response after administration of the test compound was expressed as a percent of the predrug response.

Crystal Structure of 21c. Colorless prism crystals were grown by slow evaporation of a solution in EtOH and mounted on a fully automated Rigaku AFC-5 X-ray diffractometer using Cu Kα radiation. The unit cell parameters were $a = 11.785$ (1) Å, $b = 7.4047$ (4) Å, $c = 12.687$ (1) Å, and $\beta = 103.13$ (1)° in space group $P2_1$ ($Z = 2$). Of the 2158 reflections measured with $2\theta \leq 130°$ employing a $2\theta/\omega$ scan, 1732 were independently observed at a level $F \geq 3\sigma$ (F). A partial solution was found by MULTAN78[21] and expanded into a complete structure by a series of Fourier and difference electron density syntheses. Refinement was carried out with block-diagonal least-squares with anisotropic temperature factors for nonhydrogen atoms. Hydrogen atoms were assigned equivalent isotropic temperature factors for the atoms to which they were bound and refined for positional parameter variation only. The final residual index (R factor) was 0.059. Calculations were carried out with the DIRECT-SEARCH program system.[22]

Registry No. 6, 20887-95-0; 7, 102-96-5; 8, 34312-77-1; 9, 110143-52-7; 10 (isomer 1), 72150-56-2; 10 (isomer 2), 72150-54-0; 11 (isomer 1), 110221-19-7; 11 (isomer 2), 110221-65-3; 12 (isomer 1), 110221-20-0; 12 (isomer 2), 110221-66-4; 13 (isomer 1), 110143-53-8; 13 (isomer 2), 110143-71-0; 14 (isomer 1), 110221-21-1; 14 (isomer 2), 110221-22-2; 15 (isomer 1), 110221-22-2; 15 (isomer 2), 110221-68-6; 16 (isomer 1), 110143-54-9; 16 (isomer 2), 110221-69-7; 17 (isomer 1), 110221-23-3; 17 (isomer 2), 110221-70-0; 18 (isomer 1), 110221-24-4; 18 (isomer 2), 110221-71-1; 19a, 110143-55-0; 19a·HCl, 110221-30-2; 19b, 110221-25-5; 19b·HCl, 110267-53-3; 19c, 110221-26-6; 19c·HCl, 110267-54-4; 19d, 110221-27-7; 19d·HCl, 110267-55-5; 19e, 110221-28-8; 19e·HCl, 110267-56-6; 19f, 110221-29-9; 19f·HCl, 110267-57-7; 20, 88767-98-0; 21a, 110143-56-1; 21b, 110221-31-3; 21c, 110143-57-2; 21d, 110221-32-4; 21e, 110143-58-3; 21f, 110221-33-5; 22a, 110221-35-7; 22b, 110221-36-8; 22c, 110221-37-9; 22d, 110221-38-0; 22e, 110221-39-1; 22f, 110221-40-4; 23a·HCl, 110221-42-6; 23b·HCl, 110221-43-7; 23d·HCl, 110221-45-9; 23e·HCl, 110221-46-0; 23f·HCl, 110221-47-1; 24a, 110221-51-7; 24b, 110221-52-8; 24c, 110221-53-9; 24d, 110221-54-0; 24e, 110221-55-1; 24f, 110221-56-2; 25, 110143-61-8; 26a, 110143-62-9; 26b, 102089-75-8; 27a, 110173-68-7; 27b, 110143-60-7; 28a, 110221-58-4; 28b, 110143-63-0; 29a, 110221-59-5; 29a·HCl, 110267-59-9; 29a·HBr, 110267-60-2; 29b, 110143-64-1; 29b·HBr, 110221-60-8; 30a, 110143-59-4; 30b, 110267-58-8; 30b·HCl, 110221-34-6; 31a, 110221-41-5; 31b, 102208-40-2; 32a·HCl, 110221-48-2; 32b·HCl, 110221-49-3; 33a, 110221-57-3; 33b, 102208-42-4; 34, 102089-87-2; 35, 102089-97-4; 36, 102089-98-5; 37, 102089-99-6; (3*S*,6*R*)-38, 102090-01-7; (3*S*,6*S*)-38, 110221-61-9; 39, 102090-02-8; 40, 102090-03-9; 40 maleate, 110221-62-0; 41, 102208-46-8; 42·HCl, 110221-50-6; 43, 102090-06-2; 45, 110143-65-2; -(*R*)-46, 110143-66-3; (*S*)-46, 110143-67-4; 47, 29678-81-7; 48, 90315-82-5; 49a, 110143-68-5; 49b, 110221-63-1; 49c, 110143-69-6; 49d, 110221-64-2; 50, 110143-70-9; L-cysteine, 52-90-4; diphenyl phosphorazidate, 26386-88-9; *N*-carbethoxynaphthalimide, 22509-74-6; *S*-[(*R*)-2-amino-2-phenylethyl]-*N*-[(benzyloxy)carbonyl]-L-cysteine-trifluoroacetic acid salt, 110173-70-1; *S*-[(*S*)-2-amino-2-phenylethyl]-*N*-[(benzyloxy)carbonyl]-L-cysteine-trifluoroacetic acid salt, 110173-72-3; *tert*-butyl bromoacetate, 5292-43-3; (*E*)-benzylidenepyruvic acid, 1914-59-6; *l*-menthol, 2216-51-5; angiotensin-converting enzyme, 9015-82-1.

Supplementary Material Available: Tables listing X-ray diffraction study data of 21c (5 pages). Ordering information is given on any current masthead page.

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Hybrid Bivalent Ligands with Opiate and Enkephalin Pharmacophores

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Bivalent ligands consisting of oxymorphamine and [D-Glu²]enkephalin pharmacophores linked through a spacer attached to the 6-amino group of the former and D-Glu of the latter were synthesized in an effort to investigate the possible coexistence of μ and δ recognition sites in the same opioid receptor complex. Of the two bivalent ligands (1, 2) synthesized, only 1 had substantially greater antinociceptive potency in mice than its monovalent analogues (1a, 1b). Testing of 1, 1a, and 1b in the guinea pig ileum preparation (GPI) revealed a potency profile similar to that found in vivo, whereas no correlation was observed in the mouse vas deferens (MVD). Binding data indicated the same rank-order affinities at δ receptors as the opioid activities in the GPI and in mice. However, μ binding exhibited no relationship with activity. These results are consistent with the simultaneous occupation of μ and δ by a single bivalent ligand 1, but they are also in harmony with the interaction of 1 with an opioid receptor and an accessory binding site.

Several lines of evidence suggest that μ and δ opioid receptors coexist as distinct recognition sites on an opioid receptor complex in the brain.[1-3] Moreover, it has been proposed that the observed potentiation of morphine analgesia by leucine enkephalin occurs through a coupling mechanism that links the μ receptor to the effector system.[3]

If μ and δ receptors are indeed located within the same complex, it is conceivable that a "bivalent ligand"[4] containing a μ-selective opiate and a δ-selective enkephalin pharmacophore could possess analgesic activity that is

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considerably greater than the sum of the individual activities if both opioid sites are capable of being occupied simultaneously. Here we report the synthesis and opioid activity of two bivalent ligands that contain opiate and enkephalin pharmacophores.

Design Rationale and Chemistry

A consideration in the design of the target compounds was the point of attachment of the spacer that connects the pentapeptide to the opiate structure. In view of the fact that replacement of Gly[2] of enkephalin with a variety of D-amino acids affords enhanced δ opioid activity, we have employed the γ-carboxyl group of D-Glu[2] as the point of attachment to the opiate, either directly or through a diglycyl spacer. In this regard, we avoided connecting the two pharmacophores through the terminal carboxyl group of Leu, since modification at this end has been reported[5] to confer a greater degree of μ relative to δ receptor activity to the enkephalins.

In order to determine whether or not bivalency actually enhances opioid activity, the closely related monovalent enkephalins **1a** and **2a** and the monovalent opiates **1b** and **2b**[6] were compared with their bivalent counterparts.

H-Tyr-D-Glu-Gly-Phe-Leu-OH

1, n=0
2, n=2

H-Tyr-D-Glu-Gly-Phe-Leu-OH

1a, n=0
2a, n=2

1b, n=0
2b, n=2
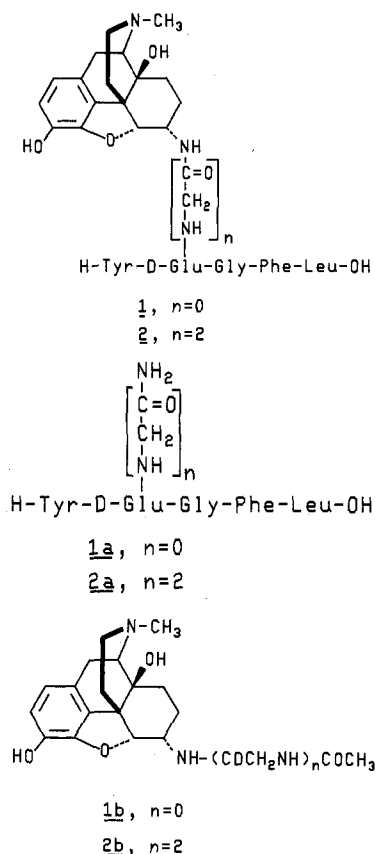
Both the bivalent ligands and the corresponding monovalent reference peptides were synthesized by the solid-phase technique. The C-terminal tripeptide segment was assembled with *tert*-butyloxycarbonyl (Boc) protection and with dicyclohexylcarbodiimide (DCC) as coupling agent. For the preparation of compounds **1**, **2**, and **2a**, N^α-Fmoc-D-glutamic acid *tert*-butyl ester was then coupled to the peptide chain. After deprotection of the D-Glu-γ-carboxy group by TFA treatment, α-oxymorphamine[7] in

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Table I. Opioid Receptor Binding of Oxymorphamine- and Enkephalin-Containing Ligands[a]

compd	[³H]DAGO: K_i^μ, nM	[³H]DSLET: K_i^δ, nM
1	19.1 ± 1.6	25.5 ± 0.3
1a	21.7 ± 0.5	47.2 ± 2.7
1b	1.81 ± 0.09	163 ± 2
2	11.1 ± 2.0	50.6 ± 1.3
2a	52.9 ± 7.7	87.8 ± 37.7
2b	5.48 ± 0.23	181 ± 40
[Leu⁵]enkephalin	9.43 ± 2.07	2.53 ± 0.35

[a] Mean of three determinations ± SEM.

Table II. Opioid Potencies of Oxymorphamine- and Enkephalin-Containing Ligands on the Guinea Pig Ileum (GPI) and Mouse Vas Deferens (MVD) Preparation[a]

compd	GPI: IC_{50}, nM	MVD: IC_{50}, nM
1	7.5 ± 0.4	26.9 ± 6.1
1a	210 ± 17	123 ± 33
1b	136 ± 48	166 ± 81
2	57.2 ± 1.5	47.7 ± 3.7
2a	597 ± 60	61.8 ± 7.5
2b	82.3 ± 19.3	78 ± 23
[Leu⁵]enkephalin	213 ± 40	8.6 ± 3.7

[a] Mean of three or more determinations ± SEM.

1.5-fold excess was coupled to the D-Glu side chain in the case of compound **1**, with DCC as coupling agent. For the synthesis of bivalent ligand **2**, D-Glu side-chain deprotection was followed by DCC coupling of glycine *tert*-butyl ester, TFA deprotection, coupling of a second glycine *tert*-butyl ester, TFA deprotection, and final coupling of α-oxymorphamine with DCC as coupling agent. Analogously, the monovalent reference compound **2a** was prepared by successive extension of the D-Glu side chain with glycine *tert*-butyl ester and glycinamide. Subsequently, N^α-Fmoc protection was removed by treatment with piperidine, and the peptide chains of compounds **1**, **2**, and **2a** were completed by coupling Boc-Tyr(OBzl)-OH. In the case of the monovalent reference peptide **1a**, the C-terminal tripeptide segment was extended with Boc-D-glutamine with DCC/HOBt as coupling agents, followed by TFA deprotection and coupling of Boc-Tyr(OBzl)-OH. After final removal of the Boc group, the peptides and peptide hybrids were cleaved from the resin and deprotected by HF treatment in the usual manner. Following gel filtration of the crude products on Sephadex G-25, the compounds were purified by reversed-phase chromatography and characterized by amino acid analysis and FAB mass spectrometry.

Biological Results

Binding. Opioid receptor affinities were determined by displacement of radiolabeled ligands from brain membrane preparations.[8] The radioligands employed were [³H]DAGO (μ-selective) and [³H]DSLET (δ-selective). The binding was expressed as K_i values (Table I).

Both the monovalent and bivalent ligands bound more strongly to μ receptors than to δ receptors. The monovalent opiates **1b** and **2b** possessed substantially more affinity than the monovalent peptides **1a** and **2a** for the μ sites. Conversely, the peptides **1a** and **2a** bound more avidly to δ sites. Neither of the bivalent ligands (**1**, **2**) possessed greater affinity than their monovalent analogues (**1a** and **1b**; **2a** and **2b**) at μ binding sites. While greater affinity was observed at δ sites for **1** and **2**, this amounted

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Table III. K_e Values for Naloxone in GPI Assay[a]

compd	K_e, nM	compd	K_e, nM
1	1.02 ± 0.04	2	1.32 ± 0.27
1a	1.63 ± 0.21	2a	2.32 ± 0.17
1b	0.88 ± 0.04	2b	1.55 ± 0.06
		[Leu⁵]enkephalin	1.53 ± 0.43

[a] Mean of three determinations ± SEM.

Table IV. Analgesic Potency of Opiate–Enkephalin Bivalent and Monovalent Ligands in Mice

compd	ED_{50},[a] pmol/mouse	compd	ED_{50},[a] pmol/mouse
1	0.7 (0.4–1.8)[b]	2	5.0 (3.4–6.7)[b]
1a	84 (64–114)[c]	2a	60 (30–100)[c]
1b	5.4 (4.4–6.5)[b]	2b	3.1 (2.6–3.8)[b]
		oxymorphone	24 (22–26)[c]

[a] Administered icv and determined at peak times for analgesia of 10 or 20 min. [b] Twenty-minute peak time. [c] Ten-minute peak time.

to a factor of only 2–3 over the corresponding more avid monomers 1a and 2a.

Smooth Muscle Preparations. The target compounds were tested on the electrically stimulated guinea pig ileum[9] (GPI) and mouse vas deferens[10] (MVD) preparations. The opioid agonist potencies expressed as IC_{50} values are listed in Table II. The K_e values for naloxone vs. these ligands in the GPI were determined in order to distinguish between μ- and δ-mediated effects (Table III). All compounds tested showed K_e values for naloxone between 0.9 and 2.3 nM, which are typical for μ-receptors interactions and rule out κ receptors, which would result in much higher K_e values (>10 nM).[11,12]

In the GPI, bivalent ligand 1 was found to be the most potent agonist among the target compounds. In this regard it was 28- and 18-fold more potent than the monovalent analogues 1a and 1b. No comparable potency enhancement of bivalent ligand 2 relative to its monomers was observed.

In the MVD, compound 1 shows a qualitatively similar profile, as it was found that both of its monovalent analogues (1a and 1b) have approximately one-sixth the potency. Also, the other bivalent ligand 2 is only slightly more potent than either of its monomeric analogues 2a and 2b.

Antinociceptive Assay. The target compounds were administered by the icv route to male Swiss-Webster mice and the antinociceptive activity was determined at peak times (10 or 20 min) by the acetic acid writhing procedure.[13] The antinociceptive potencies (Table IV) reveal that bivalent ligand 1 is substantially more potent than either of its monovalent analogues (1a, 1b). On the other hand, bivalent ligand 2 is more potent than only one (2a) of its two analogues.

In order to determine whether the potency enhancement of the bivalent ligand 1 was due to simultaneous interaction at two vicinal sites or to potentiation due to univalent interaction of the opiate and enkephalin pharmacophores of individual molecules, a mixture of the two monovalent ligands (1a and 1b) was tested.[14] This mixture, which

consisted of a 16:1 ratio of 1a:1b, displayed on ED_{50} of 0.051 (0.039–0.069) nmol/mouse at a peak activity time of 20 min. Since the theoretical[15] ED_{50} of 0.044 nmol/mouse is within the 95% confidence limits of the experimental ED_{50}, we conclude that the potencies of 1a and 1b are additive with no potentiation involved.

Discussion

In comparing the in vivo opioid potencies of the monovalent opiates (1b, 2b) it can be noted that they are comparable (Table IV). A similar relationship exists for the monovalent enkephalins 1a and 2a. This suggests that the spacer does not contribute significantly to the pharmacologic effect of the pharmacophores. Moreover, the data reveal that the opiate pharmacophore is approximately 20-fold more potent than the enkephalin pharmacophore in these monovalent ligands. Thus, any evaluation of in vivo potency enhancement in the bivalent ligand should involve a comparison with its corresponding monovalent opiate. Such a comparison (1 vs. 1b; 2 vs. 2b) indicates that there is a potency enhancement in 1 but not 2. This enhancement amounts to a factor of approximately 8. The additive effect of the combined icv administration of monovalent ligands 1a and 1b indicates that the observed potency enhancement for the bivalent ligand 1 is not due to a potentiation arising from the interaction of each of its pharmacophores with opioid receptors in a univalent mode.

The fact that bivalent ligand 2 does not exhibit enhanced in vivo potency is attributed to the glycylglycyl spacer that connects the pharmacophores. Apparently, this spacer separates the pharmacophores in such a way as to prevent interaction of the enkephalin pharmacophore with a binding site that is vicinal to the site that binds the opiate pharmacophore.

It is interesting that the rank order antinociceptive potencies of the target compounds in series 1 correlate with the opioid potencies in the GPI and MVD preparations. In fact, the structure–activity profiles of 1, 1a, and 1b in the smooth muscle preparations and in vivo closely parallel one another. This is in contrast to that found in rat brain membranes where 1b possesses about 10-fold greater affinity than either 1a or the bivalent ligand 1. Only δ binding showed correlation with the in vivo data. The reason for the absence of such correlation of the ligands in series 1 is not known. However, the fact that μ binding is greatly decreased upon attachment of the enkephalin pharmacophore to the opiate component, while the reverse occurred at δ receptors, demonstrates that the combined pharmacophores influence the selectivity of binding in a predictable way.

One explanation for the good agreement between the smooth muscle data and antinociceptive activity is that bridging between μ and peptidergic opioid receptors may occur in the smooth muscle preparations and in the brain. It is known that the MVD contains μ and δ receptors and that peptide receptors are present in the GPI.[16-18]

While the smooth muscle and in vivo data are consistent with bridging of μ and δ receptors, it is important to point

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Table V. Analytical Data of Bivalent and Monovalent Peptide-Containing Ligands

| compd | amino acid anal. | TLC: R_f | | FAB-MS[c] (MH[+]) |
		BAW[a]	BPAW[b]	
1	Tyr, 1.01; Gly, 1.01; Gly, 1.00; Phe, 0.98; Leu, 1.03	0.23	0.60	912
1a	Tyr, 1.09; Glu, 1.00; Gly, 0.97; Phe, 0.99; Leu, 1.00	0.56	0.72	
2	Tyr, 1.00; Glu, 1.01; Gly, 2.80; Phe, 1.02; Leu, 1.00	0.11	0.60	1026
2a	Tyr, 0.97; Glu, 1.00; Gly, 2.97; Phe, 1.03; Leu, 1.03	0.36	0.66	

[a] *n*-BuOH/AcOH/H$_2$O (4:1:5).   [b] *n*-BuOH/pyridine/AcOH/H$_2$O (15:10:3:12).   [c] Found values are in agreement with calculated values.

out that other mechanisms also may lead to enhanced potency. For example, the enhanced opioid potency of **1** can be explained by postulating the presence of accessory sites that are vicinal to the μ or opioid recognition sites.

Experimental Section

General Methods. Precoated plates (silica gel G, 250 μm; Analtech, Newark, DE) were used for ascending TLC in the following solvent systems (all v/v): (1) *n*-BuOH/AcOH/H$_2$O (BAW) (4:1:5, organic phase) and (2) *n*-BuOH/pyridine/AcOH/H$_2$O (BPAW) (15:10:3:12). Reversed-phase HPLC was performed on a Waters liquid chromatograph (Model 6000 solvent delivery system, Model 660 solvent programmer) equipped with a Model 450 variable-wavelength detector, utilizing a Waters column (30 × 0.78 cm) packed with C-18 Bondapak reversed-phase (10 μm) material. For amino acid analyses, compounds (0.3 mg) were hydrolyzed in 6 N HCl (0.5 mL) containing a small amount of phenol for 24 h at 110 °C in deaerated tubes. Hydrolysates were analyzed on a Beckman Model 121c amino acid analyzer equipped with a system AA computing integrator. Molecular weights of the obtained products were determined by FAB-MS on a Kratos MS-50 mass spectrometer using a DS55 computer system.

Boc and Fmoc amino acid derivatives were purchased from IAF Biochemicals, Laval, Quebec, Canada. All peptides were prepared by the manual solid-phase technique using a polystyrene/divinylbenzene resin (1% cross-linked; Pierce, Rockford, IL), substituted with Boc-Leu (0.50 mequiv/g of resin).

Symbols and abbreviations are in accordance with recommendations of the IUPAC–IUB Joint Commission on Biochemical Nomenclature: *Biochem. J.* 1984, *219*, 345. The following other abbreviations were used: Boc, *tert*-butoxycarbonyl; Bzl, benzyl; DCC, dicyclohexylcarbodiimide; FAB-MS, fast atom bombardment mass spectrometry; Fmoc, (fluoren-9-ylmethoxy)carbonyl; HOBt, 1-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; .a-Oxy, α-oxymorphamine; TFA, trifluoroacetic acid.

Solid-Phase Peptide Synthesis and Purification of Peptides and Peptide Hybrids. The bivalent and monovalent ligands were synthesized by the solid-phase method according to the schemes outlines above. Peptide **1a** and the C-terminal tripeptide segments of compounds **1**, **2**, and **2a** were assembled following a reported[8] protocol, using Boc protection and DCC as coupling agent. For the coupling of Boc-D-glutamine in **1a** DCC in combination with HOBt was used to prevent nitrile formation. In the case of compounds **1**, **2**, and **2a** N^α-Fmoc-D-glutamic acid *tert*-butyl ester was coupled with DCC in the usual manner and the side-chain *tert*-butyl protecting group was subsequently removed by treatment with 50% (v/v) TFA in CH$_2$Cl$_2$ for 30 min. For the preparation of compounds **2** and **2a** glycine *tert*-butyl ester (2.5-fold excess) was then coupled with DCC (2.5-fold excess) in CH$_2$Cl$_2$ for 6 h, followed by TFA deprotection and coupling of glycine *tert*-butyl ester and glycinamide, respectively, under identical conditions. In the syntheses of compounds **1** and **2** the D-Glu side chain and Gly carboxyl function, respectively, were deprotected by TFA treatment, and α-Oxy (1.5-fold excess) was

then coupled to the free carboxyl group with DCC (1.5-fold excess) in CH$_2$Cl$_2$ for 48 h. The assembly of compounds **1**, **2**, and **2a** was finally completed by Fmoc deprotection with 50% (v/v) piperidine in CH$_2$Cl$_2$ for 30 min, washing of the resin as described,[19] coupling of Boc-Tyr(OBzl)-OH (2.5-fold excess) with DCC (2.5-fold excess) in CH$_2$Cl$_2$ for 24 h, and removal of the Boc group by treatment with 50% (v/v) TFA in CH$_2$Cl$_2$ for 30 min. HF treatment was used for cleaving of the compounds from the resin and for concomitant deprotection of the tyrosyl residue. The reaction was carried out for 90 min at 0 °C and for 15 min at room temperature with 20 mL of HF and in the presence of 1 mL of anisole/g of resin. After evaporation of the HF, the resin was extracted three times with diethyl ether and, subsequently, three times with 7% acetic acid. Lyophilization of the acetic acid extracts provided the crude products in solid form.

Compounds were purified by gel filtration on a Sephadex-G-25 column in 0.5 N AcOH, followed by reversed-phase chromatography on an octadecasilyl silica column,[20] using a linear gradient of 0–80% MeOH in 1% TFA. Final purification was achieved by semipreparative reversed-phase HPLC (20–70% MeOH (linear gradient) in 0.1% TFA. Final products were obtained as lyophilisates. Homogeneity of the compounds was established by TLC and HPLC under conditions identical with those described above. All products were at least 95% pure, as judged from the HPLC elution profiles. On the basis of the amount of C-terminal amino acid coupled to the resin, overall reaction yields after purification to homogeneity were as follows: **1**, 4%; **1a**, 16%; **2**, 3%; **2a**, 10%. Analytical data are presented in Table V.

$N^{6\alpha}$-**Acetyl-α-oxymorphamine (1b).** To a solution of α-oxymorphamine[7] (36.3 mg, 0.12 mmol) in acetone (5 mL) was slowly added a solution of acetic anhydride (11.32 μL, 0.12 mmol) in acetone (5 mL). After 30 min, ethereal HCl (5 mL) was added and the resulting precipitate was collected, washed with ethyl ether, and crystallized from methanol/ethyl ether: yield 45 mg (90%); mp >270 °C; R_f 0.58 (silica gel GF; EtOAc/MeOH/NH$_3$, 10:3:0.4). Anal. (C$_{19}$H$_{24}$N$_2$O$_4$·HCl·2H$_2$O) H, N; C: calcd, 54.74; found, 55.35.

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