Synthesis and Biological Activities of Dynorphin A Analogues with Opioid Antagonist Properties†

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Dynorphin A, which displays a wide variety of physiological effects, binds to opioid receptors preferentially at the κ receptor type. κ-selective antagonists would be very useful as pharmacological and biochemical probes to study and better understand the action of dynorphin A at its preferred receptor. However, the development of such molecules has been elusive, and very few are known at this time. Taking these features into account, we have synthesized by the solid-phase procedure several analogues of dynorphin A containing various D-amino acid substitutions. The binding properties of the peptides have been examined at three main opioid binding sites (μ, δ, and κ) and their κ selectivity determined. Their biological activities have been tested in three specific pharmacological assays for agonist and/or antagonist properties. Introduction of D-Trp substitution leads to analogues, in particular [D-Trp^{2,8},D-Pro^{10}]-, [D-Trp^{5,8},D-Pro^{10}]-, and [D-Trp^{2,4,8},D-Pro^{10}]dynorphin(1–11), showing antagonist properties in the isolated rabbit vas deferens preparation, a κ specific bioassay. The antagonism against dynorphin A is weak, as indicated by the observed K_e values (433, 199, and 293 nM, respectively), and not very selective (κ vs. μ). Such peptide analogues derived from the endogenous ligand and endowed with antagonist properties are the first ones reported to date and could open a promising way in designing more potent and selective κ opioid antagonists.

Dynorphin (dyn) A, a 17-amino-acid opioid peptide first isolated from porcine pituitary and duodenum extracts,[1,2] is found in the hypothalamic–pituitary axis and is widely distributed within the central nervous system and peripheral organs.[3–5] A specific receptor has been proposed for dynorphin,[6] and it has been postulated that this opioid peptide is the endogenous ligand for the κ opioid receptor.[7,8] Nevertheless, dynorphin A also binds strongly to μ and δ opioid binding sites.[9,10] In addition, dynorphin elicits a wide variety of pharmacological effects,[11–13] and conflicting results have been obtained in dynorphin-induced analgesia.[14–16]

Therefore, studies on the physiological role of dynorphin A, which is mediated through its specific interaction with the κ opioid receptor, are hindered in part by the multiplicity of receptor types and the relatively poor selectivity of the endogenous ligand toward each type.

In order to better understand and clearly elucidate the in vitro and in vivo actions of dynorphin A at the κ receptor, the use of a κ-selective antagonist of dynorphin A would be of particular interest. To date, no specific antagonist of dynorphin has been designed, and very few κ-selective antagonists are known at this time.[17,18]

Taking these facts into account, and on the basis of promising results obtained in the design of antagonist analogues of neuropeptides such as substance P (SP), neurotensine (NT), or LH-RH,[19–21] we have synthesized a series of analogues of dynorphin A expected to display κ-selective antagonist properties.

The four C-terminal amino acids of dynorphin A are not necessary for the molecule to bind to opioid receptors, and dynorphin(1–13) displays a comparable binding profile.[9] Studies on shorter fragments have shown that Arg^7 is needed for interaction with κ binding sites[6] and that endogenous dynorphin(1–8) and dynorphin(1–9) can be considered κ-selective ligands.[9] However, Lys^{11} is a crucial amino acid for potency and κ specificity.[6] We have recently reported that the synthetic analogue [D-Pro^{10}]dyn(1–11) is a highly potent and selective ligand for κ opioid receptors.[10] Since triple substitution can lead to antagonists with increased affinity in the case of SP,[21] we have replaced two hydrophobic residues by D-Trp in the prototypical sequence of [D-Pro^{10}]dynorphin(1–11). Thus, Gly^2, Gly^3, Phe^4, or Leu^5, together with Ile^8, were replaced by a residue with D-Trp. In order to check the effect induced by a higher number of substitutions, Gly^2, Phe^4, and Ile^8 were

† Symbols and abbreviations are in accordance with the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature (*J. Biol. Chem.* **1971**, *247*, 977). All amino acids are in the L configuration unless otherwise stated. Other abbreviations used are as follows: DMF, dimethylformamide; DCC, dicyclohexylcarbodiimide; Boc, *tert*-butyloxycarbonyl; HOBT, *N*-hydroxybenzotriazole; TFA, trifluoroacetic acid; DEA, diisopropylethylamine; GPI, guinea pig ileum; RbVD, rabbit vas deferens; HVD, hamster vas deferens.

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Table I. Physicochemical Properties of Dynorphin A Analogues

no.	sequence	peptide	M_r	yield,[a] %
0	H_2N-Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Glu-OH	dynorphin A(1–17)		
1	H_2N-Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-OH	dynorphin(1–11)	1362.9	38
2	H_2N——D-Pro——OH	[D-Pro^{10}]dynorphin(1–11)	1362.9	45
3	H_2N——D-Trp—D-Pro——OH	[D-Trp^{8},D-Pro^{10}]dynorphin(1–11)	1435.9	32
4	H_2N—D-Trp——D-Trp—D-Pro——OH	[D-Trp^{2,8},D-Pro^{10}]dynorphin(1–11)	1565.0	36
5	H_2N——D-Trp——D-Trp—D-Pro——OH	[D-Trp^{3,8},D-Pro^{10}]dynorphin(1–11)	1565.0	36
6	H_2N——D-Trp——D-Trp—D-Pro——OH	[D-Trp^{4,8},D-Pro^{10}]dynorphin(1–11)	1474.9	29
7	H_2N——D-Trp——D-Trp—D-Pro——OH	[D-Trp^{5,8},D-Pro^{10}]dynorphin(1–11)	1509.0	47
8	H_2N—D-Trp D-Trp——D-Trp—D-Pro——OH	[D-Trp^{2,4,8},D-Pro^{10}]dynorphin(1–11)	1604.0	35

	TLC (R_f)			amino acid analysis								
no.	BAPW[b]	BAW[c]	HPLC[d] (R_t)	Gly	Ile	Leu	Tyr	Phe	Lys	Arg	Pro	Trp[e]
1	0.38	0.33	21.3	1.99	0.94	1.00	0.96	1.03	1.05	2.46	1.08	
2	0.44	0.37	21.5	2.04	0.93	1.00	0.96	1.03	1.02	3.10	0.80	
3	0.46	0.69	21.2	2.07		1.00	0.96	0.95	1.08	2.98	0.96	1.04
4	0.51	0.71	26.8	1.05		1.00	0.98	0.97	1.56	2.93	1.17	1.86
5	0.53	0.70	27.4	0.88		1.00	0.97	0.95	1.53	3.00	1.02	2.11
6	0.46	0.71	23.0	2.00		1.00	0.97		1.10	3.13	0.89	1.70
7	0.45	0.72	24.3	1.90			0.93	1.00	1.03	2.86	0.98	2.18
8	0.54	0.72	25.6	1.09		1.00	1.01		1.10	3.16	0.83	3.10

[a] Yield of final product obtained after chromatography, based on the starting quantity of C-terminal amino acid attached on the resin before synthesis. [b] 1-Butanol–acetic acid–pyridine–water (30:6:20:12, v/v). [c] 1-Butanol–acetic acid–water (4:3:3, v/v). [d] Elution conditions, using water–trifluoroacetic acid, pH 2.5 (solvent A), and methanol (solvent B); 20-min linear gradient with starting conditions A:B (80/20) and final conditions A:B (20/80); flow rate, 1 mL/min. Solvents were run through a reverse-phase column μBondapak C-18 (Waters); R_t, retention time (min). [e] Calculated values according to the method of Edelhoch (ref 35).

Table II. Opioid Binding Affinities of Dynorphin A and Its Analogues[a]

		K_I, nM (Hill coeff)[b]		
no.	peptide	[^{3}H]DAGO, μ sites	[^{3}H]DSLET, δ sites	[^{3}H]bremazocine, κ sites
0	dynorphin A(1–17)	1.55 ± 0.06 (1.18 ± 0.17)	6.3 ± 1.2 (0.95 ± 0.02)	0.23 ± 0.02 (0.79 ± 0.03)
1	dynorphin(1–11)	2.71 ± 0.96 (0.93 ± 0.04)	10.66 ± 0.67 (1.13 ± 0.14)	0.128 ± 0.038 (0.78 ± 0.06)
2	[D-Pro^{10}]dynorphin(1–11)	2.00 ± 0.68 (1.13 ± 0.03)	7.47 ± 1.29 (1.19 ± 0.15)	0.032 ± 0.008 (0.79 ± 0.07)
3	[D-Trp^{8},D-Pro^{10}]dynorphin(1–11)	0.58 ± 0.08 (0.86 ± 0.05)	5.43 ± 1.46 (0.98 ± 0.11)	0.049 ± 0.003 (1.00 ± 0.11)
4	[D-Trp^{2,8},D-Pro^{10}]dynorphin(1–11)	103 ± 14 (1.15 ± 0.17)	561 ± 88 (0.72 ± 0.11)	153 ± 15 (0.83 ± 0.02)
5	[D-Trp^{3,8},D-Pro^{10}]dynorphin(1–11)	327 ± 3 (1.31 ± 0.12)	1035 ± 37 (1.07 ± 0.04)	19.6 ± 1.7 (1.03 ± 0.06)
6	[D-Trp^{4,8},D-Pro^{10}]dynorphin(1–11)	142 ± 32 (1.06 ± 0.07)	631 ± 68 (0.92 ± 0.12)	73 ± 25 (0.85 ± 0.03)
7	[D-Trp^{5,8},D-Pro^{10}]dynorphin(1–11)	20.3 ± 2.0 (1.23 ± 0.14)	303 ± 30 (0.90 ± 0.04)	20.6 ± 1.4 (0.96 ± 0.04)
8	[D-Trp^{2,4,8},D-Pro^{10}]dynorphin(1–11)	51.5 ± 13.1 (0.89 ± 0.03)	134 ± 40 (0.86 ± 0.02)	23.8 ± 3.2 (0.85 ± 0.14)

[a] Binding properties were determined by measuring the inhibitory effects (K_I, nM) of dynorphin A and of its analogues on binding of [^{3}H]-D-Ala,MePhe^4,Glyol^5]enkephalin ([^{3}H]DAGO) (1.5 nM) and ([^{3}H]D-Ser^2,Leu^5,Thr^6]enkephalin ([^{3}H]DSLET) (2.5 nM) in rat brain membrane homogenates (μ and δ sites, respectively) and of [^{3}H]bremazocine (0.5 nM) in guinea pig cerebellum membrane homogenates (κ sites). Binding experiments were performed at 25 °C in the presence of peptidase inhibitors. [b] The values shown are the mean ± SEM of at least three independent observations.

simultaneously replaced by D-Trp. The choice of D-Trp as the replacement residue was based upon the knowledge that D-Trp imparts antagonist properties to LH–RH, NT, and SP when placed in critical positions.

However, it is well-known that one of the main consequences of multiple substitutions inside the original sequence is to drastically reduce the affinity of the peptide for the receptor. Therefore, we have synthesized an intermediate analogue in which we introduced only one D-Trp substitution, at the Ile^8 position, in the [D-Pro^{10}]-dynorphin(1–11) sequence.

The series of the dynorphin A analogues is listed in Table I.

Results and Discussion

Peptide Synthesis and Purification. The peptides were built up by the stepwise solid-phase method on chloromethylated polystyrene as a solid support. The coupling was achieved with the DCC-HOBT method.[22]

Boc was used for α-amino protection. The phenolic hydroxyl group of tyrosine and the β-indole group of tryptophan were left unprotected; the guanidino group of arginine was protected by the N^{ω}-nitro (NO_2) group and the ε-amino group of lysine by a benzyloxycarbonyl (Cbz) or 2-chlorocarbobenzoxy (2Cz) group. After completion of the synthesis, the protected peptides were cleaved from the solid support using the HF–anisole procedure to give the fully deprotected peptides. The analogues were purified to homogeneity (>98%) by ion exchange chromatography on a Whatman cation exchanger resin, CM52. The purity of the final product was checked by TLC and analytical HPLC. Amino acid compositions were confirmed by amino acid analysis and molecular weights by FAB-mass spectrometry.

Binding Properties. Receptors' Affinities and Selectivities. The binding of dynorphin A(1–17), its fragment dynorphin(1–11), and analogues was measured by determining the inhibition constants (K_I, nM) in three specific assays using crude membrane fractions from rat brain and guinea pig cerebellum. [^{3}H]DAGO ([D-Ala^2, Me-Phe^4, Gly ol^5]enkephalin) was used as a selective ligand for μ sites[23,24] and [^{3}H]DSLET ([D-Ser^2,Thr^6]-Leu^{5}enkephalin) for δ sites[25] in rat brain homogenates.

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Table III. Binding Selectivity of Dynorphin A and Its Analogues

	receptor selectivity index[a]		
no.	μ	δ	κ
0	6.7	27	1
1	22	85	1
2	62	233	1
3	12	110	1
4	1	5.5	1.5
5	17	53	1
6	1.9	8.6	1
7	1	15	1
8	2.2	5.6	1

[a] The receptor selectivity index (RSI) is defined as the ratio of the apparent K_I at a given site over the apparent K_I at the highest affinity site.

[^{3}H]Bremazocine, a nonselective ligand, was used for the determination of κ affinities in guinea pig cerebellum, an ideal and specific tissue for studies on κ receptors.[26] Binding assays were carried out at 25 °C for 60 min in the presence of peptidase inhibitors to reduce enzymatic degradation of the peptides. The affinity values of the peptides for the three opioid binding sites are listed in Table II. Taking as references the endogenous peptide **0**, its related fragment **1**, and the analogue **2**, a general loss of affinity for each type of opioid binding site is observed for the D-Trp-containing analogues **4–7**. This can be explained by triple substitution in the sequence of the peptide, with such a modification hindering recognition of the analogue by the receptor. Interestingly, a higher degree of substitution (analogue **8**) does not further enhance this phenomenon. The disubstituted analogue **3** retains affinities for δ and κ sites comparable to the ones of analogue **2**, but shows a better affinity for μ sites than does analogue **2**. In any case, for each peptide the lowest affinity is observed at the δ sites. Like dynorphin A, all of the analogues, except **4** and **7**, display a higher affinity for κ sites than for μ sites.

The respective affinities of a peptide toward each opioid binding site (μ, δ, κ) determine its selectivity profile. The binding selectivity profile of a ligand is expressed as its receptor selectivity index (RSI), defined as the ratio of its apparent K_I at a given site over its apparent K_I at the highest affinity site. The RSI values of the peptides are reported in Table III. Compared to analogue **2**, which has been shown to be highly κ selective, the D-Trp^8 monosubstituted analogue **3** retains κ selectivity, although to a lower degree. Among the D-Trp di- or trisubstituted peptides only analogue **5** shows a similar selectivity pattern, indicating that Gly^3 is not essential to preserve κ selectivity. On the other hand, substituting Gly^2 and/or Phe^4 (analogues **4**, **6**, and **8**) or Leu^5 (analogue **7**) leads to a dramatic loss of κ selectivity.

As a consequence of their binding properties, the different peptides can be classified as analogues showing (i) both good affinity and κ selectivity (**1–3**), (ii) low affinity and low selectivity (**4**, **6–8**), and (iii) low affinity and high κ selectivity (**5**).

Biological Activities. Dynorphin A and its analogues were tested in three isolated organ preparations (guinea pig ileum, hamster vas deferens, and rabbit vas deferens) that respond differently to activation of μ, δ, and κ receptors. The myenteric plexus–longitudinal muscle of the guinea pig ileum (GPI) responds to ligands acting on μ and κ receptors. The hamster vas deferens (HVD) responds preferentially to ligands that activate δ receptors,[27] whereas in the rabbit vas deferens (RbVD) inhibitory effects of κ ligands are observed.[28] In order to prevent enzymatic degradation, these assays were carried out in the presence of a mixture of peptidase inhibitors adapted from McKnight et al.[29]

Each peptide was tested for its agonist properties and for its antagonist activity against dynorphin A(1–17). The biological activities of dynorphin A are taken as references (Table IV). The IC_{50} values found in GPI (0.28 ± 0.04) and RbVD (3.07 ± 0.99) are rigorously comparable to the ones (0.29 ± 0.09 and 3.01 ± 1.6, respectively) observed by Corbett et al.[9]; the high IC_{50} value (262 ± 33) obtained in HVD confirms that dynorphin A is a weak agonist at the δ opioid receptor. Shortening the sequence (analogue **1**) and introducing D-Pro in position 10 (analogue **2**) and D-Trp in position 8 (analogue **3**) slightly reduce the potency without altering the relative activity pattern. For these analogues, no antagonism against dynorphin A is observed. However, an additional D-Trp substitution in the sequence (analogues **4–7**) results in a dramatic fall of the agonist potency in all three bioassays, with only **7** and, to a lesser extent, **5** showing some residual agonism in GPI. This overall decrease in potency can be explained in part by the well-correlated decrease in binding affinities of these analogues (see Table II), but also, interestingly, by the fact that the second D-Trp residue introduced in the sequence is able to reduce agonist potency and generate antagonist activity. Such an antagonism is observed against dynorphin A for these analogues in RbVD, a κ-specific bioassay,[28] and in GPI. In this latter organ, dynorphin A has been shown to act predominantly at κ rather than at μ opioid receptors.[30,31] Furthermore, like in RbVD, the parallel dose–response curves of dynorphin A in the absence and in the presence of the analogue (data not shown) suggest a competitive antagonism. It could be therefore possible that antagonism in the GPI is observed at the κ rather than at the μ receptors. However, interaction at a μ receptor type cannot be ruled out. In consequence, we have tested analogues **4–8** for their antagonist properties against DAGO, a μ-selective agonist, in the GPI preparation. As shown in Table IV, all of these analogues (except **4**) are able to antagonize the inhibitory effects of DAGO in GPI. In this preparation, analogues **5** and **7** show 3–4 times lower K_e values against dynorphin A than they do against DAGO, indicating some κ selectivity. On the other hand, analogue **6** shows a somewhat better antagonism at μ rather than at κ receptors. Comparable K_e values at both μ and κ receptors, i.e., no selectivity, are obtained for analogue **8**. Interestingly, analogue **4** does not show any antagonism against DAGO.

In contrast to GPI and RbVD, no antagonism is seen for all the analogues in the HVD, a δ-receptor-containing tissue.

In this study, analogues **4–8** have been shown to possess competitive antagonist properties against dynorphin A at the κ opioid receptor. However, this antagonism is weak, with very low (analogues **5** and **7**) or no (analogues **6** and **8**) selectivity. This can be easily explained by the fact that

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Table IV. Agonist and Antagonist Properties of Dynorphin A and Dynorphin A Derived Peptides[a]

		guinea pig ileum			hamster vas deferens		rabbit vas deferens	
			K_e					
no.	peptide	IC_{50}	dynA	DAGO	IC_{50}	K_e	IC_{50}	K_e
0	dynorphin A(1–17)	0.28 ± 0.04			262 ± 33		3.07 ± 0.99	
1	dynorphin(1–11)	2.88 ± 0.28	no[c]		284 ± 15	no	13.09 ± 2.15	no
2	[D-Pro[10]]dynorphin(1–11)	3.32 ± 0.23	no		929 ± 54	no	12.14 ± 3.81	no
3	[D-Trp[8],D-Pro[10]]dynorphin(1–11)	2.84 ± 0.26	no		717 ± 107	no	12.75 ± 2.95	no
4	[D-Trp[2,8],D-Pro[10]]dynorphin(1–11)	8890 ± 660	206 ± 14	no[c]	inactive[b]	no	>10000	433 ± 97
5	[D-Trp[3,8],D-Pro[10]]dynorphin(1–11)	222 ± 8	10.3 ± 0.7	40.5 ± 5.0	inactive	no	>10000	1127 ± 80
6	[D-Trp[4,8],D-Pro[10]]dynorphin(1–11)	>10000	606 ± 27	249 ± 31	inactive	no	>10000	1432 ± 237
7	[D-Trp[5,8],D-Pro[10]]dynorphin(1–11)	69 ± 7	4.9 ± 1.0	13.9 ± 1.1	4320 ± 400	no	>10000	199 ± 48
8	[D-Trp[2,4,8],D-Pro[10]]dynorphin(1–11)	>10000	61 ± 4	83 ± 13	2720 ± 820	no	>10000	293 ± 35

[a] The agonist potencies and the antagonist activities of the peptides were measured in the presence of L-Leu-L-Leu (2 mM), bestatin (30 μM), thiorphan (0.3 μM), and aprotinin (200 IU/mL). The inhibitory effects (IC_{50}) and equilibrium constants (K_e) are expressed in nM. The values are the mean ± SEM of at least four independent measurements. [b] No significant inhibitory effect on the electrically induced contractions in the dose range tested (upper concentration, 10 μM). [c] No observed antagonist effect at concentrations up to 10 μM.

these substituted analogues display 20–100 times lower affinity for the opioid receptors than does dynorphin A as well as a low κ vs. μ affinity ratio. Nevertheless, they are the first κ antagonist peptides reported to date, and they can be considered as promising prototypical molecules in the search for a potent and κ selective antagonist.

Experimental Section

(a) Reagents and Solvents. Boc amino acids were purchased from Bachem Fine Chemicals, Inc., or from Bachem Feinchemikalien AG. Their purity was tested by TLC before use. The Merrifield resin (chloromethylated copolystyrene divinylbenzene, 1% 0.75 mg of Cl/g) was obtained from UCB-Bioproducts, Belgium. The CM52 cation exchange resin was obtained from Watman Chemical Separation Ltd., England. Reagent grade solvents were bought from Prolabo, France, and purified before use; methylene chloride was distilled from sodium carbonate, and, after shaking with KOH pellets, DMF was distilled from ninhydrin in vacuo. TFA (Aldrich), DEA (Aldrich), and DCC (Aldrich) were used without further purification. Dynorphin A(1–17) was obtained from Bachem, Switzerland.

(b) Materials. (i) Peptide synthesis was carried out in either a Vega prototype or a Sempa Model SAP3 synthesizer operating in a semiautomated mode. (ii) Ion exchange chromatography was carried out by using a peristaltic pump Gilson Minipuls 2; elution was monitored with an ISCO Model 1840 spectrophotometer; and fractions were collected on an ISCO Foxy Model 2200 collector. (iii) Characterization: Silica gel plates (Merck Silica gel 60, 0.25 mm) were used for TLC and a Waters apparatus (automated gradient controller, 2 pumps Model 510, Lambda Max 481 spectrophotometer, reverse-phase column μ-Bondapack C-18) for HPLC analyses. Amino acid analyses were performed on a LKB 4400 apparatus, and mass spectra were obtained with a MM-ZAB instrument. (iv) Biological assays: Responses of the isolated organs were recorded on a Physiograph MKIII (Narco Biosystem, Inc., Houston, TX).

(c) Peptide Synthesis. (i) Protected Peptide–Resin. C-terminal Boc-Lys(Cbz) or Boc-Lys(2Cz) were coupled to the resin via the cesium salt procedure.[32] Substitutions amounted, respectively, to 0.56 and 0.38 mmol/g as determined by the picric acid method.[33]

(ii) Solid-Phase Peptide Synthesis. Boc-protected amino acid, HOBT, DCC, or TFA solutions were added manually. In order to avoid any destruction of tryptophan during synthesis of D-Trp-containing analogues, subsequent deprotections following the introduction of Boc-D-Trp were made in the presence of D,L-Met (1% w/v). Every deprotection and coupling step was monitored for completion with ninhydrin.[34]

For a typical synthesis of dynorphin(1–11) (compound 1), 1.0 g (0.56 mmol) of Boc-Lys(Cbz) resin was placed in the reaction vessel and the operations were carried out as follows: deprotection was performed for 20 min in 40% (v/v) TFA–CH_2Cl_2. After three washes with CH_2Cl_2, neutralization was carried out with 5% DEA in CH_2Cl_2 (two steps prewash (1 min) and neutralization (5 min)). Before introduction of the desired protected residue, the resin was washed with CH_2Cl_2 (2 times) and DMF (2 times). N^{α}-Boc protection was used for all the residues and N^{ω}-NO_2 side-chain protecting group for L-Arg. A solution of Boc residue (1.68 mmol) with HOBT and DCC (in stoichiometric proportions) in DMF was prepared before use and poured into the reaction vessel. The mixture was allowed to react with the deprotected peptide resin for 90 min. After the resin was washed (by alternate use of CH_2Cl_2 and MeOH), the coupling step was check by use of the ninhydrin test.[34] A negative response led to the addition of the next amino acid and a positive response to an additional coupling of the amino acid. No second positive test has been observed, so an acetylation step was not necessary. After the last amino acid (Tyr) was coupled, its Boc protecting group was removed. The peptide resin was washed successively with CH_2Cl_2 and MeOH, collected on a fritted glass funnel, dried overnight in vacuo, and weighed (1.3 g).

Analogues **2** and **3** were built in a similar way from 2.0 g of Boc-(2Cz)Lys–resin (0.38 mequiv/g) and analogues **5–8** from 5.2 g of Boc(2Cz)Lys–resin (0.36 mequiv/g).

(iii) Cleavage of the Peptide from the Support. The dried, protected dynorphin(1–11)–resin (1.3 g) was placed in the reaction vessel of a liquid HF apparatus: 2 mL of anisole was added; and HF (15 mL) was distilled from CoF_3 into the vessel. D,L-Methionine (1% w/v) was added to the mixture when tryptophan was present in the sequence of the analogue. The cleavage reaction proceeded for 25 min at −20 °C (ice–NaCl, 3:1) and at 0 °C for 30 min. HF was then rapidly evaporated in vacuo and the resin copiously washed with ether. The peptide was extracted from the resin with 250 mL of 2 N acetic acid. The solution was lyophilized to give 700 mg of crude material.

(iv) Purification of the Peptide. The crude peptide (150 mg) was dissolved in 10 mL of 5×10^{-3} M ammonium acetate buffer at pH 6.0, and the solution was loaded onto the cation exchange resin equilibrated with the same buffer. The buffer was pushed through the column at 300 mL/h using a peristaltic pump. Uncharged products were eluted immediately, and then a linear gradient of salt (5×10^{-3} to 2×10^{-1} M) and pH (6.0–7.0) was applied by using a 400–500-mL mixing volume. Elution was monitored at 280 nm, and fractions of 17.5 mL were collected. The fractions containing the purified peptide were pooled and lyophilized at least 3 times; 62 mg of pure material was obtained.

(v) Characterization of the Peptides. Purity of the peptides was checked by (1) TLC on silica gel in the following solvent systems: *n*-BuOH–AcOH–pyridine–H_2O (30:6:20:12, v/v) and *n*-BuOH–AcOH–H_2O (4:3:3, v/v), iodine vapor used for detection, and (2) HPLC using H_2O–TFA, pH 2.5 (A), and MeOH (B) as solvents under gradient conditions. A 20-min linear gradient was monitored from starting conditions A:B (80/20) to final conditions A:B (20/80). Solvents were run through the column at a flow rate of 1 mL/min.

Amino acid analyses were performed on peptide hydrolysates (distilled 6 N HCl at 110 °C in vaccuum-sealed tubes for 24 h). Tryptophan content was determined according to the method of

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Edelhoch[35] based on optical density measurements performed at 280 and 288 nm. The mass spectra of the peptides were recorded on the spectrometer operating with a FAB ion source, at +8 kV in the positive mode. Mass spectrum data are in accordance with calculated molecular weights (Table I).

(d) **Binding Assays.** Crude membrane fractions were prepared from brains of male Wistar rats and from cerebellums of guinea pigs as described by Meunier et al.[36] Conditions of equilibrium binding experiment, composition of the coktail of peptidase inhibitors, and determination of nonspecific binding were identical to the assay procedure described by Gairin et al.[10]

K_I values were calculated from the Cheng and Prusoff's equation[37] using 1.6, 1.9, and 0.07 nM for the K_D's of tritiated DAGO, DSLET, and bremazocine, respectively.

(e) **Biological Assays.** Myenteric plexus–longitudinal muscles of the guinea pig ileum were prepared according to the procedure of Gyang and Kosterlitz,[38] hamster vas deferens preparations were made according to McKnight et al.[27]; and rabbit vas deferens was prepared as described by Oka et al.[28] The tissues were suspended in a 10-mL siliconized organ bath containing Kreb's solution, heated at 37 °C and oxygenated with 95:5 O_2–CO_2. Peptidase inhibitors were added to the bathing solutions according to McKnight et al.[29]

All compounds were tested (i) for agonist activity by application of cumulative concentrations of each peptide (from 10^{-10} to 10^{-5} M) into the bath and (ii) for antagonism by introducing the compound in the bath at least 10 min before testing dynorphin A. In the test for antagonism, the concentration of the peptides used was chosen so that the height of the twitch was depressed by 20–40% as described by Kosterlitz and Watt.[39]

Potencies of the agonists were assessed by measuring IC_{50} values (concentration of the peptide inhibiting 50% of the response). Activities of the antagonists were expressed in terms of K_e (concentration of the peptide that is able to reduce the effect of a double dose of dynorphin A to that of a single dose). Concentrations of dynorphin A and its analogues are expressed in nanomolar.

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Conformation and Activity of Tetrahydrofuran Lignans and Analogues as Specific Platelet Activating Factor Antagonists

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The six (racemic or meso) isomers of 3,4-dimethyl-2,5-bis(3,4-dimethoxyphenyl)tetrahydrofuran and four corresponding desmethyl analogues were prepared and assayed as inhibitors of platelet activating factor (PAF) receptor binding to rabbit platelet plasma membranes. The inhibition by these isomers is stereodependent and varies with the gross shape of the molecules as determined by the molecular mechanics program MM2. The most potent PAF antagonist in this group of compounds is *trans*-2,5-bis(3,4,5-trimethoxyphenyl)tetrahydrofuran (L-652,731, 14) with an IC_{50} of 0.02 μM.

Platelet activating factor (PAF) is a highly potent phospholipid with a chemical structure of 1-*O*-hexadecyl/octadecyl-2-*O*-acetyl-*sn*-glyceryl-3-phosphorylcholine.[1-3] Earlier,[4] an antigen-induced histamine release from rabbit platelets was described. However, the existence of a mediator was not indicated. Platelet activating factor has been linked to various biological activities and pathways, making it one of the important mediators of physiological processes including aggregation and degranulation of platelets[5] and neutrophils, inflammation, and allergic reactions.[6] Platelet activating factor is also reported to be an endogenous hypotensive,[7] chemotactic,[8] and tumor-cytotoxic[9] agent. A specific PAF receptor site in rabbit plasma membranes[10] and human platelets[11] has also been identified and reported.

3,4-Dimethyl-2,5-bis(3,4-dimethoxyphenyl)tetrahydrofurans belong to a well-recognized subgroup of natural

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