Further Studies on Lead Compounds Containing the Opioid Pharmacophore Dmt-Tic

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Some reference opioids containing the Dmt-Tic pharmacophore, especially the δ agonists H-Dmt-Tic-Gly-NH-Ph (**1**) and H-Dmt-Tic-NH-(*S*)CH(CH_2−COOH)-Bid (**4**) (UFP-512) were evaluated for the influence of the substitution of Gly with aspartic acid, its chirality, and the importance of the −NH-Ph and N^1H-Bid hydrogens in the inductions of δ agonism. The results provide the following conclusions: (i) Asp increases δ selectivity by lowering the μ affinity; (ii) −NH-Ph and N^1H-Bid nitrogens methylation transforms the δ agonists into δ antagonists; (iii) the substitution of Gly with L-Asp/D-Asp in the δ agonist H-Dmt-Tic-Gly-NH-Ph gave δ antagonists; the same substitution in the δ agonist H-Dmt-Tic-NH-CH_2−Bid yielded more selective agonists, H-Dmt-Tic-NH-(*S*)CH(CH_2−COOH)-Bid and H-Dmt-Tic-NH-(*R*)CH(CH_2−COOH)-Bid; (iv) L-Asp seems important only in functional bioactivity, not in receptor affinity; (v) H-Dmt-Tic-NH-(*S*)CH(CH_2−COOH)-Bid(N^1-Me) (**10**) evidenced analgesia similar to **4**, which was reversed by naltrindole only in the tail flick. **4** and **10** had opposite behaviours in mice; **4** caused agitation, **10** gave sedation and convulsions.

Introduction

The prototype opioid pharmacophore Dmt-Tic,[a,1] which evolved from H-Tyr-Tic-OH[2] as a simplified form of TIP(P),[3] represents the minimum sequence that selectively interacts with δ opioid receptors as a potent δ antagonist. Extensive structure−activity studies on this prototype revealed that even minor chemical modifications changed its pharmacological profile, including a wide range of properties such as: enhanced δ antagonism,[4] appearance of mixed μ agonism/δ agonism[5] as well as mixed μ agonism/δ antagonism,[5] μ agonism,[6] μ antagonism,[6] δ inverse agonism,[7] and δ agonism.[5,8−10] Among all synthesized analogues, some lead compounds were obtained, for example the potent and selective δ antagonist *N*,*N*(Me)$_2$-Dmt-Tic-OH[11] and the δ inverse agonist *N*,*N*(Me)$_2$-Dmt-Tic-NH_2[12,13] as useful tools for pharmacological studies. Some other lead compounds endowed with potential utility as therapeutic agents, are represented by: the μ agonist/δ antagonist H-Dmt-Tic-Gly-NH-Bzl (**2**); the μ agonist/δ agonist H-Dmt-Tic-Gly-NH-Ph (**1**); and the potent δ agonists H-Dmt-Tic-NH-CH_2−Bid (**3**) (UFP-502)[27] and H-Dmt-Tic-NH-(*S*)CH(CH_2−COOH)-Bid (**4**) (UFP-512).[9] On the basis of different pharmacological studies, μ agonists/δ antagonists[14] and μ agonists/δ agonists[15] may provide new classes of analgesics with low propensity to induce tolerance, physical dependence, constipation, and other side effects. δ Opioid receptor agonists are known to produce many pharmacological effects in rodents, including analgesia,[16] antidepressant,[10,17] neuroprotection/neurogenesis,[18] and anti-Parkinson[19] activities. Moreover, peripheral δ-opioid receptors seem to be involved in cancer,[20] cardiovascular disease,[21] gastrointestinal disorders,[22] and newer paradigms for pain relief that use peripherally restricted opioids.[23] Starting from our selected lead compounds, here we report some new attempts for a better understanding of their biological profiles, especially in the light of the fact that even minor modifications can change their pharmacological characteristics.[24,25] In particular, we focused our attention once again on the importance of the C-terminal Bid (1*H*-benzimidazole-2-yl) and the anilide function (considered as an open ring surrogate of Bid); in fact, both groups are important for δ agonist activity. From previous studies, the hydrogen linked to the N^1 of Bid[24,25] (and probably the corresponding hydrogen linked to the anilide function) seems to be important for the induction of δ agonism. To ascertain this hypothesis, we synthesized some N^1−Bid and *N*-anilide methylated analogues of our selected lead compounds. Moreover, to verify the importance of the negative charge in the induction of δ selectivity and to confirm the ineffectiveness of the C-terminal chiral center,[8] we considered the substitution of Gly with L-Asp or D-Asp residues. Furthermore, such modification seems to be capable to influence the blood−brain barrier

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[a] Abbreviations. In addition to the IUPAC-IUB Commission on Biochemical Nomenclature (*J. Biol. Chem.* **1985**, *260*, 14−42), this paper uses the following additional symbols and abbreviations: AcOEt, ethyl acetate; AcOH, acetic acid; Bid, 1*H*-benzimidazole-2-yl; Boc, *tert*-butyloxycarbonyl; DAMGO, [D-Ala^2,*N*-Me-Phe^4,Gly-ol^5]enkephalin; DEL C, deltorphin II (H-Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2); DMF, *N*,*N*-dimethylformamide; DMSO-d_6, hexadeuteriodimethyl sulfoxide; Dmt, 2′,6′-dimethyl-L-tyrosine; DPDPE, (D-Pen^2,D-Pen^5)-enkephalin; Endomorphin-2, H-Tyr-Pro-Phe-Phe-NH_2; Et_2O, diethyl ether GPI, guinea-pig ileum; HOBt, 1-hydroxybenzotriazole; HPLC, high performance liquid chromatography; IBCF, isobutyl chloroformate; MALDI-TOF, matrix assisted laser desorption ionization time-of-flight. MVD, mouse vas deferens; NMM, 4-methylmorpholine; NLX, naloxone; NTI, naltrindole; pA_2, negative log of the molar concentration required to double the agonist concentration to achieve the original response; Pe, petroleum ether; PL017, H-Tyr-Pro-(N-Me)Phe-D-Pro-NH_2; TEA triethylamine; TFA, trifluoroacetic acid; Tic, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid; TLC, thin-layer chromatography; WSC, 1-ethyl-3-[3′-dimethyl)aminopropyl]-carbodiimide hydrochloride; Z, benzyloxycarbonyl.

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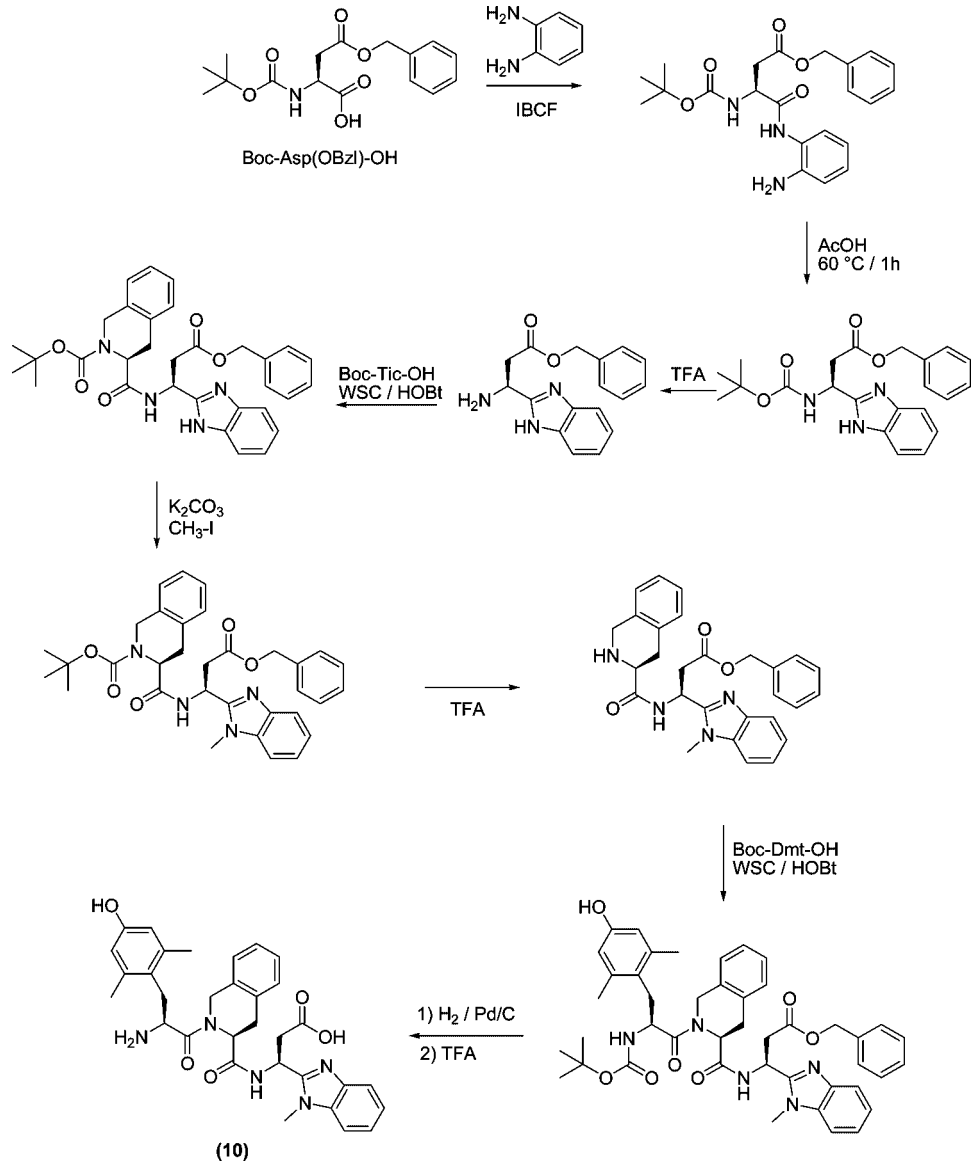
Scheme 1. Synthesis of Compound **10**



(BBB) penetration; in fact, both **3**[26,27] and **4**[10] show antidepressant activities when administered icv, but only **4** after intraperitoneus administration.

Chemistry

All peptides and pseudopeptides (**5**−**13**) were prepared stepwise in solution using conventional synthetic methods as outlined in Scheme 1 for the more representative compound (**10**). The intermediates containing Bid at their C-termini, Boc-NH-(*S*)CH(CH_2−COOBzl)-Bid[8] and Boc-NH-(*R*)CH(CH_2−COOBzl)-Bid were prepared according to the procedure of Nestor et al.[28] Briefly, mixed carbonic anhydride coupling of Boc-Asp(OBzl)-OH or Boc-D-Asp(OBzl)-OH with *o*-phenylendiamine gave the crude intermediate amides, which were converted without purification to the desired benzimidazole heterocycles (Bid) by cyclization/dehydration in acetic acid at 60 °C for 1 h.. The corresponding Boc-protected intermediate anilides, or N(Me)-anilides, or benzylamides were prepared by condensation of Boc-Gly-OH or Boc-Asp(OBzl)-OH or Boc-D-Asp(OBzl)-OH with aniline, or *N*-methyl-aniline, or benzylamine, via mixed carbonic anhydride. Each intermediate, after Boc deprotection with TFA, was condensed with Boc-Tic-OH via WSC/HOBt. N^1−Bid methylation was accomplished in DMF using K_2CO_3 as a base, followed after 1 h by the reaction with iodomethane.[24] Subsequently, Boc deprotection (TFA) and the final condensation (WSC/HOBt) with Boc-Dmt-OH gave the fully protected compounds. Final deprotections of Asp or D-Asp side chains (by catalytic hydrogenation) and N-terminal amine functions (by TFA treatment) gave the crude final compounds (**5**−**13**), which were purified by preparative reverse phase HPLC.

Results and Discussion

Receptor Affinity Analysis. Receptor binding data for δ- and μ-receptors and δ-selectivity (K_i^{μ}/K_i^{δ}) are reported in Table 1. All new compounds (**5**−**13**) had subnanomolar affinities for δ-opioid receptors (K_i^{δ} = 0.036−0.186 nM), which is in quite good accordance with the reference compounds. As expected, the introduction of a negative charge, derived from the substitution of Gly with L- or D-Asp, increased δ-selectivity essentially by decreasing μ-affinity. In fact, compounds **6**−**13** with a K_i^{μ} = 7.49−364.3 nM exhibited a δ-selectivity ranging from 101 to 5730 in comparison with references and compound **5** lacking the negative charge (δ-selectivity = 4−14). The highest increase in δ-selectivity derived from the substitution of Gly with aspartic

Table 1. Receptor Binding Affinities and Functional Bioactivities of Compounds **1**−**13**

	structure	receptor affinity[a] (nM)			functional bioactivity		
		K_i^δ	K_i^μ	K_i^μ/K_i^δ selectivity	MVD pA_2[c]	MVD IC_{50}[b] (nM)	GPI IC_{50}[b] (nM)
ref							
1	H-Dmt-Tic-Gly-NH-Ph[d]	0.042	0.16	4		3.02	2.57
2	H-Dmt-Tic-Gly-NH-Bzl[d]	0.031	0.16	5	9.25		2.69
3	H-Dmt-Tic-NH-CH_2-Bid[d]	0.035	0.50	14		0.13	26.92
4	H-Dmt-Tic-NH-(S)CH(CH_2-COOH)-Bid[e]	0.443	53.9	122		0.12	1724
compd							
5	H-Dmt-Tic-Gly-N(Me)-Ph	0.143 ± 0.024 (4)	1.75 ± 0.067 (3)	12	8.80		1466 ± 629
6	H-Dmt-Tic-Asp-NH-Ph	0.036 ± 0.002 (3)	10.8 ± 1.2 (5)	300	9.40		265 ± 10.4
7	H-Dmt-Tic-D-Asp-NH-Ph	0.058 ±0.003 (3)	7.49 ± 0.57 (4)	129	8.62		2655 ± 127.8
8	H-Dmt-Tic-Asp-N(Me)-Ph	0.186 ± 0.008 (3)	364.3 ± 37 (3)	1958	8.75		>10000
9	H-Dmt-Tic-D-Asp-N(Me)-Ph	0.084 ± 0.008 (4)	45.0 ± 4.3 (5)	536	8.06		>10000
10	H-Dmt-Tic-NH-(*S*)CH(CH_2−COOH)-Bid(N^1-Me)	0.059 ±0.008 (4)	5.93 ± 0.82 (5)	101	9.90		2886 ± 983
11	H-Dmt-Tic-NH-(*R*)CH(CH_2−COOH)-Bid(N^1-Me)	0.070 ±0.007 (4)	11.8 ± 1.4 (5)	169	9.65		>10000
12	H-Dmt-Tic- NH-(*R*)CH(CH_2−COOH)-Bid	0.067 ± 0.008 (5)	16.1 ± 1.9 (6)	240		1.21	179
13	H-Dmt-Tic-Asp-NH-Bzl	0.054 ± 0.006 (4)	309.4 ± 24 (3)	5730	8.53		>10000

[a] The K_i values (nM) were determined according to Cheng and Prusoff.[43] The mean ± SE with *n* repetitions in parenthesis is based on independent duplicate binding assays with five to eight peptide doses using several different synaptosomal preparations. [b] Agonist activity was expressed as IC_{50} obtained from dose−response curves. These values represent the mean ± SE for at least four tissue samples. DPDPE and PL017 were the internal standards for MVD (δ-opioid receptor bioactivity) and GPI (μ-opioid receptor bioactivity) tissue preparation, respectively. [c] The pA_2 values of opioid antagonists against the δ and μ agonists (deltorphin II and endomorphin-2, respectively) were determined by the method of Kosterlitz and Watt.[49] [d] Data taken from Balboni et al.[5] [e] Data taken from Balboni et al.[8]

acid as seen in H-Dmt-Tic-Gly-NH-Bzl (**2**); in fact, its selectivity ($K_i^\mu/K_i^\delta = 5$) rose over 3 orders of magnitude to 5730. The same substitution gave lower increases in selectivity when applied to the reference compounds **1** and **3**. On the basis of the C-terminal aromatic substituents, the best selectivity was induced by −NH-Bzl > −N(Me)-Ph > −NH-Ph > −Bid ≥ −Bid(N^1-Me). With regard to the aspartic acid chirality, no final conclusions can be drawn about its influence on the observed receptor selectivity.

Functional Bioactivity. Compounds **5**−**13** were tested in the electrically stimulated MVD and GPI pharmacological assays for intrinsic functional bioactivity (Table 1). As quite usually observed with compounds containing the Dmt-Tic pharmacophore, a close correlation between binding and functional bioactivity data is often lacking. Recently, a similar lack of correlation was observed by Hruby et al. with 4-anilidopiperidine analogues.[29] As expected, and partially demonstrated for **3**,[24,25] all N-methylated analogues of anilides and N^1−Bid (**5**, **8**−**11**), revealed potent and selective δ-opioid antagonist activity (MVD, $pA_2 = 8.06 - 9.90$), confirming the importance of the hydrogen of −NH-Ph and N^1H-Bid in the induction of δ agonism. Surprisingly, the substitution of Gly with L-Asp (**6**) or D-Asp (**7**) in the reference compound **1** gave two potent and quite selective δ antagonists (MVD, $pA_2 = 9.40$ and 8.62, respectively) despite the presence of the −NH-Ph hydrogen. Compound **12**, the diastereoisomer containing the D-Asp side chain of our best δ agonist **4**, indicated for the first time that better results can be obtained using L amino acids in the synthesis of compounds containing a C-terminal Bid. In fact, it shows a δ agonist activity of 1 order of magnitude lower than **4** and a μ agonist activity of almost 1 order of magnitude higher than **4**. Interestingly, compound **10**, the N^1−Bid methylated analogue of **4** gave the highest δ antagonism ($pA_2 = 9.90$) in this series of compounds, associated with a μ agonism 1.7 fold higher than **4**. The substitution of Gly with Asp (**13**) in the μ agonist/δ antagonist **2** was detrimental in its activity profile; in fact, **13** showed a selective δ antagonist activity (5 fold lower than **2**), associated with a very weak μ antagonist activity (GPI, $pA_2 = 6.26$, not reported in Table 1). Finally, in the 3 pairs of compounds (**6/7**, **8/9**, and **10/11**) and in the pair consisting of **4** and **12**, the best δ activities were always showed by the analogues containing L-aspartic acid; however, this trend is not supported by the corresponding affinity data.

In Vivo Biological Activity. In recallling the data reported by Codd et al.,[30] they demonstrated the in vivo biotrasformation of a δ opioid agonist into a μ agonist by N-deethylation. Looking at our new compounds (**5**−**13**), a similar behavior could be expected from all N-methylated analogues (**5**, **8**−**11**). On the basis of this hypothesis, we chose the potent and selective δ antagonist (**10**) as a potential prodrug of the potent and selective δ agonist **4**. Preliminary enzymatic degradation studies (Supporting Information) failed to demonstrate this assumption. In fact, both compounds **4** and **10** appeared stable to degradation for 4 and 2 h in plasma and brain homogenate, respectively. Notwithstanding the preceding negative results, we tested compound **10** for in vivo analgesia in comparison with **4**; a positive result could be tentatively considered as indirect evidence of the N-demethylation of **10** (δ antagonist) to the corresponding **4** (δ agonist). The analgesic effects of both compounds were determined by tail-flick and hot-plate tests. Results reported in Figure 1 indicated a similar dose-dependent analgesic effect for both compounds after intracerebroventricular injection. Analgesia of both compounds was reversed by the δ selective antagonist naltrindole and the nonselective antagonist naloxone in the tail-flick test but not in the hot-plate (Figures 2 and 3.) Interestingly, at the same dose, **4** and **10** provided opposite behavioural effects; namely, **4** caused excessive grooming and agitation (constant, fast moving in the cage, burrowing in the nesting material), while with **10**, the mice appeared sedated, quiet, easily handled, and moving slowly if at all. Furthermore, **4** did not induce convulsions even at the higher dosages, confirming our previous data on its antidepressant and anxiolytic studies,[9,10] which are in accord with observations about the higher convulsive effects of the nonpeptidic δ agonists in comparison to opioid peptides.[26,31,32]

Experimental Section

Chemistry. Boc-Gly-N(Me)-Ph. A solution of Boc-Gly-OH (1.00 g, 5.71 mmol) and NMM (0.62 mL, 5.71 mmol) in DMF (10 mL) was treated at −20 °C with IBCF (0.74 mL, 5.71 mmol). After 10 min at −20 °C, *N*-methylaniline (0.62 mL, 5.71 mmol) was added. The reaction mixture was allowed to stir while slowly

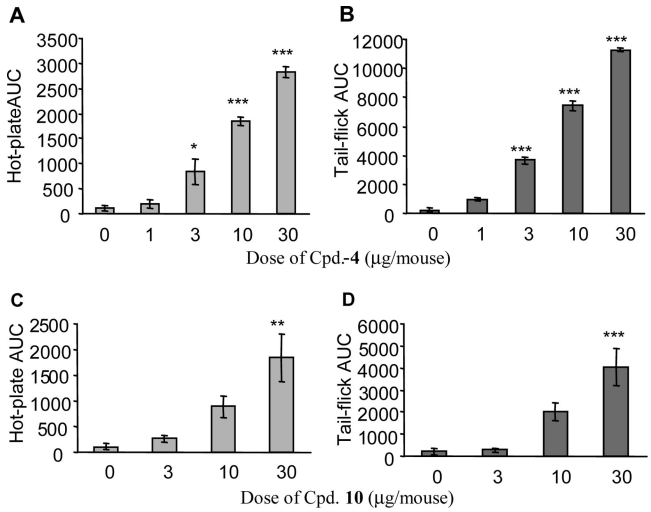


Figure 1. Dose dependent effect of icv injected **4** (A,B) and **10** (C,D) in the hot-plate (A,C) and tail-flick (B,D) tests. Each point represents the mean ± SEM ($n = 5$ mice). The asterisks denote AUC values that are significantly different from saline treated mice by Dunnett's test (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$) following ANOVA ((A) $P < 0.0001$: $F = 71.49$, d.f. 4; (B) $P < 0.0001$: $F = 251.7$, d.f. 4; (C): $P < 0.001$, $F = 9.356$, d.f. 3; (D) $p < 0.0001$: $F = 15.14$, d.f. 3).

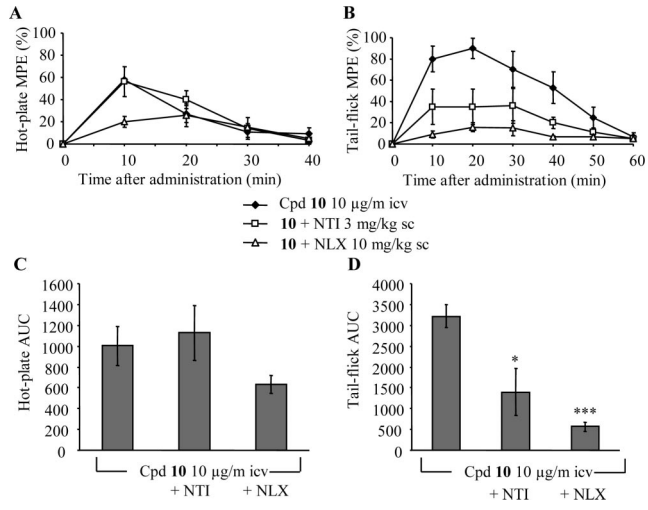


Figure 2. Effect of sc injected naltrindole (3 mg/kg) and naloxone (10 mg/kg) on **10** induced antinociception in hot-plate (A,C) and tail-flick (B,D) tests. Compound **10** was administered icv (10 μg/mouse) 20 min after administration of antagonists. (A,B) Time course; (C,D) area under the curve (AUC). Each point represents the mean ± SEM ($n = 5$ mice). The asterisks denote AUC values that are significantly different from saline treated mice by Dunett's test (*, $p < 0.05$; ***, $p < 0.001$) following ANOVA ((C) $p = 0.2138$: $F = 1.759$, d.f. 2; (D) $p < 0.001$: $F = 13.61$, d.f. 2).

warming to room temperature (1 h) and was then stirred for an additional 3 h. The solvent was evaporated, and the residue was partitioned between EtOAc and H_2O. The EtOAc layer was washed with citric acid (10% in H_2O), $NaHCO_3$ (5% in H_2O), and brine and dried over Na_2SO_4. The solution was filtered, the solvent evaporated, and the residual oil was precipitated from Et_2O/Pe (1:9, v/v): yield 1.39 g (92%); R_f (B) 0.52; HPLC K' 6.01; mp 99−101 °C; *m/z* 265 $(M + H)^+$. ^{1}H NMR (DMSO-d_6) δ 1.40 (s, 9H), 2.78 (s, 3H), 3.88−3.92 (m, 2H), 7.10−7.31 (m, 5H).

TFA˙H-Gly-N(Me)-Ph. Boc-Gly-N(Me)-Ph (1.36 g, 5.15 mmol) was treated with TFA (2.5 mL) for 0.5 h at room temperature. Et_2O/Pe (1:1, v/v) were added to the solution until the product precipitated: yield 1.36 g (95%); R_f (A) 0.49; HPLC K' 5.12; mp 115−117 °C; *m/z* 165 $(M + H)^+$.

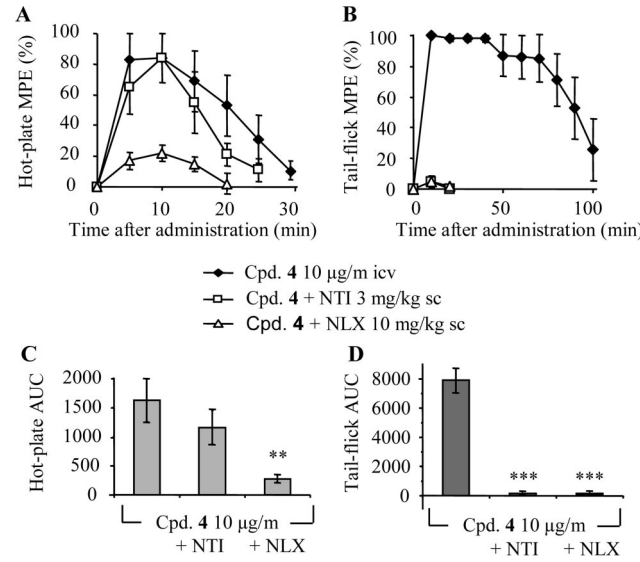


Figure 3. Effect of sc injected naltrindole (3 mg/kg) and naloxone (10 mg/kg) on **4** induced antinociception in hot-plate (A,C) and tail-flick (B,D) tests. **4** was administered icv (10 μg/mouse) 20 min after administration of antagonists. (A,B) Time course; (C,D) area under the curve (AUC). Each point represents the mean ± SEM ($n = 5$ mice). The asterisks denote AUC values that are significantly different from saline treated mice by Dunnett's test (**, $p < 0.01$; ***, $p < 0.001$) following ANOVA ((C) $p = 0.0136$: $F = 6.277$, d.f. 2; (D) $p < 0.0001$: $F = 87.13$, d.f. 2).

Boc-Tic-Gly-N(Me)-Ph. To a solution of Boc-Tic-OH (0.20 g, 0.72 mmol) and TFA˙H-Gly-N(Me)-Ph (0.20 g, 0.72 mmol) in DMF (10 mL) at 0 °C, NMM (0.08 mL, 0.72 mmol), HOBt (0.12 g, 0.79 mmol), and WSC (0.15 g, 0.79 mmol) were added. The reaction mixture was stirred for 3 h at 0 °C and 24 h at room temperature. After DMF was evaporated, the residue was dissolved in EtOAc and washed with citric acid (10% in H_2O), $NaHCO_3$ (5% in H_2O), and brine. The organic phase was dried (Na_2SO_4) and evaporated to dryness. The residue was precipitated from Et_2O/Pe (1:9, v/v): yield 0.27 g (87%); R_f (B) 0.69; HPLC K' 7.35; mp 133−135 °C; $[\alpha]^{20}_D$ −30.1; *m/z* 425 $(M + H)^+$. ^{1}H NMR (DMSO-d_6) δ 1.38−1.42 (d, 9H), 2.78 (s, 3H), 2.92−3.17 (m, 2H), 4.14−4.27 (m, 4H), 4.92 (m, 1H), 6.96−7.31 (m, 9H).

TFA˙H-Tic-Gly-N(Me)-Ph. Boc-Tic-Gly-N(Me)-Ph (0.24 g, 0.57 mmol) was treated with TFA (1.5 mL) for 0.5 h at room temperature. Et_2O/Pe (1:1, v/v) were added to the solution until the product precipitated: yield 0.24 g (95%); R_f (A) 0.39; HPLC K' 6.22; mp 158−160 °C; $[\alpha]^{20}_D$ −30.6; *m/z* 324 $(M + H)^+$.

Boc-Dmt-Tic-Gly-N(Me)-Ph. To a solution of Boc-Dmt-OH (0.10 g, 0.32 mmol) and TFA˙H-Tic-Gly-N(Me)-Ph (0.14 g, 0.32 mmol) in DMF (10 mL) at 0 °C, NMM (0.03 mL, 0.32 mmol), HOBt (0.05 g, 0.35 mmol), and WSC (0.07 g, 0.35 mmol) were added. The reaction mixture was stirred for 3 h at 0 °C and 24 h at room temperature. After DMF was evaporated, the residue was dissolved in EtOAc and washed with citric acid (10% in H_2O), $NaHCO_3$ (5% in H_2O), and brine. The organic phase was dried (Na_2SO_4) and evaporated to dryness. The residue was precipitated from Et_2O/Pe (1:9, v/v): yield 0.17 g (87%); R_f (B) 0.68; HPLC K' 10.01; mp 141−43 °C; $[\alpha]^{20}_D$ −18.5; *m/z* 616 $(M + H)^+$. ^{1}H NMR (DMSO-d_6) δ 1.38−1.42 (d, 9H), 2.35 (s, 6H), 2.78 (s, 3H), 2.92−3.17 (m, 4H), 4.14−4.92 (m, 6H), 6.29 (s, 2H), 6.96−7.31 (m, 9H).

TFA˙H-Dmt-Tic-Gly-N(Me)-Ph (5). Boc-Dmt-Tic-Gly-N(Me)-Ph (0.14 g, 0.23 mmol) was treated with TFA (1 mL) for 0.5 h at room temperature. Et_2O/Pe (1:1, v/v) were added to the solution until the product precipitated: yield 0.14 g (97%); R_f (A) 0.45; HPLC K' 7.25; mp 150−152 °C; $[\alpha]^{20}_D$ −20.3; *m/z* 516 $(M + H)^+$. ^{1}H NMR (DMSO-d_6) δ 2.35 (s, 6H), 2.78 (s, 3H), 2.92−3.17 (m, 4H), 3.95−4.92 (m, 6H), 6.29 (s, 2H), 6.96−7.31 (m, 9H); Anal. $C_{32}H_{35}F_3N_4O_6$: C; H; N.

Boc-Asp(OBzl)-NH-Ph. This intermediate was obtained by condensation of Boc-Asp(OBzl)-OH with aniline via mixed anhydride as reported for Boc-Gly-N(Me)-Ph: yield 0.57 g (93%); R_f (B) 0.73; HPLC K' 7.02; mp 129−132 °C; $[\alpha]^{20}_D$ +12.5; *m/z* 399 (M + H)$^+$. ^{1}H NMR (DMSO-d_6) δ 1.40 (s, 9H), 2.72−2.97 (d, 2H), 5.17−5.34 (m, 3H), 7.19−7.60 (m, 10H).

TFA˙H-Asp(OBzl)-NH-Ph. Boc-Asp(OBzl)-NH-Ph was treated with TFA as reported for TFA˙H-Gly-N(Me)-Ph: yield 0.54 g (96%); R_f (A) 0.75; HPLC K' 5.02; mp 138−140 °C; $[\alpha]^{20}_D$ +15.2; *m/z* 299 (M + H)$^+$.

Boc-Tic-Asp(OBzl)-NH-Ph. This intermediate was obtained by condensation of Boc-Tic-OH with TFA˙H-Asp(OBzl)-NH-Ph via WSC/HOBt as reported for Boc-Tic-Gly-N(Me)-Ph: yield 0.21 g (88%); R_f (B) 0.82; HPLC K' 6.43; mp 147−149 °C; $[\alpha]^{20}_D$ +5.2; *m/z* 559 (M + H)$^+$. ^{1}H NMR (DMSO-d_6) δ 1.38−1.42 (d, 9H), 2.72−3.17 (m, 4H), 4.17−4.27 (m, 2H), 4.92−5.34 (m, 4H), 6.96−7.64 (m, 14H).

TFA˙H-Tic-Asp(OBzl)-NH-Ph. Boc-Tic-Asp(OBzl)-NH-Ph was treated with TFA as reported for TFA˙H-Tic-Gly-N(Me)-Ph: yield 0.18 g (96%); R_f (A) 0.73; HPLC K' 4.21; mp 145−147 °C; $[\alpha]^{20}_D$ +5.3; *m/z* 459 (M + H)$^+$.

Boc-Dmt-Tic-Asp(OBzl)-NH-Ph. This intermediate was obtained by condensation of Boc-Dmt-OH with TFA˙H-Tic-Asp(OBzl)-NH-Ph via WSC/HOBt as reported for Boc-Dmt-Tic-Gly-N(Me)-Ph: yield 0.14 g (87%); R_f (B) 0.78; HPLC K' 9.31; mp 165−167 °C; $[\alpha]^{20}_D$ −2.5; *m/z* 750 (M + H)$^+$. ^{1}H NMR (DMSO-d_6) δ 1.38−1.42 (d, 9H), 2.35 (s, 6H), 2.72−3.17 (m, 6H), 4.41−4.51 (m, 2H), 4.92−5.34 (m, 5H), 6.29 (s, 2H), 6.96−7.64 (m, 14H).

Boc-Dmt-Tic-Asp-NH-Ph. To a solution of Boc-Dmt-Tic-Asp(OBzl)-NH-Ph (0.11 g, 0.15 mmol) in methanol (30 mL) was added Pd/C (10%, 0.07 g), and H_2 was bubbled for 1 h at room temperature. After filtration, the solution was evaporated to dryness. The residue was crystallized from Et_2O/Pe (1:9, v/v): yield 0.09 g (95%); R_f (B) 0.67; HPLC K' 7.19; mp 169−171 °C; $[\alpha]^{20}_D$ −6.4; *m/z* 660 (M + H)$^+$.

TFA˙H-Dmt-Tic-Asp-NH-Ph (6). Boc-Dmt-Tic-Asp-NH-Ph was treated with TFA as reported for TFA˙H-Dmt-Tic-Gly-N(Me)-Ph: yield 0.09 g (96%); R_f (A) 0.67; HPLC K' 4.21; mp 158−160 °C; $[\alpha]^{20}_D$ −7.3; *m/z* 560 (M + H)$^+$. ^{1}H NMR (DMSO-d_6) δ 2.35 (s, 6H), 2.72−3.17 (m, 6H), 3.95−4.51 (m, 3H), 4.86−4.92 (m, 2H), 6.29 (s, 2H), 6.96−7.64 (m, 9H); Anal. $C_{33}H_{35}F_3N_4O_8$: C; H; N.

Boc-D-Asp(OBzl)-NH-Ph. This intermediate was obtained by condensation of Boc-D-Asp(OBzl)-OH with aniline via mixed anhydride as reported for Boc-Gly-N(Me)-Ph: yield 0.38 g (92%); R_f (B) 0.73; HPLC K' 7.02; mp 129−132 °C; $[\alpha]^{20}_D$ −12.5; *m/z* 399 (M + H)$^+$. ^{1}H NMR (DMSO-d_6) δ 1.40 (s, 9H), 2.72−2.97 (d, 2H), 5.17−5.34 (m, 3H), 7.19−7.60 (m, 10H).

TFA˙H-D-Asp(OBzl)-NH-Ph. Boc-D-Asp(OBzl)-NH-Ph was treated with TFA as reported for TFA˙H-Gly-N(Me)-Ph: yield 0.25 g (96%); R_f (A) 0.75; HPLC K' 5.02; mp 138−140 °C; $[\alpha]^{20}_D$ −15.2; *m/z* 299 (M + H)$^+$.

Boc-Tic-D-Asp(OBzl)-NH-Ph. This intermediate was obtained by condensation of Boc-Tic-OH with TFA˙H-D-Asp(OBzl)-NH-Ph via WSC/HOBt as reported for Boc-Tic-Gly-N(Me)-Ph: yield 0.17 g (87%); R_f (B) 0.80; HPLC K' 6.38; mp 147−149 °C; $[\alpha]^{20}_D$ +8.4; *m/z* 559 (M + H)$^+$. ^{1}H NMR (DMSO-d_6) δ 1.38−1.42 (d, 9H), 2.73−3.19 (m, 4H), 4.21−4.29 (m, 2H), 4.90−5.31 (m, 4H), 6.96−7.64 (m, 14H).

TFA˙H-Tic-D-Asp(OBzl)-NH-Ph. Boc-Tic-D-Asp(OBzl)-NH-Ph was treated with TFA as reported for TFA˙H-Tic-Gly-N(Me)-Ph: yield 0.15 g (97%); R_f (A) 0.71; HPLC K' 4.17; mp 146−148 °C; $[\alpha]^{20}_D$ +10.1; *m/z* 459 (M + H)$^+$.

Boc-Dmt-Tic-D-Asp(OBzl)-NH-Ph. This intermediate was obtained by condensation of Boc-Dmt-OH with TFA˙H-Tic-D-Asp(OBzl)-NH-Ph via WSC/HOBt as reported for Boc-Dmt-Tic-Gly-N(Me)-Ph: yield 0.11 g (89%); R_f (B) 0.75; HPLC K' 8.98; mp 160−162 °C; $[\alpha]^{20}_D$ +8.6; *m/z* 750 (M + H)$^+$. ^{1}H NMR (DMSO-d_6) δ 1.38−1.42 (d, 9H), 2.35 (s, 6H), 2.70−3.20 (m, 6H), 4.40−4.53 (m, 2H), 4.93−5.32 (m, 5H), 6.29 (s, 2H), 6.96−7.64 (m, 14H).

Boc-Dmt-Tic-D-Asp-NH-Ph. Boc-Dmt-Tic-D-Asp(OBzl)-NH-Ph was treated with Pd/C and H_2 as reported for Boc-Dmt-Tic-Asp-NH-Ph: yield 0.08 g (95%); R_f (B) 0.64; HPLC K' 6.95; mp 164−166 °C; $[\alpha]^{20}_D$ +12.8; *m/z* 660 (M + H)$^+$.

TFA˙H-Dmt-Tic-D-Asp-NH-Ph (7). Boc-Dmt-Tic-D-Asp-NH-Ph was treated with TFA as reported for TFA˙H-Dmt-Tic-Gly-N(Me)-Ph: yield 0.04 g (93%); R_f (A) 0.64; HPLC K' 4.15; mp 150−152 °C; $[\alpha]^{20}_D$ +12.5; *m/z* 560 (M + H)$^+$. ^{1}H NMR (DMSO-d_6) δ 2.35 (s, 6H), 2.70−3.19 (m, 6H), 3.96−4.53 (m, 3H), 4.85−4.90 (m, 2H), 6.29 (s, 2H), 6.96−7.66 (m, 9H); Anal. $C_{33}H_{35}F_3N_4O_8$: C; H; N.

Boc-Asp(OBzl)-N(Me)-Ph. This intermediate was obtained by condensation of Boc-Asp(OBzl)-OH with *N*-methylaniline via mixed anhydride as reported for Boc-Gly-N(Me)-Ph: yield 0.43 g (90%); R_f (B) 0.75; HPLC K' 7.11; mp 126−128 °C; $[\alpha]^{20}_D$ +10.3; *m/z* 413 (M + H)$^+$. ^{1}H NMR (DMSO-d_6) δ 1.40 (s, 9H), 2.72−2.97 (m, 5H), 5.17−5.34 (m, 3H), 7.10−7.31 (m, 10H).

TFA˙H-Asp(OBzl)-N(Me)-Ph. Boc-Asp(OBzl)-N(Me)-Ph was treated with TFA as reported for TFA˙H-Gly-N(Me)-Ph: yield 0.40 g (97%); R_f (A) 0.77; HPLC K' 5.11; mp 135−139 °C; $[\alpha]^{20}_D$ +14.7; *m/z* 313 (M + H)$^+$.

Boc-Tic-Asp(OBzl)-N(Me)-Ph. This intermediate was obtained by condensation of Boc-Tic-OH with TFA˙H-Asp(OBzl)-N(Me)-Ph via WSC/HOBt as reported for Boc-Tic-Gly-N(Me)-Ph: yield 0.21 g (85%); R_f (B) 0.84; HPLC K' 6.45; mp 144−146 °C; $[\alpha]^{20}_D$ +4.8; *m/z* 573 (M + H)$^+$. ^{1}H NMR (DMSO-d_6) δ 1.38−1.42 (d, 9H), 2.73−3.19 (m, 7H), 4.17−4.27 (m, 2H), 4.92−5.34 (m, 4H), 6.96−7.31 (m, 14H).

TFA˙H-Tic-Asp(OBzl)-N(Me)-Ph. Boc-Tic-Asp(OBzl)-N(Me)-Ph was treated with TFA as reported for TFA˙H-Tic-Gly-N(Me)-Ph: yield 0.18 g (97%); R_f (A) 0.75; HPLC K' 4.25; mp 141−143 °C; $[\alpha]^{20}_D$ +4.6; *m/z* 473 (M + H)$^+$.

Boc-Dmt-Tic-Asp(OBzl)-N(Me)-Ph. This intermediate was obtained by condensation of Boc-Dmt-OH with TFA˙H-Tic-Asp(OBzl)-N(Me)-Ph via WSC/HOBt as reported for Boc-Dmt-Tic-Gly-N(Me)-Ph: yield 0.13 g (88%); R_f (B) 0.81; HPLC K' 9.36; mp 160−162 °C; $[\alpha]^{20}_D$ −3.5; *m/z* 764 (M + H)$^+$. ^{1}H NMR (DMSO-d_6) δ 1.38−1.42 (d, 9H), 2.35 (s, 6H), 2.70−3.17 (m, 9H), 4.43−4.51 (m, 2H), 4.92−5.34 (m, 5H), 6.29 (s, 2H), 6.96−7.31 (m, 14H).

Boc-Dmt-Tic-Asp-N(Me)-Ph. Boc-Dmt-Tic-Asp(OBzl)-N(Me)-Ph was treated with Pd/C and H_2 as reported for Boc-Dmt-Tic-Asp-NH-Ph: yield 0.08 g (94%); R_f (B) 0.69; HPLC K' 7.23; mp 165−167 °C; $[\alpha]^{20}_D$ −7.3; *m/z* 674 (M + H)$^+$.

TFA˙H-Dmt-Tic-Asp-N(Me)-Ph (8). Boc-Dmt-Tic-Asp-N(Me)-Ph was treated with TFA as reported for TFA˙H-Dmt-Tic-Gly-N(Me)-Ph: yield 0.04 g (94%); R_f (A) 0.69; HPLC K' 4.26; mp 152−154 °C; $[\alpha]^{20}_D$ −8.2; *m/z* 574 (M + H)$^+$. ^{1}H NMR (DMSO-d_6) δ 2.35 (s, 6H), 2.70−3.17 (m, 9H), 3.95−4.51 (m, 3H), 4.86−4.92 (m, 2H), 6.29 (s, 2H), 6.96−7.31 (m, 9H); Anal. $C_{34}H_{37}F_3N_4O_8$: C; H; N.

Boc-D-Asp(OBzl)-N(Me)-Ph. This intermediate was obtained by condensation of Boc-D-Asp(OBzl)-OH with *N*-methylaniline via mixed anhydride as reported for Boc-Gly-N(Me)-Ph: yield 0.42 g (88%); R_f (B) 0.75; HPLC K' 7.11; mp 126−128 °C; $[\alpha]^{20}_D$ −10.3; *m/z* 413 (M + H)$^+$. ^{1}H NMR (DMSO-d_6) δ 1.40 (s, 9H), 2.72−2.97 (m, 5H), 5.17−5.34 (m, 3H), 7.10−7.31 (m, 10H).

TFA˙H-D-Asp(OBzl)-N(Me)-Ph. Boc-D-Asp(OBzl)-N(Me)-Ph was treated with TFA as reported for TFA˙H-Gly-N(Me)-Ph: yield 0.39 g (97%); R_f (A) 0.77; HPLC K' 5.11; mp 135−139 °C; $[\alpha]^{20}_D$ −14.7; *m/z* 313 (M + H)$^+$.

Boc-Tic-D-Asp(OBzl)-N(Me)-Ph. This intermediate was obtained by condensation of Boc-Tic-OH with TFA˙H-D-Asp(OBzl)-N(Me)-Ph via WSC/HOBt as reported for Boc-Tic-Gly-N(Me)-Ph: yield 0.19 g (87%); R_f (B) 0.83; HPLC K' 6.44; mp 145−147 °C; $[\alpha]^{20}_D$ +7.8; *m/z* 573 (M + H)$^+$. ^{1}H NMR (DMSO-d_6) δ 1.38−1.42 (d, 9H), 2.73−3.19 (m, 7H), 4.21−4.29 (m, 2H), 4.90−5.31 (m, 4H), 6.96−7.35 (m, 14H).

TFA·H-Tic-D-Asp(OBzl)-N(Me)-Ph. Boc-Tic-D-Asp(OBzl)-N(Me)-Ph was treated with TFA as reported for TFA·H-Tic-Gly-N(Me)-Ph: yield 0.16 g (96%); R_f (A) 0.73; HPLC K' 4.24; mp 142−144 °C; $[\alpha]^{20}_D$ +9.2; *m/z* 473 $(M + H)^+$.

Boc-Dmt-Tic-D-Asp(OBzl)-N(Me)-Ph. This intermediate was obtained by condensation of Boc-Dmt-OH with TFA·H-Tic-D-Asp(OBzl)-N(Me)-Ph via WSC/HOBt as reported for Boc-Dmt-Tic-Gly-N(Me)-Ph: yield 0.12 g (88%); R_f (B) 0.79; HPLC K' 9.08; mp 156−158 °C; $[\alpha]^{20}_D$ +7.8; *m/z* 764 $(M + H)^+$. ^{1}H NMR (DMSO-d_6) δ 1.38−1.42 (d, 9H), 2.35 (s, 6H), 2.70−3.20 (m, 9H), 4.40−4.53 (m, 2H), 4.93−5.32 (m, 5H), 6.29 (s, 2H), 6.96−7.35 (m, 14H).

Boc-Dmt-Tic-D-Asp-N(Me)-Ph. Boc-Dmt-Tic-D-Asp(OBzl)-N(Me)-Ph was treated with Pd/C and H_2 as reported for Boc-Dmt-Tic-Asp-NH-Ph: yield 0.08 g (93%); R_f (B) 0.68; HPLC K' 7.03; mp 155−157 °C; $[\alpha]^{20}_D$ +11.3; *m/z* 674 $(M + H)^+$.

TFA·H-Dmt-Tic-D-Asp-N(Me)-Ph (9). Boc-Dmt-Tic-D-Asp-N(Me)-Ph was treated with TFA as reported for TFA·H-Dmt-Tic-Gly-N(Me)-Ph: yield 0.04 g (96%); R_f (A) 0.68; HPLC K' 4.23; mp 145−147 °C; $[\alpha]^{20}_D$ +11.7; *m/z* 574 $(M + H)^+$. ^{1}H NMR (DMSO-d_6) δ 2.35 (s, 6H), 2.70−3.19 (m, 9H), 3.96−4.53 (m, 3H), 4.85−4.90 (m, 2H), 6.29 (s, 2H), 6.96−7.35 (m, 9H); Anal. $C_{34}H_{37}F_3N_4O_8$: C; H; N.

(*S*)-*tert*-butyl 3-((*S*)-2-((benzyloxy)carbonyl)-1-(1-methyl-1*H*-benzo[*d*]imidazol-2-yl)ethylcarbamoyl)-3,4-dihydroisoquinoline-2(1*H*)-carboxylate [Boc-Tic-NH-(*S*)CH(CH_2−COOBzl)-Bid(N^1-Me)]. To a solution of Boc-Tic-NH-(*S*)CH(CH_2−COOBzl)-Bid[8] (0.27 g; 0.40 mmol) in DMF (10 mL) at room temperature, K_2CO_3 (0.25 g; 1.8 mmol) and, after 1 h, iodomethane (0.03 mL; 0.42 mmol), were added. The reaction mixture was stirred for 24 h at room temperature. After DMF was evaporated, the residue was dissolved in EtOAc and washed with $NaHCO_3$ (5% in H_2O) and brine. The organic phase was dried (Na_2SO_4) and evaporated to dryness. The residue was precipitated from Et_2O/Pe (1:9, v/v): yield 0.20 g (90%); R_f (B) 0.81; HPLC K' 6.15; mp 151−153 °C; $[\alpha]^{20}_D$ +9.7; *m/z* 570 $(M + H)^+$. ^{1}H NMR (DMSO-d_6) δ 1.38−1.42 (d, 9H), 2.80−3.17 (m, 4H), 3.63 (s, 3H), 4.17−4.27 (m, 2H), 4.92−5.51 (m, 4H), 6.96−7.70 (m, 13H).

2TFA·H-Tic-NH-(*S*)CH(CH_2−COOBzl)-Bid(N^1-Me). Boc-Tic-NH-(*S*)CH(CH_2−COOBzl)-Bid(N^1-Me) was treated with TFA as reported for TFA·H-Tic-Gly-N(Me)-Ph: yield 0.21 g (93%); R_f (A) 0.71; HPLC K' 5.52; mp 144−146 °C; $[\alpha]^{20}_D$ +10.1; *m/z* 470 $(M + H)^+$.

Boc-Dmt-Tic-NH-(*S*)CH(CH_2−COOBzl)-Bid(N^1-Me). To a solution of Boc-Dmt-OH (0.08 g, 0.26 mmol) and 2TFA·H-Tic-NH-(*S*)CH(CH_2−COOBzl)-Bid(N^1-Me) (0.18 g, 0.26 mmol) in DMF (10 mL) at 0 °C, NMM (0.06 mL, 0.52 mmol), HOBt (0.04 g, 0.29 mmol), and WSC (0.05 g, 0.29 mmol) were added. The reaction mixture was stirred for 3 h at 0 °C and 24 h at room temperature. After DMF was evaporated, the residue was dissolved in EtOAc and washed with $NaHCO_3$ (5% in H_2O), and brine. The organic phase was dried (Na_2SO_4) and evaporated to dryness. The residue was precipitated from Et_2O/Pe (1:9, v/v): yield 0.17 g (86%); R_f (B) 0.68; HPLC K' 9.23; mp 165−167 °C; $[\alpha]^{20}_D$ +3.8; *m/z* 760 $(M + H)^+$. ^{1}H NMR (DMSO-d_6) δ 1.38−1.42 (d, 9H), 2.35 (s, 6H), 2.92−3.20 (m, 6H), 3.63 (s, 3H), 4.41−4.51 (m, 2H), 4.92−5.51 (m, 5H), 6.29 (s, 2H), 6.96−7.70 (m, 13H).

Boc-Dmt-Tic-NH-(*S*)CH(CH_2−COOH)-Bid(N^1-Me). Boc-Dmt-Tic-NH-(*S*)CH(CH_2−COOBzl)-Bid(N^1-Me) was treated with Pd/C and H_2 as reported for Boc-Dmt-Tic-Asp-NH-Ph: yield 0.11 g (94%); R_f (B) 0.55; HPLC K' 7.26; mp 166−168 °C; $[\alpha]^{20}_D$ +4.6; *m/z* 671 $(M + H)^+$.

2TFA·H-Dmt-Tic-NH-(*S*)CH(CH_2−COOH)-Bid(N^1-Me) (10). Boc-Dmt-Tic-NH-(*S*)CH(CH_2−COOH)-Bid(N^1-Me) was treated with TFA as reported for TFA·H-Dmt-Tic-Gly-N(Me)-Ph: yield 0.05 g (96%); R_f (A) 0.69 HPLC K' 4.56; mp 152−154 °C; $[\alpha]^{20}_D$ +7.3; *m/z* 571 $(M + H)^+$. ^{1}H NMR (DMSO-d_6) δ 2.35 (s, 6H), 2.92−3.20 (m, 6H), 3.63−3.95 (m, 4H), 4.41−4.51 (m, 2H), 4.92−5.20 (m, 2H), 6.29 (s, 2H), 6.96−7.70 (m, 8H). Anal. $C_{36}H_{37}F_6N_5O_9$: C; H; N.

***tert*-Butyl-(*R*)-2-((benzyloxy)carbonyl)-1-(1*H*-benzo[d]imidazol-2-yl)ethylcarbamate [Boc-NH-(*R*)CH(CH_2−COOBzl)-Bid].** A solution of Boc-D-Asp(OBzl)-OH (1 g, 3.1 mmol) and NMM (0.34 mL, 3.1 mmol) in DMF (10 mL) was treated at −20 °C with IBCF (0.4 mL, 3.1 mmol). After 10 min at −20 °C, *o*-phenylendiamine (0.33 g, 3.1 mmol) was added. The reaction mixture was allowed to stir while slowly warming to room temperature (1 h) and was then stirred for 3 h. The solvent was evaporated, and the residue was partitioned between EtOAc and H_2O. The AcOEt layer was washed with $NaHCO_3$ (5% in H_2O) and brine and dried over Na_2SO_4. The solution was filtered, the solvent was evaporated, and the residual solid was dissolved in glacial acetic acid (10 mL). The solution was heated at 60 °C for 1 h. After the solvent was evaporated, the residue was precipitated from Et_2O/Pe (1:9, v/v): yield 1 g (82%); R_f (B) 0.52; HPLC K' 6.50; mp 136−138 °C; $[\alpha]^{20}_D$ −15.8; *m/z* 396 $(M + H)^+$. ^{1}H NMR (DMSO-d_6) δ 1.40 (s, 9H), 2.67−2.92 (m, 2H), 5.34−5.51 (m, 3H), 7.19−7.70 (m, 9H).

2TFA·H_2N-(*R*)CH(CH_2−COOBzl)-Bid. Boc-NH-(*R*)CH(CH_2−COOBzl)-Bid was treated with TFA as reported for TFA·H-Gly-N(Me)-Ph: yield 0.51 g (96%); R_f (A) 0.78; HPLC K' 4.20; mp 142−144 °C; $[\alpha]^{20}_D$ −17.3; *m/z* 296 $(M + H)^+$.

Boc-Tic-NH-(*R*)CH(CH_2−COOBzl)-Bid. To a solution of Boc-Tic-OH (0.28 g, 1 mmol) and 2TFA·H_2N-(*R*)CH(CH_2−COOBzl)-Bid (0.52 g, 1 mmol) in DMF (10 mL) at 0 °C, NMM (0.2 mL, 2 mmol), HOBt (0.17 g, 1.1 mmol), and WSC (0.21 g, 1.1 mmol) were added. The reaction mixture was stirred for 3 h at 0 °C and 24 h at room temperature. After DMF was evaporated, the residue was dissolved in EtOAc and washed with $NaHCO_3$ (5% in H_2O), and brine. The organic phase was dried (Na_2SO_4) and evaporated to dryness. The residue was precipitated from Et_2O/Pe (1:9, v/v): yield 0.48 g (87%); R_f (B) 0.72; HPLC K' 5.92; mp 155−157 °C; $[\alpha]^{20}_D$ −1.6; *m/z* 556 $(M + H)^+$. ^{1}H NMR (DMSO-d_6) δ 1.38−1.42 (d, 9H), 2.80−3.17 (m, 4H), 4.17−4.27 (m, 2H), 4.92−5.51 (m, 4H), 6.96−7.70 (m, 13H).

(*S*)-*tert*-Butyl 3-((*R*)-2-((Benzyloxy)carbonyl)-1-(1-methyl-1*H*-benzo[d]imidazol-2-yl)ethylcarbamoyl)-3,4-dihydroisoquinoline-2(1*H*)-carboxylate [Boc-Tic-NH-(*R*)CH(CH_2−COOBzl)-Bid(N^1-Me)]. This intermediate was obtained by alkylation of Boc-Tic-NH-(*R*)CH(CH_2−COOBzl)-Bid with K_2CO_3 and iodomethane as reported for Boc-Tic-NH-(*S*)CH(CH_2−COOBzl)-Bid(N^1-Me): yield 0.22 g (88%); R_f (B) 0.77; HPLC K' 6.07; mp 154−156 °C; $[\alpha]^{20}_D$ +4.3; *m/z* 570 $(M + H)^+$. ^{1}H NMR (DMSO-d_6) δ 1.38−1.42 (d, 9H), 2.82−3.20 (m, 4H), 3.63 (s, 3H), 4.15−4.29 (m, 2H), 4.90−5.49 (m, 4H), 6.96−7.70 (m, 13H).

2TFA·H-Tic-NH-(*R*)CH(CH_2−COOBzl)-Bid(N^1-Me). Boc-Tic-NH-(*R*)CH(CH_2−COOBzl)-Bid(N^1-Me) was treated with TFA as reported for TFA·H-Tic-Gly-N(Me)-Ph: yield 0.21 g (92%); R_f (A) 0.68; HPLC K' 5.39; mp 148−150 °C; $[\alpha]^{20}_D$ +5.4; *m/z* 470 $(M + H)^+$.

Boc-Dmt-Tic-NH-(*R*)CH(CH_2−COOBzl)-Bid(N^1-Me). This intermediate was obtained by condensation of Boc-Dmt-OH with 2TFA·H-Tic-NH-(*R*)CH(CH_2−COOBzl)-Bid(N^1-Me) via WSC/HOBt as reported for Boc-Dmt-Tic-NH-(*S*)CH(CH_2−COOBzl)-Bid(N^1-Me): yield 0.17 g (86%); R_f (B) 0.64; HPLC K' 8.28; mp 169−171 °C; $[\alpha]^{20}_D$ −2.7; *m/z* 760 $(M + H)^+$. ^{1}H NMR (DMSO-d_6) δ 1.38−1.42 (d, 9H), 2.35 (s, 6H), 2.94−3.22 (m, 6H), 3.63 (s, 3H), 4.40−4.52 (m, 2H), 4.92−5.48 (m, 5H), 6.29 (s, 2H), 6.96−7.70 (m, 13H).

Boc-Dmt-Tic-NH-(*R*)CH(CH_2−COOH)-Bid(N^1-Me). Boc-Dmt-Tic-NH-(*R*)CH(CH_2−COOBzl)-Bid(N^1-Me) was treated with Pd/C and H_2 as reported for Boc-Dmt-Tic-Asp-NH-Ph: yield 0.10 g (92%); R_f (B) 0.50; HPLC K' 6.98; mp 172−174 °C; $[\alpha]^{20}_D$ −1.9; *m/z* 671 $(M + H)^+$.

2TFA·H-Dmt-Tic-NH-(*R*)CH(CH_2−COOH)-Bid(N^1-Me) (11). Boc-Dmt-Tic-NH-(*R*)CH(CH_2−COOH)-Bid(N^1-Me) was treated with TFA as reported for TFA·H-Dmt-Tic-Gly-N(Me)-Ph: yield 0.07 g (96%); R_f (A) 0.58 HPLC K' 4.02; mp 161−163 °C; $[\alpha]^{20}_D$ +2.2; *m/z* 571 $(M + H)^+$. ^{1}H NMR (DMSO-d_6) δ 2.35 (s, 6H), 2.90−3.22 (m, 6H), 3.61−3.93 (m, 4H), 4.42−4.53 (m, 2H), 4.90−5.18 (m, 2H), 6.29 (s, 2H), 6.96−7.70 (m, 8H). Anal. $C_{36}H_{37}F_6N_5O_9$: C; H; N.

2TFA·H-Tic-NH-(*R*)CH(CH_2−COOBzl)-Bid. Boc-Tic-NH-(*R*)CH(CH_2−COOBzl)-Bid was treated with TFA as reported for TFA·H-Tic-Gly-N(Me)-Ph: yield 0.18 g (93%); R_f (A) 0.66; HPLC K' 5.35; mp 149−151 °C; $[\alpha]^{20}_D$ +6.3; *m/z* 456 (M + H)$^+$.

Boc-Dmt-Tic-NH-(*R*)CH(CH_2−COOBzl)-Bid. This intermediate was obtained by condensation of Boc-Dmt-OH with 2TFA·H-Tic-H_2N-(*R*)CH(CH_2−COOBzl)-Bid via WSC/HOBt as reported for Boc-Dmt-Tic-NH-(*S*)CH(CH_2−COOBzl)-Bid(N^1-Me): yield 0.15 g (87%); R_f (B) 0.61; HPLC K' 8.01; mp 174−176 °C; $[\alpha]^{20}_D$ −3.5; *m/z* 747 (M + H)$^+$. ^{1}H NMR (DMSO-d_6) δ 1.36−1.40 (d, 9H), 2.35 (s, 6H), 2.63−3.17 (m, 6H), 4.42−4.53 (m, 2H), 4.92−5.51 (m, 5H), 6.29 (s, 2H), 6.96−7.70 (m, 13H).

Boc-Dmt-Tic-NH-(*R*)CH(CH_2−COOH)-Bid. Boc-Dmt-Tic-NH-(*R*)CH(CH_2−COOBzl)-Bid was treated with Pd/C and H_2 as reported for Boc-Dmt-Tic-Asp-NH-Ph: yield 0.09 g (93%); R_f (B) 0.46; HPLC K' 6.84; mp 176−178 °C; $[\alpha]^{20}_D$ −2.8; *m/z* 657 (M + H)$^+$.

2TFA·H-Dmt-Tic-NH-(*R*)CH(CH_2−COOH)-Bid (12). Boc-Dmt-Tic-NH-(*R*)CH(CH_2−COOH)-Bid was treated with TFA as reported for TFA·H-Dmt-Tic-Gly-N(Me)-Ph: yield 0.06 g (96%); R_f (A) 0.52; HPLC K' 3.92; mp 166−168 °C; $[\alpha]^{20}_D$ +3.5; *m/z* 557 (M + H)$^+$. ^{1}H NMR (DMSO-d_6) δ 2.35 (s, 6H), 2.90−3.17 (m, 6H), 3.95−4.52 (m, 3H), 4.90−5.21 (m, 2H), 6.29 (s, 2H), 6.96−7.70 (m, 8H). Anal. $C_{35}H_{35}F_6N_5O_9$: C; H; N.

Boc-Asp(OBzl)-NH-Bzl. This intermediate was obtained by condensation of Boc-Asp(OBzl)-OH with benzylamine via mixed anhydride as reported for Boc-Gly-N(Me)-Ph: yield 0.45 g (90%); R_f (B) 0.77; HPLC K' 7.18; mp 126−128 °C; $[\alpha]^{20}_D$ −11.2; *m/z* 413 (M + H)$^+$. ^{1}H NMR (DMSO-d_6) δ 1.40 (s, 9H), 2.63−2.88 (d, 2H), 4.46 (s, 2H), 5.17−5.34 (m, 3H), 7.06−7.19 (m, 10H).

TFA·H-Asp(OBzl)-NH-Bzl. Boc-Asp(OBzl)-NH-Bzl was treated with TFA as reported for TFA·H-Gly-N(Me)-Ph: yield 0.42 g (96%); R_f (A) 0.78; HPLC K' 5.18; mp 135−137 °C; $[\alpha]^{20}_D$ +14.3; *m/z* 313 (M + H)$^+$.

Boc-Tic-Asp(OBzl)-NH-Bzl. This intermediate was obtained by condensation of Boc-Tic-OH with TFA·H-Asp(OBzl)-NH-Bzl via WSC/HOBt as reported for Boc-Tic-Gly-N(Me)-Ph: yield 0.22 g (87%); R_f (B) 0.86; HPLC K' 6.51; mp 144−146 °C; $[\alpha]^{20}_D$ +4.7; *m/z* 573 (M + H)$^+$. ^{1}H NMR (DMSO-d_6) δ 1.38−1.42 (d, 9H), 2.63−3.17 (m, 4H), 4.17−4.46 (m, 4H), 4.92−5.34 (m, 4H), 6.96−7.19 (m, 14H).

TFA·H-Tic-Asp(OBzl)-NH-Bzl. Boc-Tic-Asp(OBzl)-NH-Bzl was treated with TFA as reported for TFA·H-Tic-Gly-N(Me)-Ph: yield 0.18 g (97%); R_f (A) 0.77; HPLC K' 4.29; mp 140−142 °C; $[\alpha]^{20}_D$ +4.7; *m/z* 473 (M + H)$^+$.

Boc-Dmt-Tic-Asp(OBzl)-NH-Bzl. This intermediate was obtained by condensation of Boc-Dmt-OH with TFA·H-Tic-Asp(OBzl)-NH-Bzl via WSC/HOBt as reported for Boc-Dmt-Tic-Gly-N(Me)-Ph: yield 0.13 g (88%); R_f (B) 0.82; HPLC K' 9.38; mp 160−162 °C; $[\alpha]^{20}_D$ −1.9; *m/z* 764 (M + H)$^+$. ^{1}H NMR (DMSO-d_6) δ 1.38−1.42 (d, 9H), 2.35 (s, 6H), 2.63−3.17 (m, 6H), 4.41−4.51 (m, 4H), 4.92−5.34 (m, 5H), 6.29 (s, 2H), 6.96−7.19 (m, 14H).

Boc-Dmt-Tic-Asp-NH-Bzl. Boc-Dmt-Tic-Asp(OBzl)-NH-Bzl was treated with Pd/C and H_2 as reported for Boc-Dmt-Tic-Asp-NH-Ph: yield 0.09 g (96%); R_f (B) 0.71; HPLC K' 7.24; mp 164−166 °C; $[\alpha]^{20}_D$ −5.2; *m/z* 674 (M + H)$^+$.

TFA·H-Dmt-Tic-Asp-NH-Bzl (13). Boc-Dmt-Tic-Asp-NH-Bzl was treated with TFA as reported for TFA·H-Dmt-Tic-Gly-N(Me)-Ph: yield 0.05 g (96%); R_f (A) 0.69; HPLC K' 4.29; mp 154−156 °C; $[\alpha]^{20}_D$ −6.7; *m/z* 574 (M + H)$^+$. ^{1}H NMR (DMSO-d_6) δ 2.35 (s, 6H), 2.63−3.17 (m, 6H), 3.95−4.51 (m, 5H), 4.86−4.92 (m, 2H), 6.29 (s, 2H), 6.96−7.14 (m, 9H). Anal. $C_{34}H_{37}F_3N_4O_8$: C; H; N.

Pharmacology. Competitive Binding Assays. Opioid receptor affinities were determined under equilibrium conditions [2.5 h room temperature (23 °C)] in competition assays using brain P_2 synaptosomal membranes prepared from Sprague−Dawley rats.[40,41] Synaptosomes were preincubated to remove endogenous opioid peptides and stored at −80 °C in buffered 20% glycerol.[40,42] Each analogue was analyzed in duplicate assays using 5−8 dosages and 3−5 independent repetitions with different synaptosomal preparations (*n* values are listed in Table 1 in parenthesis and results are mean ± SE). Unlabeled peptide (2 μM) was used to determine nonspecific binding in the presence of 1.9 nM [^{3}H]deltorphin II (45.0 Ci/mmol, Perkin-Elmer, Boston, MA; K_D = 1.4 nM) for δ-opioid receptors and 3.5 nM [^{3}H]DAMGO (50.0 Ci/mmol), Amersham Bioscience, Buckinghamshire, UK; K_D = 1.5 nM) for μ-opioid receptors. Glass fiber filters (Whatman GFC) were soaked in 0.1% polyethylenimine in order to enhance the signal-to-noise ratio of the bound radiolabeled-synaptosome complex, and the filters were washed thrice in ice-cold buffered BSA,[40] and the affinity constants (K_i) were calculated according to Cheng and Prusoff..[43]

Biological Activity in Isolate Tissue Preparations. The myenteric plexus longitudinal muscle preparations (2−3 cm segments) from the small intestine of male Hartley strain of guinea pigs (GPI) measured μ-opioid receptor agonism, and a single mouse vas deferens (MVD) was used to determine δ-opioid receptor agonism as described previously.[44] The isolated tissues were suspended in organ baths containing balanced salt solutions in a physiological buffer, pH 7.5. Agonists were tested for the inhibition of electrically evoked contraction and expressed as IC_{50} (nM) obtained from the dose−response curves. The IC_{50} values represent the mean ± SE of five or six separate assays, and the δ-antagonist potencies in the MVD assay were determined against the δ agonist deltorphin-II, while μ antagonism (GPI assay) used the μ agonist endomorphin-2. Antagonism is expressed as pA_2 determined using the Schild Plot.[45]

Animals for in Vitro and in Vivo Studies. Laboratory animals were used under protocols approved and governed by the Animal Care and Use Committees of Tohoku Pharmaceutical University, University of Ferrara and the National Institute of Environmental Health Sciences.

In Vivo Assessment of Opioid Bioactivity. Male Swiss−Webster mice (20−25 g, Taconic, Germantown, NY) were housed on a 12 h light/dark cycle with free access to food and water and experimental protocol approved by the NIEHS Animal Care and Use Committee.

Intracerebroventricular injection (icv) used a Hamilton microsyringe with disposable 26 gauge needle that was inserted 2.3−3 mm into the ventricular sinus at the bregma as described;[46] the total volume administered was 5 μL (peptide in saline). Upon completion of the study, mice were sacrificed according ACUC protocols and those animals having needle tract 2 mm lateral from the midline were counted as being valid.

Hot-plate test consisted of animals placed on an electrically heated plate (55 ± 0.1 °C, IITC MODEL 39D Hot Plate analgesia meter, IITC Inc., Woodland Hills, CA) 10 min after icv administration. Hot-plate latency (HPL) is the interval between placement of mice onto the hot plate and observing movement consisting of either jumping, licking, or shaking their hind paws; a baseline latency of 5−10 s (preresponse time) and maximal cut off time of 30 s. Measurements were repeated every 10 min and testing was terminated when the HPL was close to the preresponse time.

Spinal effects used of a tail-flick instrument (Columbus Instruments, Columbus, OH). Radiant heat was applied on the dorsal surface of the tail and the latency before removal of the tail from the onset of the radiant heat is defined as the tail-flick latency. The baseline was to 2−3 s (preresponse time) and a cut off time was set at 8 s to avoid external heat-related damage. The time sequence was the same as described for the HPL test[47,48]

Statistical Analysis. Statistical significance of the data was estimated by one-way analysis of variance (ANOVA) followed by Dunnett's test using the computer software program JMP (SAS Institute Inc., Cary, NC) and considered significant at $p < 0.05$. Minimum effective dose (MED) is the minimum dose of compound showing statistically significant antinociceptive effect expressed as the area under the time-response curve (AUC) value compared to a saline-treated group. The AUC was obtained by plotting the response time (sec) on the ordinate and time (min) on the abscissa after administration of the compounds. The percent maximum possible effect (% MPE) was calculated as follows: ([postdrug response latency − predrug response latency]/[cutoff time (30 s) − predrug response latency]) × 100.

Conclusions

Starting from the assumption that even minor chemical modifications can change the pharmacological profile of opioids, such as peptides and pseudopeptides containing the Dmt-Tic pharmacophore or nonpeptide derivatives related to morphine,[33] we selected some reference compounds, especially our δ agonists **1** and **4**, and evaluated the influence of aspartic acid and its chirality and the importance of the −NH-Ph and N^1H-Bid hydrogens in the inductions of δ agonism. The results obtained confirm some expectations: (i) Asp increases the δ selectivity by lowering μ affinity; (ii) Methylation of −NH-Ph and N^1H-Bid nitrogens transforms potent δ agonists in potent δ antagonists. However other conclusions are quite unexpected: (iii) The substitution of Gly with L-Asp or D-Asp in the δ agonist **1** gave potent and selective δ antagonists; in contrast, the same substitution made in the δ agonist **3**, produced the more selective δ agonists **4** and **12**; (iv) Asp chirality seems to be important only in terms of functional bioactivity because analogues **4**, **6**, **8**, and **10**, containing L-Asp, are more active than the corresponding diastereoisomers **12**, **7**, **9**, and **11**; but the same is not true for receptor affinity. Finally, and totally unexpected and in our opinion of potential interest, the potent and selective δ antagonist **10** yielded an analgesic effect similar to **4** that was reversed by naltrindole only when it was tested by the tail flick method, and not in the hot plate test. Furthermore, **4** and **10** gave opposite behavioral effects in mice; **4** caused grooming and agitation (constant, fast moving in the cage, burrowing in the nesting material), while with **10**, mice appeared sedated, quiet, easily handled, moving slowly if at all; convulsions were reported only in animals treated with the δ antagonist at high doses icv. Considering the novelty of such a compound, more detailed pharmacological studies are in progress both in vivo (as an analgesic for neuropatic and inflammatory pain, antidepressant. and anxiolytic) and in vitro, considering also its potential interaction with different receptors,[34] or with heterodimers involving δ receptors.[35] The last consideration is reserved to preliminary enzymatic degradation studies that failed to demonstrate the N-demethylation of **10** to the corresponding **4**; more detailed studies (involving the use of different methods[36] and/or other tissue preparations, such as kidney and liver[30]) are just planned, also in collaboration with other laboratories. If we will be able to demonstrate such enzymatic N-demethylation, **10** could be considered a new lead compound of potential pharmacological utility for in vivo studies connected to δ opioid receptors trafficking at the membrane level.[37−39]

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Supporting Information Available: Chemistry general methods, enzymatic degradation general methods, elemental analysis, MS, and HPLC data. This material is available free of charge via the Internet at http://pubs.acs.org.

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