipid-insoluble trigonellyl (T^+) salt that is retained ("locked-in") in the brain. Thus, unlike a lipophilic peptide prodrug,[23] T^+-S-DADLE-OLpf cannot leave the brain. It has been shown[13,14] that the trapped peptide conjugate undergoes enzymatic cleavages releasing the parent peptide in a slow and sustained manner. The lipophilic ester group is cleaved by lipases and/or esterases after or during penetration through the BBB.

Synthesis. The CDSs of DADLE for brain-targeted delivery were obtained by two routes; both applied the segment-coupling peptide synthesis strategy. Initially,[13,15] peptide CDSs were prepared by DCC/HOBt coupling via a stepwise elongation of the peptide chain in the C to N direction starting with the lipophilic cholesteryl ester of the C-terminal residue. This coupling method was time-consuming and inconvenient because frequent purification was required. We have improved this initial procedure according to Scheme 2 (method A) by combining the step by step elongation of the peptide chain with segment condensation and using active esters instead of DCC activation. Peptides esters having a Cho ester group on the C-terminus and a Nic residue on the N-terminus (**8a,b**) were obtained after a dipeptide−pentapeptide (2 + 5) coupling. The pentapeptide represented the lipophilic ester of the enkephalin DADLE-OLpf (**7**, an enkephalin prodrug). It was prepared by stepwise elongation of the peptide chain with *N*-hydroxysuccinimide esters of the Boc-protected amino acids. The first step in the synthesis of **7** involved the preparation of the lipophilic ester of D-leucine (**3**) that was easily achieved by either DCC/DMAP or PyBOP/DIEA[24] activation. Then, the Boc protecting group was removed in a customary manner,

Scheme 2. Synthesis of **10a,b** by Method A[a]



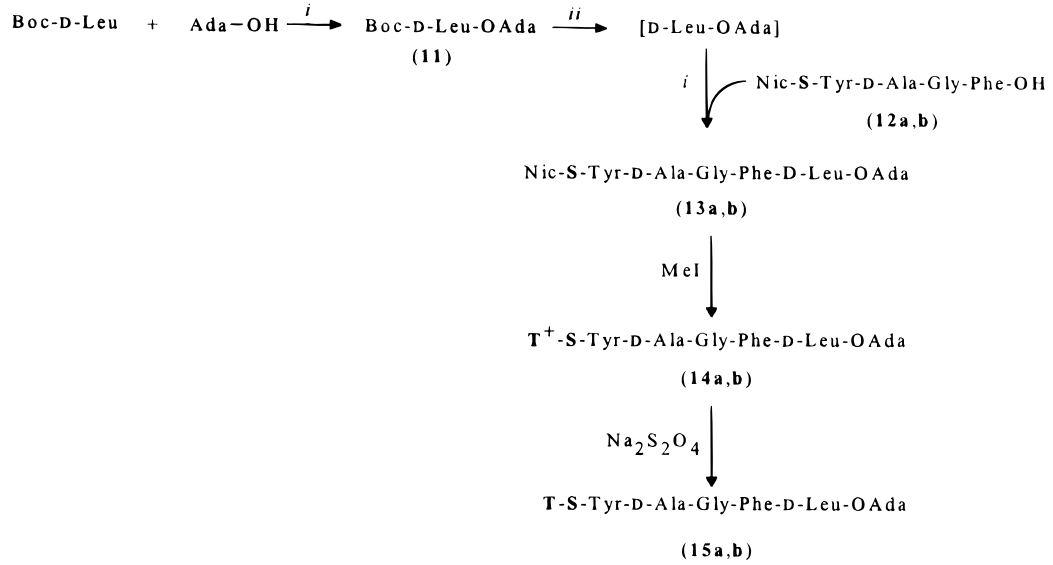
[a] (i) PyBOP/DIEA; (ii) TFA/TEA. **a**: S = Pro, **b**: S = Ala.

and triethylamine was used for neutralization. Compound **3** was elongated with a slight excess of the active ester of the relevant Boc-protected amino acid. The progress of the coupling reactions was monitored by TLC and fast-atom bombardment mass spectrometry (FAB-MS). The intermediate peptides obtained after recrystallization were of sufficient purity (FAB-MS, TLC) to be used directly for the next peptide bond formation. Therefore, no attempts have been made to further characterize and/or purify the pendant peptide chain leading to **7**.

The dipeptide segment in this procedure was the pentafluorophenyl ester of the N-terminal dipeptide, Nic-S-OPfp (**2a,b**), prepared from Nic-S-OH (**1a,b**) and pentafluorophenol with DCC.[25] Compounds **1a,b** were synthesized from Nic-Cl and Ala/Pro-O^{t}Bu followed by *in situ* hydrolysis of the ester group with TFA/DCM (1:1, v/v). The two fragments, **2a,b** and **7**, were assembled in DMF with excellent yield without any auxiliary additives resulting in compounds **8a,b**. The reaction was complete within 1 day using a 2-fold excess of **2a** or **2b**. At this stage, we applied the only chromatographic purification in this procedure for ensuring the purity of the final products, the peptide CDSs. The peptide esters **8a,b** were purified by column chromatography on silica gel. Because they showed limited solubility in the chromatographic solvents, they were dissolved in chloroform/MEOH (6:4, v/v), evaporated on silica gel, and put on the top of the column. Using chloroform/MEOH (9:1, v/v) containing 0.1% TEA as an eluent, pure products **8a,b** were obtained. After alkylation on the pyridine ring with methyl iodide, the obtained pyridinium salts **9a,b** were reduced with sodium dithionite[26] to the corresponding peptide CDS **10a,b**.

For an alternative route (method B), we chose the hexapeptide−single amino acid (6 + 1) segment coupling and combined solid phase peptide synthesis (SPPS) with solution phase synthesis. As Scheme 3 shows, the two individual segments in this case were the lipophilic ester of the C-terminal amino acid **11** and the residual fragment of the peptide chain Nic-S-Tyr-D-Ala-Gly-Phe-OH (**12a,b**), respectively. This approach is illustrated with the synthesis of CDSs containing Ada ester group on the C-terminus (**15a,b**). The hexapeptides were prepared by SPPS using an automated Fmoc chemistry protocol. The DCC-mediated coupling was accelerated with HOBt. After cleavage from the resin, the crude peptide compounds **13a,b** were identified by FAB-MS and used without further purification. Esterification of D-Leu was carried out the same way as in method A for compound **3**. The PyBOP/DIEA system was also selected for the preparation of **13a,b**. The segment coupling was complete (monitored by TLC and FAB-MS) within 1 day using approximately 1.5-fold excess of

Scheme 3. Preparation of **15a,b** by Method B[a]



[a] (i) PyBOP/DIEA; (ii) TFA/TEA. **a**: S = Pro, **b**: S = Ala.

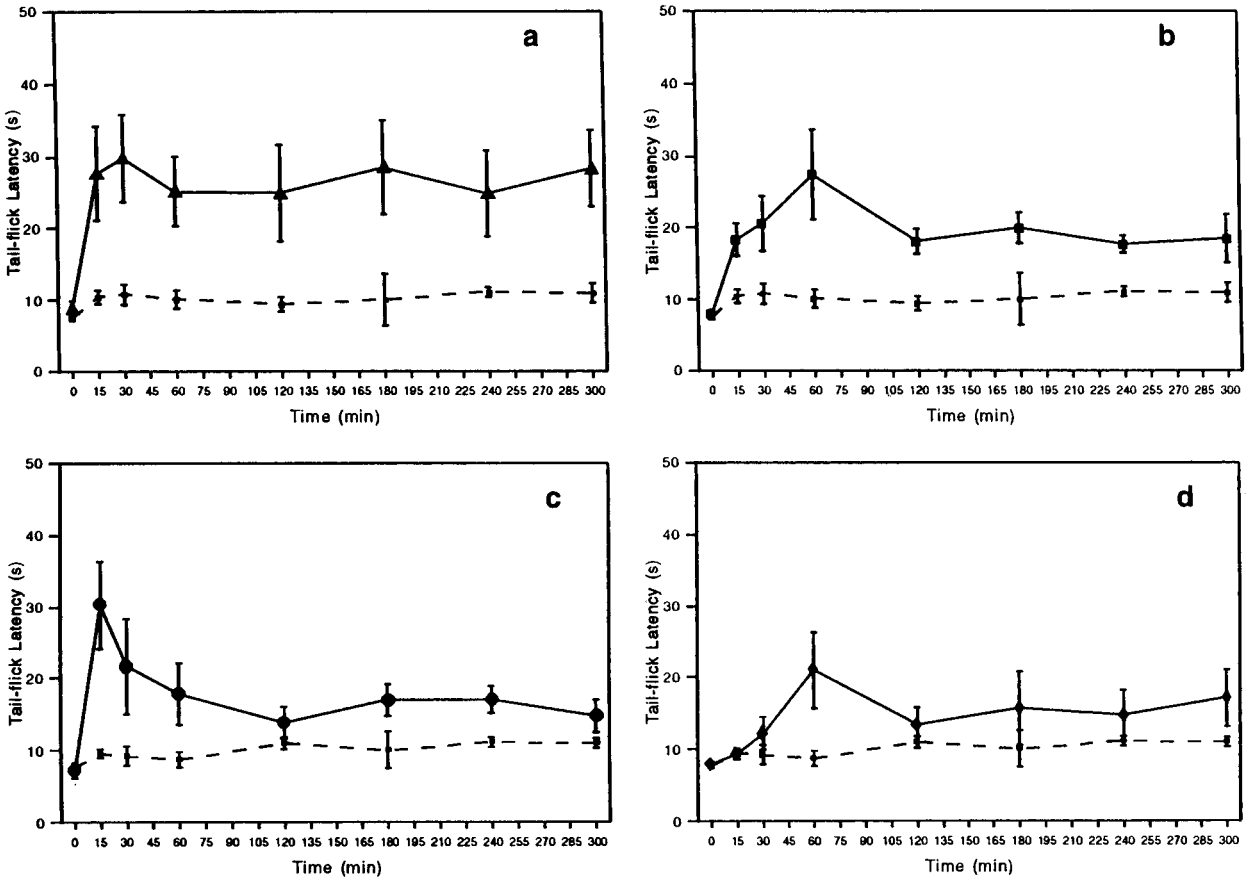


Figure 1. Analgesic activity of DADLE CDSs in rats, measured by the tail-flick latency, at 4.2 μmol/kg of body weight and intravenous injection: (a) compound **10a** (Lpf = Cho, S = Pro), (b) compound **10b** (Lpf = Cho, S = Ala), (c) compound **15a** (Lpf = Ada, S = Pro), and (d) compound **15b** (Lpf = Ada, S = Ala). Data are represented as average ± SEM. Dashed lines represent the control group (injection of vehicle).

12a,b in a solvent mixture of DMF/dioxane. After removing the volatiles, the crude products **13a,b** were carefully purified by column chromatography the same way as **8a,b**. Methylation of the pyridine ring (**14a,b**) and the subsequent reduction resulting in the CDSs **15a,b** followed the same routine we applied for method A.

We have also investigated another approach for the preparation of peptide CDSs, in which the direct esterification on the C-terminus of the entire heptapeptide Nic-S-DADLE-OH was studied. However, the ester bond formation either with cholesterol or with 1-adamantaneethanol proved to be very inefficient and not reproducible. Therefore we did not consider this route for obtaining peptide CDS.

***In Vitro* Stability and Receptor Binding.** Stability studies of the CDSs and their predicted biotransformation products have been performed in phosphate buffer, whole blood, and 20% (w/w) brain homogenate. An experimental formulation that involved inclusion complex of the CDS with a chemically modified cyclodextrin has also been developed. The CDSs were unstable, as expected, in biological fluid due to oxidation. All the quaternary intermediates (T^+-S-DADLE-OLpf (**9a,b/14a,b**) and T^+-S-DADLE-OH (**16a,b**)) were stable in the pH range 4−9; no decomposition occurred over 6 h. The oxidized CDS **9** had half-lives of around 2−3 min in whole blood and around 35−40 min in brain homogenate. The quaternary acids **16** were stable in whole blood and had a half-life of 4.9 h for **16a**, while **16b** was more stable ($t_{1/2}$ > 12 h). The sustained release of DADLE was unequivocally shown by electrospray ionization mass spectrometric analysis, as given in the Supporting Information.

The receptor binding study (competition with [^{3}H]-diprenorphine, a μ- and δ-receptor agonist) confirmed that **9** had very low ($IC_{50} \approx 6$ μM) affinity, while we measured weak binding (IC_{50} > 200 nM)[27] to opioid receptors for **16**, as compared to DADLE (IC_{50} = 36 nM). Competitive binding that involved a μ-selective radioligand ([^{3}H]DAGO) also showed about 2 orders of magnitude difference in affinity between DADLE and **16**. Therefore, it can be concluded that only a "real" chemical delivery of DADLE results in a profound antinociceptive response.

Analgesic Activity. The antinociceptive properties of the CDSs of DADLE (**10a,b** and **15a,b**) were evaluated by tail-flick latency measurement upon iv administration (4.2 μmol/kg of body weight), similar to those in the preliminary studies.[12,13] We observed a statistically significant and prolonged (more than 5 h) increase in the tail-flick latency for all of the tested CDSs, as revealed in Figure 1d. Compounds having proline as a spacer (**10a** and **15a**) reached their maximum analgesic effect earlier (between 15 and 30 min after the administration of the drug) than those containing an alanine spacer. The CDSs with the smaller, thus less lipophilic, ester group on the C-terminus (**15a,b**) showed lower analgesic activity compared to those with Cho.

We selected compound **10a** (S = Pro, Lpf = Cho), as the most potent CDS among those tested, for further study. Naloxone, an opiate antagonist,[28] was injected subcutaneously at a dose of 2.7 μmol/kg of body weight, and the tail-flick latency was monitored. The analgesic

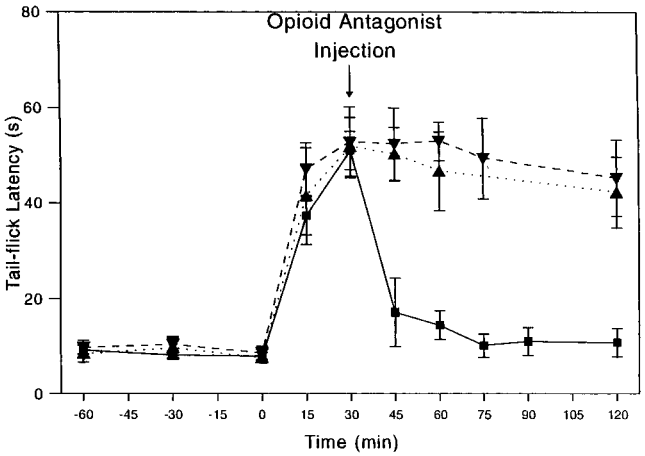


Figure 2. Reversal of the analgesia of **10a** (4.2 μmol/kg of body weight, iv, dotted line) by naloxone (2.7 μmol/kg of body weight, sc, solid line), but not by naloxonium (2.1 μmol/kg of body weight, sc, dashed line), in rats.

effect was completely antagonized by naloxone (Figure 2), providing evidence for the centrally mediated analgesia induced by the peptide CDS. This observation was further confirmed by administering naloxone methyl iodide, the quaternary derivative of naloxone that does not cross the BBB, under the same experimental conditions (Figure 2). However, the antinociceptive effect of the CDS was not reversed by the peripherally acting opioide antagonist.

Discussion

For the further refinement of an already successful targeting of DADLE to the brain[12,13] by a CDS approach, we have evaluated the spacer (Ala or Pro) and the size/lipophilicity of the ester group (Cho or Ada) for the efficacy of the CDSs to induce CNS-mediated analgesia upon iv administration. For these studies, it was necessary to develop an effective synthesis strategy for obtaining peptide CDSs. We have successfully applied the segment-coupling strategy for a fast and straightforward synthesis of this type of peptide conjugate. Both methods reported here (methods A and B) are also equally effective and reproducible for large scale preparation without frequent, time-consuming purifications of the pendant peptide chain, independently of the size of Lpf. Even using a solely solution phase approach involving active ester (method A), the side chain protection of tyrosine can be avoided,[29] which further simplifies the procedure and the purification of the key compounds Nic-S-DADLE-OLpf. The purity of these peptide esters with a Nic residue on the N-terminus (**8a,b**, and **13a,b**) is crucial for obtaining pure CDSs. Although the remaining two subsequent reaction steps (methylation and reduction) leading to the peptide CDS are straightforward, the chromatographic purification of their product, **9a,b/14a,b** and **10a,b/15a,b**, respectively, is cumbersome and very inefficient. Using pure precursors (Nic-S-DADLE-OLpf), however, the CDSs with excellent purity can be obtained without troublesome purification.

The CDSs were tested for analgesic efficacy based on tail-flick latency measurements in rats. All of the compounds showed a profound, long-lasting analgesia as illustrated in Figures 1 and 2. Our studies revealed that the packaged peptide and the intermediates formed during the sequential metabolism ultimately leading to the release of DADLE had weak affinity to opioid receptors. Thus, the observed analgesia was mostly due to the enkephalin analogue liberated at the site of action. The selection of S and Lpf can greatly influence the efficacy of the CDS. The lipophilicity/size of Lpf is evidently a basic requirement for the successful delivery of the peptide through the BBB. Compounds with Cho on the C-terminus produced higher tail-flick latencies compared to those having Ada. Consequently, the size of Lpf *per se* has an important contribution to the successful delivery of DADLE. (Ada should provide enough lipid solubility to the peptide conjugate; the calculated log *P* values are >2 for **15a,b**.)[30] On the other hand, the rate of the release of DADLE is most significantly influenced by the spacer. For the proline spacer containing CDSs, the maximum of the antinociceptive effect was reached earlier than for those containing the Ala spacer, independently of the size of Lpf, due to the different affinity of Pro and Ala to the dipeptidyl peptidases.[20,21] The *in vitro* experiments with the relevant biotransformed intermediates **16a,b** complemented these observations. The measured $t_{1/2}$ in 20% brain homogenate is 3−4 times shorter for **16a** (S = Pro) than for **16b** (S = Ala). By manipulating the spacer, the analgesic efficacy of the CDS can, thus, be modified and predicted according to the therapeutic objective. Based on our experiments, for a potentially strong, fast-acting analgesic, the molecular packaging should contain S = Pro and Lpf = Cho functions. We have also shown that the antinociceptive effect of DADLE brain targeted by a CDS can completely be antagonized by naloxone, an opioid antagonist that crosses the BBB (Figure 2). This observation was complemented by an experiment using a quaternary analogue of naloxone that cannot cross the BBB and thus may interact only with peripheral opioid receptors. Indeed, the naloxonium salt did not reverse the analgesic effect of the tested compound. These results provide evidence for the exclusively centrally mediated analgesia induced by the CDS due to its specific design that delivered, retained, and then released DADLE in the brain.

In conclusion, the brain-targeted delivery of DADLE can be refined and optimized according to the therapeutic objective by manipulating the lipophilic moiety (Lpf), and the spacer (S) function that affects the rate of peptide release in the CNS. Furthermore, due to the rational design of the CDS, the induced analgesia is solely centrally mediated.

Experimental Section

Instruments and Materials. All chemicals used were of reagent or peptide synthesis grade. *N*-Hydroxysuccinimide esters of Boc-protected amino acids were obtained from Bachem Bioscience, Inc. (Philadelphia, PA). Solvents and other chemicals were purchased from Fisher Scientific, Inc. (Pittsburgh, PA). Fmoc-phenylalanine *p*-benzyl alcohol resin, 100−200 mesh, 0.78 mequiv/g on 1% DVB was purchased from Chem-Impex Int. (Wood Dale, IL). Naloxone hydrochloride and naloxone methiodide were obtained from Research Biochemicals International (Natick, MA). Thin layer chromatography was performed on silica gel-coated (E. Merck Kieselgel 60 F-254, 0.2-mm thickness) plates and developed with chloroform/MEOH, 9:1, v/v. Spots were located either with UV light or by treatment of the plate with HCl fumes followed by heating and subsequent spraying with ninhydrin. Organic solutions that had been previously extracted with aqueous solution were dried over anhydrous Na_2SO_4. Column chromatographies were performed using Davisil grade 643 silica gel. Melting points were determined on a Fisher-Johns

apparatus and uncorrected. Elemental analyses were supplied by Atlantic Microlabs, Inc. (Norcross, GA). FAB mass spectra were recorded on a Kratos MS80RFA instrument (Kratos Analyticals, Manchester, U.K.), xenon beam (8 keV energy), by dissolving the sample in 3-nitrobenzyl alcohol matrix with or without Na.[31] UV spectra were recorded in methanol on a Lambda 11 UV/vis Perkin Elmer (Perkin Elmer Anal. Inst., Norwalk, CT) spectrophotometer. A Synthor 2000, Peptide International (Louisville, KY), instrument was used for SPPS. RP-HPLC purification was performed on a system consisting of a ThermoSeparation/SpectraPhysics (Fermont, CA) Spectra Series P200 binary gradient pump, a Rheodyne (Cotati, CA) Model 7125 injector equipped with a 5-mL loop, and a SpectraPhysics Spectra 100 UV/vis detector set at 216 nm. The preparative column (25 mm × 100 mm cartridge with a Guard-Pak insert from Waters Chromatography) was packed with 15-μm Delta-Pak C_{18}. The mobile phase was mixed from 0.1% (v/v) TFA in H_2O and 0.08% (v/v) TFA in CH_3CN at a 5.0 mL/min flow rate.

Synthesis. Nicotinoylproline (Nic-Pro-OH, 1a). In 100 mL of DCM, 1.95 g (11 mmol) of nicotinoyl chloride hydrochloride and 2.1 g (10 mmol) of Pro-*t*Bu hydrochloride were dissolved. Under ice cooling, 4.32 mL (21 mmol) of TEA was added, and the cooling was removed. The solution was stirred overnight and then extracted with 40 mL of 1 N HCl (2×), 40 mL of saturated $NaHCO_3$ (2×), and brine. The organic layer was dried and concentrated *in vacuo*. The residue (*tert*-butyl ester) was diluted with an equivalent volume of TFA and stirred overnight. After evaporation of the volatiles, the residue was dissolved in water and neutralized with $NaHCO_3$. Then the aqueous phase was exhaustively extracted with EtOAc. The combined organic layer was dried and evaporated to a white, hygroscopic solid. After recrystallization from EtOAc/ether 2.83 g (92% yield) of white solid was obtained. **1a**: mp 88−92 °C; MS $[M + H]^+$ *m/z* 221. Anal. ($C_{11}H_{12}N_2O_3$) C, H, N.

Nicotinoylalanine (Nic-Ala-OH, 1b): prepared similarly to **1a** with 89% yield after recrystallization from EtOAc/ether; white, solid; mp 140−142 °C; MS $[M + H]^+$ *m/z* 195. Anal. ($C_9H_{10}N_2O_3$) C, H, N.

Nicotinoylproline Pentafluorophenyl Ester (Nic-Pro-OPfp, 2a). To the solution of 0.99 g (4.5 mmol) of **1a** in 40 mL of EtOAc was added 0.91 g (4.9 mmol) of pentafluorophenol under ice cooling followed by 1.00 g (4.9 mmol) of DCC. A precipitate formed almost immediately. After 30 min, the cooling was removed, and the solution was stirred for 2 h. The precipitate (urea) was filtered off and discarded. The filtrate was washed with 20 mL of 0.1 N HCl (2×), 20 mL of saturated $NaHCO_3$ (2×), and 20 mL of brine (2×). The organic layer was dried and evaporated. The obtained solid was recrystallized from EtOAc/ether resulting in 1.46 g (84% yield) of white solid: mp 65−68 °C; R_f = 0.88; MS $[M + H]^+$ *m/z* 387. Anal. ($C_{17}H_{11}N_2O_3F_5$) C, H, N,

Nicotinoylalanine pentafluorophenyl ester (Nic-Ala-OPfp, 2b): prepared as **2a** with 89% yield; recrystallized from EtOAc/hexane; white solid; mp 110 °C; R_f = 0.79; MS $[M + H]^+$ *m/z* 361. Anal. ($C_{15}H_9N_2O_3F_5$) C, H, N.

Boc-D-leucine Cholesteryl Ester (Boc-D-Leu-OCho, 3). The solution of 2.31 g (10.0 mmol) of Boc-D-leucine, 5.72 g (11 mmol) of PyBOP, and 4.4 mL (25 mmol) of DIEA in 200 mL of DCM was stirred under ice for 1 h; then 3.5 g (9 mmol) of cholesterol was added. The solution was stirred at room temperature overnight and then extracted with 50 mL of 0.1 N HCl (2×) and 50 mL of saturated $NaHCO_3$. The organic layer was dried and evaporated to an oil that slowly crystallized on standing. The crude product was recrystallized from EtOAc/hexane (89% yield) and used without further purification. An analytical sample was obtained after column chromatographic purification using chloroform containing 0.05% of TEA: white solid; mp 107−109 °C; R_f = 0.58; MS $[M + Na]^+$ *m/z* 623. Anal. ($C_{38}H_{65}NO_4 \cdot H_2O$) C, H, N.

General Procedure for Peptide Chain Elongation Using Amino Acid Active Ester (4−7). The Boc-protected compound (**3**−**6**) was deprotected with TFA/DCM (1:1, v/v) for 30 min at ice temperature; then the volatiles were removed by exhaustive evaporation. The residue was dissolved in the minimum amount of DCM and/or DMF and neutralized with TEA under ice cooling. The Boc-amino acid-OSu (15−20% excess to the lipophilic ester) was added, the cooling was removed, and the reaction mixture was stirred at room temperature for 1−2 days (monitored by TLC and/or FAB-MS). The solution was diluted with DCM and extracted with ice cold 1 N HCl (2×), saturated $NaHCO_3$ (2×), and brine (2×), respectively. The organic layer was dried and evaporated *in vacuo*. The crude peptide product was identified by FAB-MS and used after recrystallization from chloroform/ether. The yields (for crude peptides) were 70−80%.

Boc-DADLE-OCho (7). An analytical sample was obtained after column chromatographic purification using chloroform/MeOH (95:5, v/v) containing 0.1% TEA as an eluent; white solid; mp 121−122 °C dec; R_f = 0.42; MS $[M + Na]^+$ *m/z* 1060. Anal. ($C_{61}H_{91}N_5O_9 \cdot 1.5\ H_2O$) C, H, N.

Method A: (2 + 5) Segment Coupling (8a,b). Compound **7** (1 equiv) was deprotected with TFA/DCM (1:1, v/v) for 30 min at ice temperature; then the solvent was removed *in vacuo*. The residue was dissolved in the minimum amount of DMF and neutralized with TEA under ice cooling. Then 2 equiv of **2a** or **2b** dissolved in a small amount of DMF was added. The cooling was removed, and the solution was stirred at room temperature. The reaction was monitored by TLC. Then the volatiles were removed *in vacuo*, and the residual oily material was purified by column chromatography using chloroform/MEOH (9:1, v/v) containing 0.1% TEA.

Nic-Pro-DADLE-OCho (8a): white solid; mp 175−179 °C; R_f = 0.41; MS $[M + H]^+$ *m/z* 1140. Anal. ($C_{67}H_{93}N_7O_9 \cdot H_2O$) C, H, N.

Nic-Ala-DADLE-OCho (8b): white solid; mp >200 °C; R_f = 0.36; MS $[M + H]^+$ *m/z* 1114. Anal. ($C_{65}H_{91}N_7O_9 \cdot H_2O$) C, H, N.

Boc-D-leucine Adamantylethyl Ester (Boc-D-Leu-OAda, 11). 11 was Prepared similarly to **3** with 92% yield. An analytical sample was obtained after column chromatographic purification: oily material; R_f = 0.49; MS $[M + Na]^+$ *m/z* 415. Anal. ($C_{23}H_{39}NO_4 \cdot 0.25\ H_2O$) C, H, N.

Solid Phase Peptide Synthesis (12a,b). Fmoc-Phe resin (3.0 g, 2.34 mmol) was placed in a 250-mL reaction vessel, and the stepwise SPPS was carried out as follows: (1) deprotection with a 9-min cycle of piperidine/NMP (1:1, v/v), (2) washing, NMP (3 × 2 min), MeOH (3 × 2 min), and NMP (3 × 2 min), (3) coupling, Fmoc-amino acid (2.5 equiv), DCC (2.5 equiv), and HOBt (2.5 equiv) in NMP, and (4) washing, NMP (3 × 2 min), MeOH (3 × 2 min), and NMP (3 × 2 min). The coupling time was set for 4−10 h, and usually double coupling was applied. The completeness of the reaction was monitored by the Kaiser test.[32] After the final coupling the resin was washed as before and dried *in vacuo*. Cleavage was effected by TFA/H_2O (95:5, v/v) for 90 min. The solution was concentrated; the residue was dissolved in glacial acetic acid and freeze-dried. The crude peptide was shown to be about 90% pure by analytical RP-HPLC. Compound **12** was used without purification. Analytical samples were obtained after preparative RP-HPLC purification using gradient elution.

Nic-Pro-Tyr-D-Ala-Gly-Phe-OH trifluoroacetate (12a): no definite melting point; MS $[M + H]^+$ *m/z* 659. Anal. ($C_{36}H_{38}N_6O_{10}F_3 \cdot 0.25TFA \cdot 0.75H_2O$) C, H, N.

Nic-Ala-Tyr-D-Ala-Gly-Phe-OH trifluoroacetate (12b): no definite melting point; MS $[M + H]^+$ *m/z* 633. Anal. ($C_{34}H_{36}N_6O_{10}F_3 \cdot 0.5\ TFA \cdot H_2O$) C, N, H.

Method B: (6 + 1) Segment Coupling (13a,b). In a solvent mixture of DMF and dioxane, 1 equiv of unpurified **12a** or **12b**, 1.1. equiv of PyBOP, and 3.5 equiv of DIEA were dissolved. The solution was stirred at ice temperature for ca. 1 h. In the meantime, in a separate flask **11** was deprotected in a customary manner. After removing the solvents, the residue (TFA salt of Leu ester) was dissolved in a minimum amount of DMF and neutralized with DIEA. This solution was added to the coupling milieu. The reaction mixture was stirred at room temperature overnight and then evaporated to an oily residue and purified similarly to **8a,b**.

Nic-Pro-DADLE-OAda (13a): white solid; mp 155−165 °C dec; R_f = 0.56; MS $[M + H]^+$ *m/z* 934. Anal. ($C_{52}H_{67}N_7O_9 \cdot 1.75\ H_2O$) C, H, N.

Nic-Ala-DADLE-OAda (13b): white solid; mp 220−223 °C; $R_f = 0.53$; MS $[M + H]^+$ m/z 908. Anal. ($C_{50}H_{65}N_7O_9 \cdot 1.5\ H_2O$) C, H, N.

General Procedure for Preparation of T$^+$-S-DADLE-OLpf (9a,b and 14a,b). Compound Nic-S-DADLE-OLpf (1 equiv) was dissolved in chloroform/MeOH, and ca. 20 equiv of MeI was added. The solution was stirred at room temperature overnight. Ether was added, and the obtained yellow, pasty precipitate was filtered off and recrystallized (2×) from chloroform/MeOH (8:2, v/v) and EtOAc. The product was obtained quantitatively.

Trigonellyl-Pro-DADLE-OCho iodide (9a): yellow solid; mp 195−197 °C dec; $R_f = 0$; MS Q^+ (quaternary pyridinium ion) m/z 1154; UV_{max} 264 nm. Anal. ($C_{68}H_{96}N_7O_9I \cdot 1.5\ H_2O \cdot 0.5$ MeI) C, H, N, I.

Trigonellyl-Ala-DADLE-OCho iodide (9b): yellow solid; mp 164−166 °C dec; $R_f = 0$; MS Q^+ m/z 1128; UV_{max} 264 nm. Anal. ($C_{66}H_{94}N_7O_9I \cdot 1.5\ H_2O \cdot 0.5$ MeI) C, H, N, I.

Trigonellyl-Pro-DADLE-OAda iodide (14a): yellow solid; mp 145−149 °C (dec); $R_f = 0$; MS Q^+ m/z 948; UV_{max} 264 nm. Anal. ($C_{53}H_{70}N_7O_9I \cdot 1.75\ H_2O$) C, H, N, I.

Trigonellyl-Ala-DADLE-OAda iodide (14b): yellow solid; mp 173−173 °C (dec); $R_f = 0$; MS Q^+ m/z 922; UV_{max} 264 nm. Anal. ($C_{51}H_{68}N_7O_9I \cdot 1.5\ H_2O$) C, H, N, I.

General Procedure for Sodium Dithionite Reduction (10a,b and 15a,b). The pyridinium salts (1 equiv), **9a,b**, and **14a,b** were dissolved in a mixture of ethanol/water (ca. 1:1, v/v). The solution was kept under ice cooling and degassed with continuous nitrogen bubbling for ca. 20 min before the mixture of $Na_2S_2O_4$ (4−5 equiv) and Na_2CO_3 (6−7 equiv) was added. The pH of the reaction mixture was kept around 7.0−7.2. The cooling and the nitrogen bubbling continued during the reduction (3−4 h) monitored by TLC and UV spectroscopy. Then the reaction mixture was diluted with ice cold, degassed chloroform and extracted with ice cold, degassed water. The layers were separated under a nitrogen atmosphere. The organic layer was dried and carefully evaporated to a pale yellow solid that was recrystallized from degassed chloroform/MEOH (8:2, v/v) and ether in an inert atmosphere. The yield was around 60%.

1,4-Dihydrotrigonellyl-Pro-DADLE-OCho (10a): $R_f = 0.53$; MS $[M + Na]^+$ m/z 1177; UV_{max} 360 nm.

1,4-Dihydrotrigonellyl-Ala-DADLE-OCho (10b): $R_f = 0.48$; MS $[M + Na]^+$ m/z 1151; UV_{max} 360 nm.

1,4-Dihydrotrigonellyl-Pro-DADLE-OAda (15a): $R_f = 0.34$; MS $[M + Na]^+$ m/z 971; UV_{max} 360 nm.

1,4-Dihydrotrigonellyl-Ala-DADLE-OAda (15b): $R_f = 0.34$; MS $[M + Na]^+$ m/z 945; UV_{max} 360 nm.

Trigonellyl-Pro-DADLE-OH trifluoroacetate (16a): prepared by SPPS similarly to **12**; MS Q^+ m/z 786. Anal. ($C_{43}H_{52}N_7O_{11}F_3 \cdot 2\ H_2O$) C, H, N.

Trigonellyl-Ala-DADLE-OH trifluoroacetate (16b): prepared by SPPS similarly to **12b**; MS Q^+ m/z 760. Anal. ($C_{41}H_{50}N_7O_{11}F_3 \cdot 1.5\ H_2O$) C, H, N.

***In Vitro* Stability and Metabolism Studies**. Approximately 250 nmol of CDS or its oxidized form (T^+), dissolved in DMSO, or 30 nmol of **16** was added to 1 mL of rat brain homogenate (20%, w/w, in pH 7.4 Tris buffer), respectively, and the mixture was incubated at 37 °C in a temperature-controlled, shaking water bath. Aliquots (100 μL) were removed after 5, 15, 30, 45, 60, and 90 min of incubation, respectively, and transferred to 1.5-mL plastic centrifuge tubes containing 200 μL of ice cold 1 M aqueous acetic acid. After centrifugation at 12500*g* for 15 min, the supernatant was removed and analyzed by microbore HPLC. Analyses were done on a system consisting of a ThermoSeparation/Spectra-Physics (Fremont, CA) Spectra Series P200 binary gradient solvent delivery system, a Rheodyne (Cotati, CA) Model 7125 injector valve equipped with a 5-μL sample loop, a Spectroflow 757 variable wavelength UV/vis detector (Kratos Analytical, Manchester, U.K.) operated at 216 nm, and a Hewlett-Packard Model HP 3395 computing integrator (Palo Alto, CA). A 30 cm × 1.0 mm i.d. Supelcosil LC-18 (5 μm) reversed phase column (Supelco, Bellefonte, PA) was used at a flow rate of 50 μL/min that was maintained by using a dynamic split (before the injection valve) via a balance column (25 mm × 4.6 mm i.d) connected in parallel with the microbore column. The mobile phase was mixed from 0.1% (v/v) TFA in water and 0.08% (v/v) TFA in acetonitrile, and gradient elution was done from 5% to 95% organic modifier that changed in a linear profile at a rate of 1%/min. HPLC calibration curves for determining concentrations were obtained by adding known amounts of peptide into aliquots of brain homogenate transferred into ice cold 1 M acetic acid solution and analyzing the supernatant after centrifugation. Concetration−time profiles were analyzed by exponential fitting, assuming a pseudo-first-order degradation. Half-lives ($t_{1/2}$) were calculated from the rate constants (k) as $0.693/k$. Representative samples were desalted on a reversed phase minicolumn (1-mL Supelclean LC-18, Supelco, Bellefonte, PA) and analyzed by electrospray ionization mass spectrometry (Vestec/PerSeptive Biosystems, Model 200 ES instrument) to ascertain the release of DADLE from **16**.

Opioid Receptor Binding. Opioid receptor assays used rat brain membrane preparations and measured binding affinities versus known potent ligands with standardized rapid-filtration techniques. Adult male Sprague−Dawley rats (200−220 g) were decapitated, the brain was removed and chilled on ice, and then tissue was suspended in about 30 mL of cold 50 mM Tris HCl buffer containing 100 mM NaCl and adjusted to pH 7.4 with HCl. The suspension was homogenized in a motor-driven Potter-Evjen tissue grinder, and the homogenate was centrifuged at 2000 rpm for 15 min at 5 °C. The pellet was resuspended in sodium-free Tris HCl buffer, washed twice in the same media before the suspension was aliquoted, and stored frozen at −50 °C. Receptor binding affinity was determined from competitive displacement with potent opioid agonists [^{3}H]diprenorpine (μ and δ) or [^{3}H]DAGO (μ-selective). The brain preparation was diluted with Tris HCl buffer to a final concentration of 0.5% and preincubated to remove endogenous opioids. The brain receptor preparation (100 μL aliquots) was then incubated with labeled opioid agonist (0.5−1.0 nM) in a total volume of 1 mL of 50 mM Tris HCl, pH 7.4, containing 30 μM bestatin, 0.6 μM tiorphan, 10 μM captopril, and 2 μM Leu-Leu for 3 h at 25 °C.[33] The samples were filtered with a Brandel cell harvester through GF/B Whatman glass-fiber filter strips pretreated with 0.1% poly(ethylenimine) solution. Three washes with ice cold saline were included before assaying bound radioactivity by liquid scintillation spectrophotometry. Nonspecific binding was determined in the presence of excess naloxone (1 μM). The IC_{50} values were calculated from standard curves of $\%R^x$ versus concentration of the added peptide:

$$\%R^x = 100[(Cpm^x - Cpm^N)/(Cpm^L - Cpm^N)]$$

where Cpm^L is the mean value with only the radiolabeled ligand and $\%R^L = 0$ and Cpm^N is the mean value with ligand and excess naloxone and $\%R^N = 100$.

Pharmacology. CDSs were evaluated for CNS-mediated analgesia in Sprague−Dawley rats (250−350 g of body weight). The latency of the tail-flick response to radiant heat (focused in the middle of the tail) was measured by using a Tail-Flick Analgesia Meter 0570-001L (Columbus Instruments International Corp., Columbus, OH). Base-line measurements (2−3) were collected prior to drug administration, and tail-flick latency was measured at various times up to 6 h following drug administration. Groups of 5−10 animals were treated with vehicle control, the solution of the unmodified peptide, and its CDSs, respectively. The peptide and CDSs were administered in 4.2 μmol/kg of body weight doses. For the study involving the opioid antagonist naloxone and its quaternary methyl analogue, 15 rats (divided into three groups of five) were injected with a dose of 4.2 μmol/kg of body weight CDS, iv, in 0.1 mL of vehicle solution. After 30 min, animals in two groups were injected subcutaneously with naloxone and naloxone methiodide at a dose of 1 mg/kg of body weight, respectively, and the tail-flick latency was monitored for an additional 90 min in 30-min intervals. Data generated from the studies comparing treatment groups were subjected to analysis of variance (ANOVA) followed by Dunnett's procedure to detect significant differences between groups with $p > 0.05$ selected as the level of significance.

Formulation of the CDS. The freshly prepared compounds **10a,b** and **15a,b** were dissolved in the mixture (9:1,

v/v) of (2-hydroxypropyl)-β-cyclodextrin (HPβCD)[34] (50%, w/v, aqueous solution) and DMSO[35] for the tail-flick latency measurements. We have also studied the solubility of our peptide CDSs in HPβCD for optimizing their formulation. The cyclodextrin inclusion complexes were prepared by equilibrating an excess of CDSs with a 50% (w/v) aqueous solution of HPβCD by the following way: To the degassed solution of HPβCD was added a known amount of CDS. The suspension was sonicated for 30 min under ice cooling and then filtered, and the filtrate was lyophilized. The CDS content of the complexes was analyzed. The degree of complexation was around 20 mg/g.

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Supporting Information Available: Electrospray ionization mass spectrometric analysis showing the release of DADLE in rat brain homogenate *in vitro* (1 page). Ordering information is given on any current masthead page.

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