Solid-Phase Synthesis and Opioid Activities of [D-Ala^2]Deltorphin II Analogs[1)]

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[D-Ala^2]Deltorphin II (DL-II) analogs having various aliphatic amino acids at positions 5 and 6 were synthesized by a solid-phase method and their opioid activities on electrically induced guinea pig ileum (GPI) and mouse *vas deferens* (MVD) preparations were determined. During the synthesis of an analog, [*tert*-leucine(Tle)^{5,6}]DL-II, we encountered difficulty in the coupling reaction between Tle^5 and Tle^6 with the usual diisopropylcarbodiimide (DIPCDI)-mediated *tert*-butoxycarbonyl (Boc) strategy, though the other analogs could be successfully synthesized. We found that the fluorenylmethoxycarbonyl (Fmoc)-Tle/DIPCDI/1-hydroxybenztriazole method was very useful for the synthesis of such a peptide having a sterically hindered sequence. Acid hydrolysis studies of the synthetic analogs suggested that the steric hindrance of consecutive aliphatic amino acid sequences depend upon the degree of branching at the β-carbon atom of the amino acids. In the MVD assay, two analogs, [Ala^{5,6}] and [Tle^{5,6}]DL-II showed remarkably low potencies while other analogs with Nva^{5,6}, Nle^{5,6}, Ile^{5,6}, Leu^{5,6} and Mle^{5,6} substituted for Val^{5,6}(DL-II) showed comparable or slightly lower potencies than DL-II. In the GPI assay, no remarkable changes in potency were observed between DL-II and this series of analogs. Conformational aspects of synthetic analogs were examined by comparing the circular dichroism spectra.

Keywords [D-Ala^2]deltorphin II analog; *in vitro* bioactivity; solid phase synthesis; aliphatic amino acid substitution (residues 5,6); steric hindrance; circular dichroism

[D-Ala^2]Deltorphin II (DL-II: Tyr–D-Ala–Phe–Glu–Val–Val–Gly–NH_2) is one of the deltorphins, a new family of δ-opioid receptor-selective peptides recently isolated from the skin of South American frogs.[2)] In a recent study,[3)] we have demonstrated that some DL-II analogs, in which the Val^{5,6} residues were replaced by other more hydrophobic amino acids, had higher affinity and selectivity for δ-opioid receptor than those of the parent peptide. In the course of the study, we encountered synthetic difficulty with a peptide containing *tert*-leucine (Tle) residues, Tyr–D-Ala–Phe–Glu–Tle–Tle–Gly–NH_2 (**8**), due to the sterically hindered sequence, –Tle–Tle–, while analogs in which positions 5 and 6 were replaced by other amino acids with alkyl side chains, *i.e.*, [Ala^{5,6}](**1**), [Ala^5](**2**), [Ala^6](**3**), [Nva^{5,6}](**4**), [Nle^{5,6}](**5**), [Ile^{5,6}](**6**), [Leu^{5,6}](**7**) and [γ-Me-Leu (Mle)^{5,6}](**9**), could be successfully synthesized by the usual solid-phase peptide method (SPPS).

The structure–activity study also suggested that the residues at positions 5 and 6 of DL-II play an important role in generating the bioactive conformations at the receptor site. The present paper deals with the synthetic details of these DL-II analogs in SPPS, their opioid activities to inhibit electrically induced contraction of isolated longitudinal muscle strips of guinea pig ileum (GPI) and mouse *vas deferens* (MVD) and conformational studies based on comparisons of the circular dichroism (CD) spectra.

Peptides were synthesized by the usual SPPS with the DIPCDI-mediated Boc strategy except that 0.5 M MSA, a novel reagent for N^{α}-Boc deblocking in SPPS,[4)] was used. The peptide was constructed on a benzhydrylamine (BHA) resin by using a single coupling reaction for each Boc-amino acid and was cleaved from the resin and simultaneously deprotected by treatment with an anisole/HF mixture. Purification of the peptide was achieved by medium-pressure HPLC. However, in the case of the synthesis of analog **8**, the desired peptide was obtained in a low yield (9% overall yield) as a minor product along with the des-Tle^5-peptide as the main product by the usual method (Fig. 1A), possibly due to inefficient coupling reaction of Boc-Tle with Tle–Gly–BHA resin. After incorporation of the first Tle residue on the solid support, we therefore examined the incorporation rates of the second Tle residue with several coupling methods involving the use of Fmoc-Tle as shown in Table I. The usual Boc/DIPCDI method (A) resulted in a very low coupling rate as expected. The reaction with an additive, HOBt, or with BOP reagent showed a slight improvement but still resulted in unsatisfactory reaction rates. The coupling reaction of Fmoc-Tle by the DIPCDI/HOBt method (D) gave the best result among the methods tested. These observations are in accordance with the conclusion recently reported by several groups[5)] that the Fmoc strategy is superior to the Boc one in terms of coupling efficiency in SPPS. The usual DIPCDI-mediated peptide assembly following method D gave **8** with an overall yield of 46% (Fig. 1B), demonstrating the usefulness of the Fmoc/HOBt/

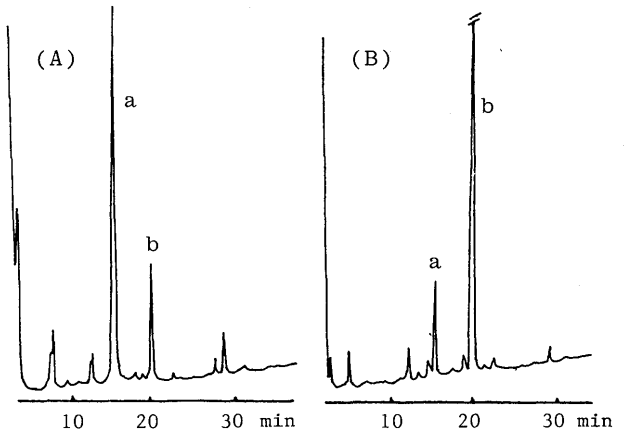

Fig. 1. HPLC Profiles of the Crude Analog **8** Obtained by the Boc-Tle/DIPCDI Method (A) and the Fmoc-Tle/HOBt/DIPCDI Method (B)

a: des-Tle^5-**8**. b: analog **8**.

DIPCDI method for the synthesis of peptides containing sterically hindering amino acids. No synthetic difficulty was observed with **9**, which contained amino acids having a *tert*-butyl group at the β-carbon (Mle). Yields and analytical data of the synthetic analogs are shown in Tables II and III.

Table IV shows the recovery of hydrophobic amino acids on acid hydrolyses of synthetic analogs. It is noteworthy that the recovery of Tle from **8** was less than 50% after 6 N HCl hydrolysis at 110 °C for 22 h. DL-II and **6** also gave poor recoveries of the hydrophobic amino acids as compared to other analogs, **4**, **5**, **7** and **9** (Tables III and IV). These results suggest that the steric hindrance in consecutive aliphatic amino acid sequences depends greatly upon the degree of branching at the amino acid β-carbon atom and affects the peptide bond cleavage or peptide bond formation between the two residues. Unusual behavior of the Tle residue in the synthesis of Tle-containing peptides has also been reported by others.[6] The hydrophobicity of isomeric analogs assessed from the retention times (t_R) on reversed-phase HPLC was in the order of DL-II < **4**, and **8** < **6** < **7** < **5** < **9** (Table II). Since analogs **5**, **6**, **7** and **9** have higher δ-receptor affinities than that of DL-II (Table V), these data support the concept that the hydrophobicity at the address domain in deltorphins is an important factor for high δ-receptor affinity.[3,7]

The opioid activities of these synthetic analogs were determined using the GPI and MVD assays, which are used as *in vitro* bioassays for determining subtype preferences of μ- and δ-opioid receptors, respectively. The results are shown in Table V. In the GPI assay, no remarkable changes in potency were observed with this series of analogs, though analogs **1** and **2** showed slightly enhanced potencies as compared to DL-II. In the MVD assay, analogs **1** and **8** showed remarkably reduced potencies and, for the most part, the results with all analogs were basically consistent with the receptor binding profiles determined using a rat brain homogenate.[3] However, some unexpected results were observed with analogs **5**, **6**, **7** and **9**, which were equipotent or slightly less potent in the MVD assay as compared to DL-II, even though these analogs had significantly higher δ-receptor affinities in the rat brain than DL-II. Discrepancies between the opioid activities and receptor binding data are often observed with deltorphins and their analogs.[2a,c,8] Concerning the results in the MVD assay given above, at present we can only speculate that there may be subtle differences between the central and peripheral opioid receptors.

The CD spectra of all analogs were measured in 10 mM phosphate buffer at pH 7.60 and in 50% TFE/10 mM phosphate buffer (pH 7.60) and compared with those of DL-II and deltorphin[2a,c] (or dermenkephalin[2b]), Tyr–D-Met–Phe–His–Leu–Met–Asp–NH_2 (Fig. 2). The CD spectra of these analogs in the buffer solution (Fig. 2A) showed characteristic curves indicative of random structure, having a positive band at about 221 nm with a shoulder at about 212 nm and a negative band near to 200 nm. The CD spectra in the hydrophobic medium (Fig. 2B) showed similar spectral patterns to those observed in the buffer solution except for the intensities of the positive and negative maxima, which were somewhat shifted to higher wavelength, while the shoulder at about 212 nm was unaltered. It is noteworthy that the relative intensities of the shoulder at 212 nm and positive maximum of analog **8** between the two solvent systems were almost unaltered whereas those of the other analogs clearly increased in the hydrophobic medium. The weaker spectral changes of

TABLE I. Coupling Reaction of N^{α}-Protected Tle with Tle–Gly–BHA Resin

Resin	Tle/Gly ratio[a]
Boc–Tle–Gly–BHA Resin	0.86
Boc(Fmoc)–Tle–Tle–Gly–BHA resin	
Methods, A: Boc–Tle/DIPCDI	1.27
B: Boc–Tle/DIPCDI/HOBt	1.58
C: Boc–Tle/BOP/DIPEA	1.61
D: Fmoc–Tle/DIPCDI/HOBt	1.87

a) See Experimental.

TABLE II. Physico-Chemical Properties of Synthetic Analogs

Analog	Yield (%)	$[\alpha]_D$[a] (°)	TLC[b] *Rf* (A)	TLC[b] *Rf* (B)	HPLC[b] t_R[c] (min)	FAB-MS $(M+H)^+$
1, [$Ala^{5,6}$]	40	−3.3	0.35	0.69	6.8	—
2, [Ala^{5}]	39	+6.1	0.44	0.75	10.6	—
3, [Ala^{6}]	39	−9.5	0.42	0.74	11.1	—
4, [$Nva^{5,6}$]	45	+0.8	0.62	0.82	18.1	783
5, [$Nle^{5,6}$]	42	+1.1	0.61	0.82	27.9	811
6, [$Ile^{5,6}$]	38	−8.3	0.61	0.82	21.9	811
7, [$Leu^{5,6}$]	40	−6.5	0.60	0.81	25.8	811
8, [$Tle^{5,6}$]	46[d]	−1.8	0.60	0.82	20.5	811
9, [$Mle^{5,6}$]	34	−4.4	0.65	0.82	32.3	839

a) Optical rotation was measured in 50% MeOH ($c=0.5$) at 23 °C. *b*) See Experimental. *c*) Retention time (DL-II: 14.4 min). *d*) By using the Fmoc-Tle/DIPCDI/HOBt method for the incorporation of Tle^5 residue.

TABLE III. Amino Acid Analysis of Synthetic Analogs[a]

Analog	Glu	Gly	Ala	Val	Nva	Nle	Ile	Leu	Tle	Mle	Tyr	Phe	NH_3
1	1.03	1.00	3.07	—	—	—	—	—	—	—	0.80	1.04	1.06
2	1.03	1.00	2.03	0.98	—	—	—	—	—	—	0.74	1.08	0.98
3	1.02	1.00	1.95	0.96	—	—	—	—	—	—	0.73	1.02	0.92
4	1.12	1.00	1.10	—	2.10[b]	—	—	—	—	—	0.97	1.17	1.15
5	1.00	1.00	1.00	—	—	2.01[c]	—	—	—	—	0.73	0.93	1.13
6	1.00	1.00	1.00	—	—	—	1.48	—	—	—	0.72	0.93	0.96
7	1.00	1.00	1.04	—	—	—	—	2.04	—	—	0.86	0.86	0.89
8	0.95	1.00	1.03	—	—	—	—	—	0.86[d]	—	0.88	0.92	0.91
9	0.98	1.00	1.03	—	—	—	—	—	—	1.86[c]	0.83	0.90	0.96

a) After acid hydrolysis with 6 N HCl at 110 °C for 20—24 h. *b*) Eluted just after Met. *c*) Eluted just before Tyr. *d*) Eluted just before Met.

8 suggest a restricted conformational flexibility of the molecule. This was supported by examination of a Dreiding stereomodel, which indicated that conformational freedom around the Tle[5,6] residues was significantly restricted, and also by the apparently greater steric hindrance of the Tle–Tle sequence than those of the Val–Val (DL-II) and Ile–Ile (**6**) sequences as described before (Table IV). The steric effects appear to disfavor the required conformation of the δ-selective ligand at the receptor site. The facts that **8** has considerably low δ-affinity in binding assay and low potency in MVD assay may also support this interpretation. These observations accord with the results obtained using [aminoisobutyric acid[5,6]]DL-II by Misicka *et al.*[9] However, further studies are required to elucidate the bioactive conformation of DL-II at the receptor site.

In conclusion, single or multiple substitution of amino acid residues with sterically hindered Tle residues appears to result in restriction of the conformational freedom around the residues due to severe steric hindrance.[6b] Consequently, introduction of a Tle–Tle moiety into other biologically active peptides could lead to the preparation of conformationally restricted analogs which may have improved biological activity or receptor selectivity, as was observed with somatostatin,[10] LHRH[11] and opioids peptides.[12]

Experimental

Melting points were determined with a Yanaco MP-S3 apparatus and are uncorrected. Optical rotations were measured with a JASCO DIP-140 polarimeter. TLC was carried out on silica gel plates (Merck, Kiesel gel $60F_{254}$, 5 × 10 cm) with the following solvent systems: *Rf*(A), 1-BuOH–AcOH–H_2O (4:1:5, upper phase); *Rf*(B), 1-BuOH–AcOH–pyridine–H_2O. Analytical HPLC was performed on a Cosmosil 5C18-AR column (4.6 × 250 mm) by gradient elution using the following solvent systems, A: 0.06% TFA and B: 80% acetonitrile containing 0.06% TFA. A linear gradient from 25% B to 50% B over 40 min at a flow rate of 1 ml/min was used and the eluate was monitored at 220 nm. FAB-MS was measured with a JEOL JMS-DX303 instrument. The CD measurement was performed at room temperature with a JASCO J-720 spectropolarimeter equipped with a data processor, using a cell having 1 cm optical path length. CD curves depicted are averages of five runs. Amino acid analyses were carried out with a Hitachi 835 amino acid analyzer.

TABLE IV. Stability of Hydrophobic Domains of Some Synthetic Analogs to Acid Hydrolysis

Peptide	Hydrolysis	Amino acid ratio	Recovery (%)
DL-II	6 N HCl, 110 °C, 22 h	Val/Gly = 1.60	80 (Val)
6	6 N HCl, 110 °C, 24 h	Ile/Gly = 1.48	74 (Ile)
8	6 N HCl, 110 °C, 22 h	Tle/Gly = 0.93	47 (Tle)
8	c. HCl–propionic acid (1:1),[a] 130 °C, 22 h	Tle/Gly = 1.98	99 (Tle)
Des-Tle[5]-**8**	6 N HCl, 110 °C, 22 h	Tle/Gly = 1.04	104 (Tle)
9	6 N HCl, 110 °C, 22 h	Mle/Gly = 2.05	102 (Mle)

a) Containing 2% tricresol.

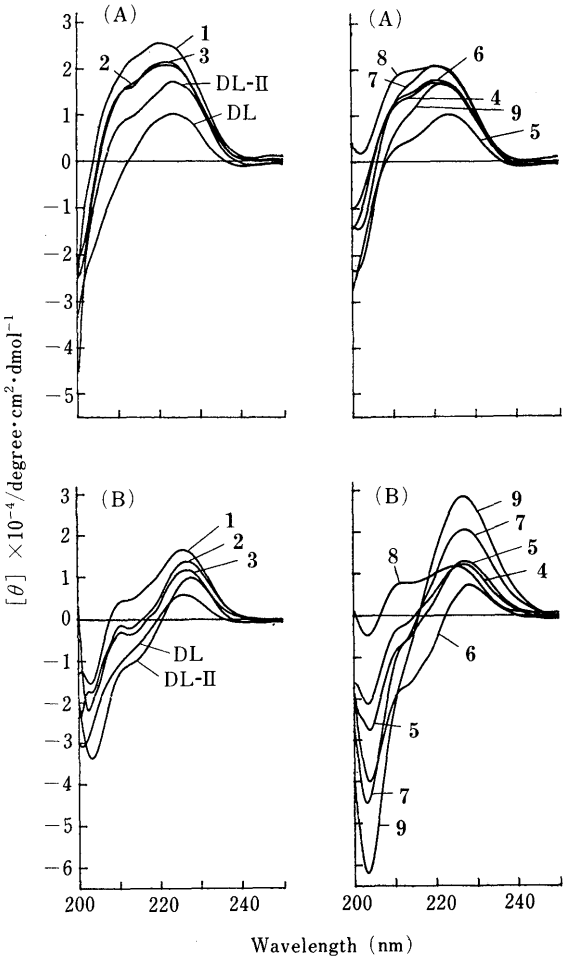

Fig. 2. CD Spectra of Deltorphins and DL-II Analogs in 10 mM Phosphate Buffer at pH 7.60 (A) and in 50% TFE/10 mM Phosphate Buffer at pH 7.60 (B)

1—**9**: analogs **1**—**9**, DL: deltorphin, DL-II: [D-Ala[2]]deltorphin II.

TABLE V. Inhibitory Potency of DL-II Analogs on Electrically Evoked Contractions of GPI and MVD Preparations

Peptide	IC_{50}, nM (mean ± S.E.M.)		IC_{50} ratio (GPI/MVD)	[^{3}H]DAGO[a] K_i, nM	[^{3}H]DADLE[a] K_i, nM	K_i ratio[a] ([^{3}H]DAGO/[^{3}H]DADLE)
	GPI	MVD				
DL-II	3232 ± 281	0.86 ± 0.14	3758	418 ± 56	0.54 ± 0.11	774
1, [Ala[5,6]]DL-II	2220 ± 721	70.24 ± 4.73	32	568 ± 186	21.0 ± 1.9	27
2, [Ala[5]]DL-II	1110 ± 121	2.52 ± 0.67	439	200 ± 20	1.60 ± 0.34	125
3, [Ala[6]]DL-II	1990 ± 552	2.94 ± 0.28	677	582 ± 95	1.07 ± 0.06	543
4, [Nva[5,6]]DL-II	2510 ± 612	0.55 ± 0.07	4563	217 ± 27	0.74 ± 0.22	293
5, [Nle[5,6]]DL-II	2340 ± 260	1.00 ± 0.14	2340	275 ± 18	0.085 ± 0.021	3235
6, [Ile[5,6]]DL-II	4080 ± 1190	1.56 ± 0.06	2615	251 ± 20	0.067 ± 0.021	3746
7, [Leu[5,6]]DL-II	2050 ± 300	0.54 ± 0.18	3796	250 ± 32	0.227 ± 0.040	1101
8, [Tle[5,6]]DL-II	5650 ± 620	13.87 ± 0.87	407	352 ± 59	6.20 ± 0.80	56
9, [Mle[5,6]]DL-II	3340 ± 621	2.62 ± 0.37	1275	433 ± 85	0.157 ± 0.042	2758

a) Receptor binding assay data cited from ref. 3.

Fmoc-Tle Fmoc-ONSu (1.96 g) was added to a solution of Tle (787 mg) and Na_2CO_3 (636 mg) in acetone–H_2O (1 : 1, 15 ml). The mixture was stirred overnight, then the acetone was evaporated off, EtOAc (20 ml) was added, and the mixture was acidified with 2 N HCl. The organic phase was separated, washed with H_2O (×3), dried over $MgSO_4$ and evaporated to dryness *in vacuo*. Storage of the resulting oil in the cold gave fine needles; yield 2.01 g (95%), mp 123—125 °C, $[\alpha]_D^{20}$ −11.0° (c = 1, MeOH). *Anal.* Calcd for $C_{21}H_{23}NO_4$: C, 71.37; H, 6.56; N, 3.96. Found: C, 70.85; H, 6.47; N, 3.88.

Boc-Mle Boc-ON (1.35 g) was added to a solution of Mle (726 mg) and Et_3N (1.1 ml) in DOX–H_2O (1 : 1, 15 ml). The mixture was stirred overnight, then the solution was diluted with H_2O (30 ml), washed twice with EtOAc, acidified with citric acid to pH 4 and extracted twice with EtOAc. The extract was washed with H_2O (×3), dried over $MgSO_4$ and evaporated to dryness *in vacuo*. The product obtained was reprecipitated from EtOAc–pet. ether; yield 1.01 g (87%), mp 100—102 °C, $[\alpha]_D^{20}$ −17.5° (c = 1, MeOH). *Anal.* Calcd for $C_{12}H_{23}NO_4$: C, 58.75; H, 9.45; N, 5.71. Found: C, 58.85; H, 9.93; N, 5.71.

Solid-Phase Synthesis The peptide was constructed on a benzhydrylamine resin (0.6 mmol/g, 1% cross-linked) according to the following schedule: 1) DOX–DCM (1 : 9, ×2), 2) 0.5 M MSA/DOX–DCM (1 : 9) (×2, 5 and 30 min), 3) DOX–DCM (1 : 3, ×2), 4) DCM (×3), 5) 10% DIPEA/DCM (×2, 2 and 3 min), 6) DCM (×6), 7) Boc-amino acid (4 eq)/DMF and DIPCDI (4 eq)/DCM (×1, 120 min), 8) DCM–DMF (1 : 1, ×2), 9) DCM (×3). For the synthesis of **8**, the Tle[5] residue was incorporated using Fmoc-Tle (4 eq) in the presence of HOBt (4 eq) at step 7 and the schedule at the next cycle was change as follows: 4) DMF (×3), 5) 20% piperidine/DMF (×2, 5 and 30 min), 6) DMF (×6). The protected peptide resin was treated with HF in the presence of 10% anisole at 0 °C for 60 min. After evaporation of HF *in vacuo*, 5% AcOH and ether (1 : 1) were added to the reaction vessel and the mixture was vigorously stirred for 10 min. The resin was filtered off. The aqueous phase was washed with ether and evaporated to dryness *in vacuo* below 40 °C. The crude product was applied to a column (2.4 × 36 cm) of Develosil Lop ODS 24S, which was eluted with CH_3CN/0.06% TFA linear gradient systems over 150 min at a flow rate of 3 ml/min. The eluate was monitored at 280 nm. Fractions around the main peak were checked by analytical HPLC and the pure portions were collected and freeze-dried. Analytical data for peptides synthesized are given in Tables II and III.

The des-Tle[5]-analog **8** (peak a in Fig. 1A) was isolated after the usual solid phase synthesis as described above in an overall yield of 30%. Amino acid analysis (6 N HCl): Glu 1.06; Gly 1.00; Ala 1.10; Tyr 0.87; Phe 0.99; Tle 1.04, FAB-MS m/z: 698 $(M+H)^+$.

Coupling Reaction of N^{α}-Protected Tle with Tle–Gly-Resin Boc-Tle (4 eq, methods A—C) or Fmoc-Tle (4 eq, method D) was allowed to react with Tle–Gly–BHA resin (200 mg) in the absence (method A) or presence (methods B and D) of HOBt (4 eq) with the aid of DIPCDI (4 eq, methods A, B and D) for 2 h according to the schedule described above. For method C, Boc-Tle, HOBt (4 eq) and BOP (4 eq) in DMF were added, followed by addition of DIPEA (4.8 eq) and the coupling was performed for 2 h. The protected tripeptide resin was thoroughly washed with DMF and EtOH and dried. An aliquot of the resin was hydrolyzed with propionic acid–concentrated HCl (1 : 1) containing 2% tricresol at 130 °C for 20 h and subjected to the amino acid analysis. Tle/Gly ratios were calculated from the equation, Tle value/(0.86 × Gly value).

Opioid Activity For the GPI assay, the myenteric plexus-longitudinal muscle was obtained from male Hartley strain guinea-pig (250—300 g) ileum as described by Rang.[13] The tissue was mounted in a 20 ml bath containing 37 °C aerated (95% O_2, 5% CO_2) Krebs–Henseleit solution (composition in mM: 118 NaCl, 5.4 KCl, 0.57 $MgSO_4 \cdot 7H_2O$, 0.9 NaH_2PO_4, 2.5 $CaCl_2$, 24 $NaHCO_3$, 11 glucose). The tissue was stimulated transmurally between platinum wire electrodes with pulses of 0.5 ms duration at a frequency of 0.1 Hz using supramaximal voltage. Longitudinal contractions were recorded *via* an isometric transducer.

For the MVD assay, the MVD was prepared as described by Hughes *et al.*[14] from male mouse of ddY strain weighing 25—35 g. A pair of *vasa* was mounted in a 20 ml bath containing 37 °C aerated (95% O_2, 5% CO_2), modified Krebs solution (Mg^{2+}-free) containing ascorbic acid (0.1 mM) and EDTA·4Na (0.027 mM).[14] The tissue was stimulated transmurally with trains of rectilinear pulses of 1 ms. Stimulation trains were given at intervals of 20 s and consisted of seven stimuli of 1 ms duration with intervals of 10 ms. In both assays, three to four washings were done with intervals of 15 min between each dose. Dose–response curves were constructed and IC_{50} values (concentration causing a 50% reduction of the electrically induced twitches) were calculated graphically.

References and Notes

1) Amino acids and peptides are of L-configuration unless otherwise noted. Symbols for amino acids and peptides used are those recommended by the IUPAC-IUB Commission of Biochemical Nomenclature in *Eur. J. Biochem.*, **138**, 9 (1984). Other abbreviations used are: Tle = *tert*-leucine, Mle = γ-methyl-leucine, Boc = *tert*-butoxycarbonyl, Fmoc = fluorenylmethoxycarbonyl, Br-Z = 2-bromobenzyloxycarbonyl, cHex = cyclohexyl, ONSu = *N*-hydroxysuccinimide ester, Boc-ON = *tert*-butoxycarbonyl-oxyimino-2-phenylacetonitrile, DIPCDI = *N*,*N*′-diisopropylcarbodiimide, DIPEA = *N*,*N*′-diisopropylethylamine, HOBt = 1-hydroxybenzotriazole, BOP = benzotriazoleyloxy-trisdimethylaminophosphonium hexafluorophosphate, MSA = methanesulfonic acid, TFE = trifluoroethanol, LHRH = luteinizing hormon-releasing hormon, DMF = *N*,*N*-dimethylformamide, HPLC = high performance liquid chromatography, TLC = thin layer chromatography, DOX = dioxane, SPPS = solid-phase peptide synthesis, DCM = dichloromethane.
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