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Transformation of μ-opioid receptor agonists into biologically potent μ-opioid receptor antagonists

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Abstract—*N*-Allylation (–CH_2–CH=CH_2) of [Dmt[1]]endomorphins yielded the following: (i) [*N*-allyl-Dmt[1]]endomorphin-2 (Dmt = 2′,6′-dimethyl-L-tyrosine) (**12**) and [*N*-allyl-Dmt[1]]endomorphin-1 (**15**) (K_iμ = 0.45 and 0.26 nM, respectively) became μ-antagonists (pA_2 = 8.59 and 8.18, respectively) with weak δ-antagonism (pA_2 = 6.32 and 7.32, respectively); (ii) intracerebroventricularly administered **12** inhibited morphine-induced CNS-mediated antinociception in mice [AD_{50} (0.148 ng/mouse) was 16-fold more potent than naloxone], but not spinal antinociception, and (iii) **15** reversed the alcohol-elevated frequency in spontaneous inhibitory post-synaptic currents (IPSC) in hippocampal CA1 pyramidal cells in rat brain slices (P = 0.0055). Similarly, *N*-allylation of the potent μ-opioidmimetic agonists, 1,6-bis-[H-Dmt-NH]-hexane and 3,6-bis-[Dmt-NH-propyl]-2(1*H*)-pyrazinone, converted them into μ-antagonists (pA_2 = 7.23 and 7.17 for the *N*-allyl-derivatives **17** and **19**, respectively), and exhibited weak δ-antagonism. Thus, *N*-allylation of Dmt containing opioid peptides or opioidmimetics continues to provide a facile means to convert selective μ-opioid agonists into potent μ-opioid antagonists.

1. Introduction

Whereas the existence of potent opioid peptide agonists is well known for the major opioid-receptor classes (μ, δ, κ), the development of selective opioid antagonists continues to be a key objective in pharmacology. In particular, μ-opioid antagonists are important pharmacological tools not only to delineate critical biochemical, pharmacological, and physiological roles played by these receptors, but also to serve as clinically and therapeutically relevant agents.[1] Interestingly, the addictive behavior associated with morphine in animal studies when treated by some alkaloid-derived antagonists can result in severe withdrawal symptoms due to their ability to act as inverse agonists.[1] Furthermore, the predilection to alcoholism, in part, depends on the presence of intact μ-opioid receptors evidenced by the existence of substantial data using knock-out mice and selective opiate antagonists which verify that these receptors are intimately associated with a neural reward pathway(s) in the CNS (central nervous system) that is affected by alcohol consumption[2] as well as morphine and its cognates.

Opioid antagonists do not occur naturally and modification of the alkaloid architecture of morphine led to the

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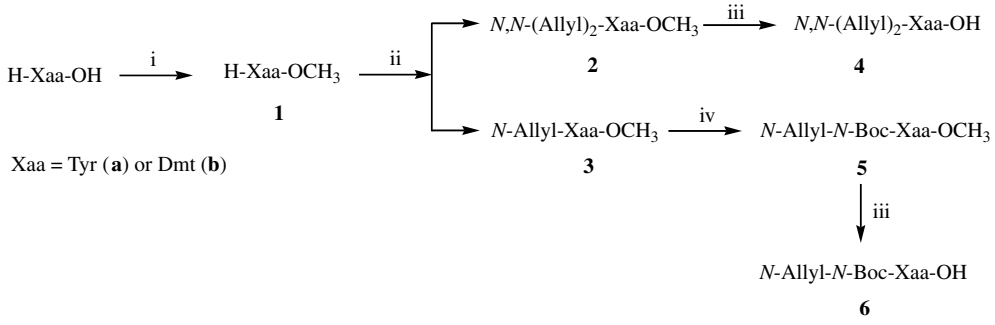
elucidation of structural requirements for their development; however, the conversion was neither straightforward nor predictable. In particular, an allyl ($–CH_2–CH=CH_2$) substitution[3] yielded either non-specific antagonist for μ-, δ-, and κ-opioid receptors (naloxone), or a mixed agonist/antagonist (nalorphine); a comparable dichotomy in pharmacological activity with N-allylated opioid peptides occurred: for example N,N-diallylation of the multisubstituted [Leu^5]enkephalin framework yielded two distinct δ-opioid antagonists, namely *N*,*N*-(allyl)$_2$-Tyr-Gly-Gly-ψ-(CH_2S)-Phe-Leu-OH (ICI 154129)[4] and *N*,*N*-(allyl)$_2$-Tyr-Aib-Aib-Phe-Leu-OH (ICI 174864),[5] while N-allylation produced mixed agonist/antagonist opioids.[6,7] Similarly, [*N*,*N*-(allyl)$_2$-Tyr^1,D-Pro^{10}]dynorphin A (1-11) imparted antagonism toward both κ- and μ-opioid receptors with weak κ-opioid receptor selectivity;[8] however, [*N*-allyl-Tyr^1, D-Pro^{10}]dynorphin A (1-11) retained the κ-agonism of the parent compound.[3]

Inasmuch as that many specific δ-antagonists exist (e.g., ICI 174864,[5] naltrindole,[9] the TIP(P) family,[10] and Dmt-Tic pharmacophore derivatives[11]), a majority of the μ-antagonists used pharmacologically lack a high degree of biological specificity or potency, such as naloxone, naltrexone, nalmefene or CTAP (D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2).[12] On the other hand, while the alkaloids naloxone and naltrexone are non-selective opioid receptor antagonists, β-funaltrexamine behaves as an irreversible μ-antagonist. Nonetheless, a host of other opioid peptides sporadically exhibit weak μ-antagonism, but fail to meet the criteria for potent bioactivity.[13–15]

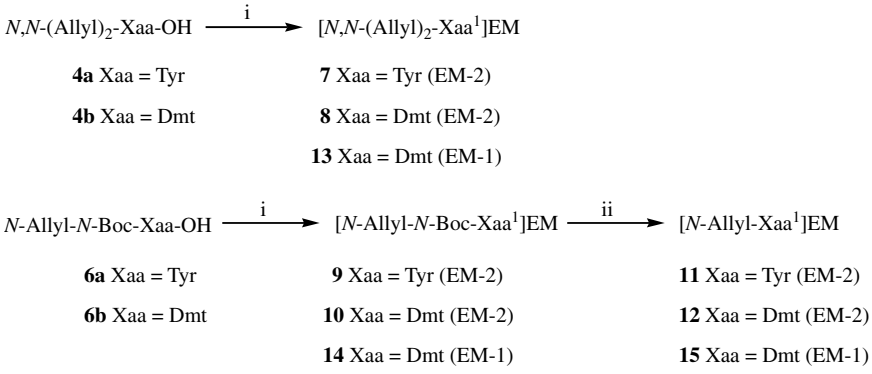
The μ-opioid agonists endomorphin-1 (EM-1: H-Tyr-Pro-Trp-Phe-NH_2) and endomorphin-2 (EM-2: H-Tyr-Pro-Phe-Phe-NH_2) exhibit the highest selectivity for the μ-opioid receptor,[16] and thus represent a potential opioid framework for modification into μ-antagonists. In this communication, μ-agonists containing 2′,6′-dimethyl-L-tyrosine (Dmt) in lieu of Tyr, such as [Dmt^1]-endomorphin,[17] the bis-Dmt opioidmimetic ligands linked by an alkyldiamine[18] or 3,6-bis-(aminoalkyl)-2(1*H*)-pyrazinone,[19] were transformed by N-allylation into potent μ-opioid antagonists.

2. Chemistry

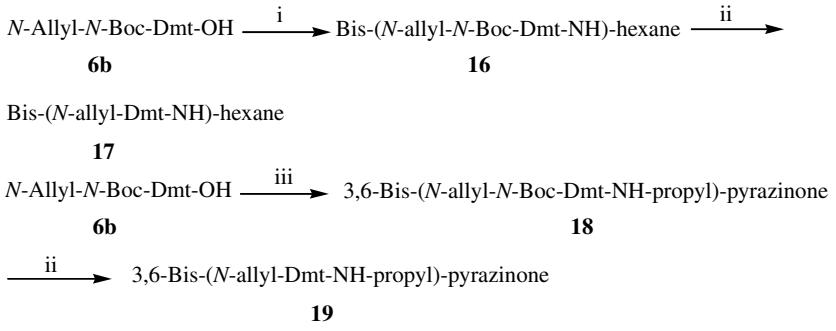
The key intermediates (Scheme 1) of *N*-allyl and *N*,*N*-(allyl)$_2$-Xaa-OCH_3 (**3** and **2**, Xaa = Tyr or Dmt) were prepared in one pot from allylbromide (2.5 equiv) and H-Xaa-OMe (**1**) in the presence of diisopropylethylamine (DIPEA) (2.7 equiv) at 50 °C for 5 h. *N*,*N*-(Allyl)$_2$-Xaa-OCH_3 (**2**) was hydrolyzed to give *N*,*N*-(allyl)$_2$-Xaa-OH (**4**). N^{α}-H of *N*-allyl-Xaa-OCH_3 (**3**) was protected with *tert*-butyloxycarbonyl (Boc) group in dioxane to produce *N*-allyl-*N*-Boc-Xaa-OCH_3 (**5**), and then hydrolyzed with NaOH to *N*-allyl-*N*-Boc-Xaa-OH (**6**). As shown in Scheme 2, [*N*,*N*-(allyl)$_2$-



Scheme 1. Synthesis of N-terminal mono- and diallyl-substituted amino acid derivatives. Reagents and conditions: (i) $SOCl_2$/MeOH; (ii) allyl-Br, DIPEA, MeOH, 50 °C, 5 h; (iii) 1 N NaOH; (iv) $(Boc)_2O$, Et_3N, dioxane.



Scheme 2. Synthesis of N-terminal *N*-allyl- and *N*,*N*-(allyl)$_2$-substituted endomorphin-1 and endomorphin-2 analogues. Reagents: (i) H-Pro-Phe/Trp-Phe-NH_2, DIPEA, PyBop, DMF; (ii) TFA/anisole.



Scheme 3. Synthesis of N-terminal bis-(allyl-Dmt)-opioidmimetic ligands. Reagents and conditions: (i) 1,6-diaminohexane, DIPEA, PyBop, DMF; (ii) TFA/anisole; (iii) 3,6-bis-(3′-aminopropyl)-pyrazinone, DIPEA, PyBop, DMF.

Xaa^1]- (**7** and **8**) and (*N*-allyl-Xaa^1)EM-2 analogues (**11** and **12**) were prepared in solution using Boc-protection; [*N*,*N*-(allyl)$_2$-Dmt^1]- (**13**) and [*N*-allyl-Dmt^1]EM-1 (**15**) were prepared identically. After deprotection of Boc-Pro-Phe-Phe-NH$_2$[17a] with HCl/dioxane, the resulting amino component was condensed with **4** or **6** using benzotriazol-1-yloxytris-(pyrrolidino)phosphate (PyBop) as the coupling reagent to give **7** and **8**, and Boc-protected **9** and **10**, respectively. Removal of the Boc-group (**9** and **10**) with trifluoroacetic acid (TFA) gave **11** and **12**. Compound **15** was synthesized using the same method for **11**, but using [*N*-allyl-*N*-Boc-Dmt^1]EM-1 (**14**). As shown in Scheme 3, *N*-allyl-*N*-Boc-Dmt-OH (**6b**) was coupled with 1,6-diaminohexane or 3,6-bis-(aminopropyl)-2(1*H*)-pyrazinone to produce bis(*N*-allyl-*N*-Boc-Dmt)-compounds, followed by removal of Boc group with TFA to give **17** and **19**, respectively. All final compounds were purified by semi-preparative reversed-phase HPLC to >98% purity, and verified using mass spectrometry, ^{1}H and ^{13}C NMR, optical rotation, thin-layer chromatography, analytical HPLC, and elemental analysis.

3. Results and discussion

3.1. Opioid receptor affinity

Compared to the standard opioids (**1′**–**6′**, Table 1), the allylated derivatives decreased affinity toward μ-opioid receptors in the following order: parent peptides > *N*-allyl > *N*,*N*-diallyl derivatives; of these analogues, however, **12** and **15** exhibited the highest μ-opioid affinities (K_iμ = 0.45 and 0.26 nM, respectively) (Table 1). The effect of N-allylation of EM-2 on μ-opioid affinity was more sensitive than that of [H-Dmt^1]EM-2; that is, *N*-allyl-EM-2 (**11**) fell 98-fold compared to only 3-fold with [*N*-allyl-Dmt^1]EM-2 (**12**). Although the δ-opioid receptor affinities are lower than those for the μ-opioid receptor, the order was similar, except **7** and **11**. In all cases, the δ-opioid affinities were substantially less than those observed for the μ-opioid receptor (Table 1).

The primary, secondary or tertiary amino function at the N-terminus is apparently necessary for the expression of high affinity and bioactivity presumably due to the formation of an ionic bond to a group in the receptor,[20] since deletion reduces ligand efficacy.[13] On the other hand, the impairment of binding by allyl groups[3–5,7–9] might be attributed to steric hindrance preventing the formation of an ionic bond required for agonism, yet permitting the ligand to function as an antagonist through interaction with a different binding region within the receptor.[11a] Furthermore, that Dmt overcame the detrimental effect of N-allylation (Table 1) implies that hydrophobic factors also play a dominant role in anchoring the opioid in the receptor by aligning or stabilizing the hydroxyl group to form a H-bond, enhancing π–π interactions or aromatic ring stacking.[11a]

3.2. Functional bioactivity

N-Allylation completely eliminated the μ-agonism of [H-Dmt^1]EM-1 (**4′**) and [H-Dmt^1]EM-2 (**2′**) to yield **15** and **12** with potent μ-antagonism (pA_2 = 8.18–8.59) (Table 1). Furthermore, both compounds are 6.6- and 17-fold, respectively, more effective than the published data on CTAP;[12] even the *N*,*N*-diallyl ligand **13** has nearly twice the potency of this molecule.[12] As shown in Table 1, [H-Dmt^1]EM-2 (**2′**) is an agonist for both μ- and δ-opioid receptors, while [H-Dmt^1]EM-1 (**4′**) exhibited potent μ-agonism/δ-antagonism (GPI: IC_{50} = 0.27 nM; MVD: pA_2 = 8.60). Aromatic moiety of Trp at position 3 in **4′** is bulkier and more hydrophobic than that of Phe^3 in **2′**. It can be deduced that aromatic properties of the moiety at position 3 of [H-Dmt^1]EM-1 or -2 are an important factor that determines whether [H-Dmt^1]EM exhibits δ-agonism or δ-antagonism. This phenomenon is compatible with the following results: ligand **12** exhibited high μ-opioid antagonism with an A_2 ratio of 186, while ligand **15** had only an A_2 ratio of 7.2. This difference in the A_2 ratio (26-fold) between **12** and **15** is mainly due to the weaker δ-antagonism exhibited by **12** (pA_2 = 6.32) compared with that of **15** (pA_2 = 7.32) which can be explained by the hypothesis given above.

In general, N-modification profoundly affects opioid peptide activities: for example, elimination of the N-terminal amino group of [Met^5]enkephalin abolished δ-opioid agonism;[21] N-formylation of H-Dmt-c[-D-Orn-2-Nal-D-Pro-Gly] resulted in a moderately active μ-opioid antagonist;[13] *N*-allyl-enkephalin[7] and *N*-allyl-

Table 1. Receptor affinities and functional bioactivities of opioid ligands

No.	Compound	$K_i\mu$ (nM)	$K_i\delta$ (nM)	δ/μ	GPI (μ)[d] IC_{50}, nM	pA_2[e]	MVD (δ)[d] IC_{50}, nM	pA_2[e]	$[A_2]$ ratio[f] MVD/GPI
1′	Endomorphin-2 (EM-2)	1.33 ± 0.15 (3)	6080 ± 1,215 (3)	4571	6.88 ± 0.94	—	344 ± 93	—	—
7	[*N*,*N*-(Allyl)$_2$]EM-2	1045 ± 175 (6)	5755 ± 1224 (4)	6	NT	—	NT	—	—
11	*N*-Allyl-EM-2	130 ± 9.3 (6)	7358 ± 259 (3)	57	NT	—	NT	—	—
2′	[H-Dmt^1]EM-2[a]	0.15 ± 0.004	28.2 ± 8.1	188	0.07 ± 0.016	—	1.87 ± 0.61	—	—
8	[*N*,*N*-(Allyl)$_2$-Dmt^1]EM-2	13.1 ± 1.3 (6)	1416 ± 50 (4)	108	>10,000 (33.9%)	7.07 (7.33–7.02)	>10,000 (13.3%)	6.13 (6.34–5.72)	8.7
12	[*N*-Allyl-Dmt^1]EM-2	0.45 ± 0.08 (5)	560 ± 49 (4)	1244	>10,000 (49.3%)	8.59 (9.05–8.33)	>10,000 (42.8%)	6.32 (7.35–5.48)	186
3′	Endomorphin-1 (EM-1)	0.56 ± 0.04 (4)	1750 ± 146 (4)	3125	4.03 ± 1.14	—	283 ± 39	—	—
4′	[H-Dmt^1]EM-1	0.054 ± 0.01 (5)	5.60 ± 0.67 (3)	104	0.27 ± 0.08	—	>10,000 (21.6%)	8.60 (9.58–8.01)	—
13	[*N*,*N*-(Allyl)$_2$-Dmt^1]EM-1	4.60 ± 0.32 (4)	217 ± 23 (5)	47	>10,000 (23.4%)	7.60 (7.68—7.51)	>10,000 (19.8%)	7.15 (7.40–6.90)	2.8
15	[*N*-Allyl-Dmt^1]EM-1	0.26 ± 0.01 (4)	10.3 ± 1.1 (3)	40	>10,000 (49.0%)	8.18 (8.28–8.16)	>10,000 (37.5%)	7.32 (7.48–7.16)	7.2
5′	1,6-Bis-[H-Dmt-NH]-hexane[b]	0.053 ± 0.01 (6)	46.1 ± 8.8 (5)	870	3.08 ± 0.53	—	>10,000	6.1	—
17	1,6-Bis-[*N*-allyl-Dmt-NH]-hexane	12.4 ± 1.1 (3)	51.5 ± 4.1 (3)	4	>10,000 (<1%)	7.23 (7.36–6.99)	>10,000 (<1%)	6.83 (7.56–5.58)	2.5
6′	3,6-Bis-[H-Dmt-NH-propyl]-2(1*H*)-pyrazinone[c]	0.042 ± 0.003	13.2 ± 1.7	314	1.33 ± 0.2	—	>10,000	—	—
19	3,6-Bis-[*N*-allyl-Dmt-NH-propyl]-2(1*H*)-pyrazinone	6.94 ± 1.4 (3)	77.8 ± 14 (4)	11	>10,000 (<1%)	7.17 (7.82–6.84)	>10,000 (<1%)	6.38 (6.56–6.21)	6.2

The affinity of each compound was assayed with 5–8 graded dosages of peptide in independent repetitions (*n*) and data are presented as means ± SE. The functional bioactivity is the mean ± SE (*n* = 5–6 separate tissue preparations). NT, not tested (due to the very weak interaction with opioid receptors, K_i). A dash (−) indicates non-relevancy.

[a] Data from Ref. 17.

[b] Data from Ref. 18.

[c] Data from Ref. 19.

[d] Values in parentheses are maximum inhibition of the tissue contraction at the concentration of 10,000 nM.

[e] pA_2 is the negative log of the molar concentration required to double the agonist's IC_{50} value in order to achieve the original response, and the 95% confidence limits are in parentheses.

[f] $[A_2]$ ratio is the ratio of molar concentrations at the pA_2 values (MVD/GPI). Antagonism was determined using endomorphin-2 and deltorphin II as μ- and δ-receptor agonists, respectively.

dynorphin A-(1-11)[8] had mixed biological properties; and N,N-dimethylation of bis-Dmt-Tic-derivatives (δ-opioid antagonists) yielded potent dual μ-/δ-opioid antagonists.[22] On the other hand, replacement of Tyr[1] in endomorphin by Mdp [(2*S*)-2-methyl-3-(2′,6′-dimethyl-4′-hydroxyphenyl)-propionic acid] or Dhp [3-(2′,6′-dimethyl-4′-hydroxyphenyl)-propionic acid] gave moderately weak μ-opioid antagonists.[23] The parent compounds of **17** and **19** are μ-opioid agonists[18,19] and the allylated derivatives exhibited μ-opioid antagonism (pA_2 = 7.23 and 7.17, respectively) (Table 1) comparable to that of CTAP (pA_2 = 7.36).[12] While the δ-opioid affinity of **17** remained similar to its parent ligand, the 6.6-fold improvement in δ-opioid antagonism yielded a μ-/δ-opioid antagonist profile similar to that of **19**. Our group,[17–19] as well other investigators,[11] published considerable data demonstrating discrepancies and inconsistencies between receptor affinity and bioactivity without an acceptable explanation; however, tentative answers may, in part, involve inherent differences between the assay systems, which include the artificial nature of the synaptosomes used to determine affinity (K_i) and the bioactivity in isolated tissue preparations for estimation of functional bioactivity, and the differential solubility of the ligand in the membrane lipid milieu that depends on the hydrophobic properties of opioids. Another possible reason for the inconsistencies regarding endomorphin analogues may be related to their interactions with tachykinin NK1 and NK2 receptors,[24] which mediate antiopioid activity.

3.3. Inhibition of morphine antinociception

The intracerebroventricular (icv) administration of a drug is the first analysis of its potential central role in affecting morphine-induced antinociception using the classic hot-plate or tail-flick tests. [*N*-Allyl-Dmt[1]]EM-2 (**12**) alone failed to show any significant icv effect at the same dosages used under identical conditions (hot-plate test) for the morphine-induced antinociception; on the other hand, **12** was a potent μ-opioid antagonist in a dose-dependent manner against morphine, such that the observed effect implies that supraspinally (CNS) μ-opioid receptors are involved (Fig. 1) (infra vide).[25] The %MPE (percent of maximal possible effect) at 10 min post-injection of **12** revealed that the AD_{50} **[12]** (0.148 ng/mouse, 95% CL_{95}: 0.02–0.993 ng/mouse) was 16 times more potent than naloxone ($AD_{50}^{naloxone}$ = 2.38 ng/mouse, 95% CL_{95}: 0.935–6.00 ng/mouse). Although, there is little difference between the antinociception produced by the highest dose for **12** and naloxone in Figure 1c, a further increase in naloxone completely blocked morphine antinociception; however, **12** failed to show this effect. Moreover, in contrast to many alkaloid opiates and peptidic μ-opioid agonists, **12** only acts supraspinally (CNS) due to the absence of μ-opioid antagonism in the classic and accepted pharmacological tail-flick test which measures spinal nociceptive mechanisms (Fig. 2a and b); that is, the tail-flick test indicates whether a spinal mechanism is involved in pain perception by activating a descending mechanism regardless of the type of injection or route of administration.

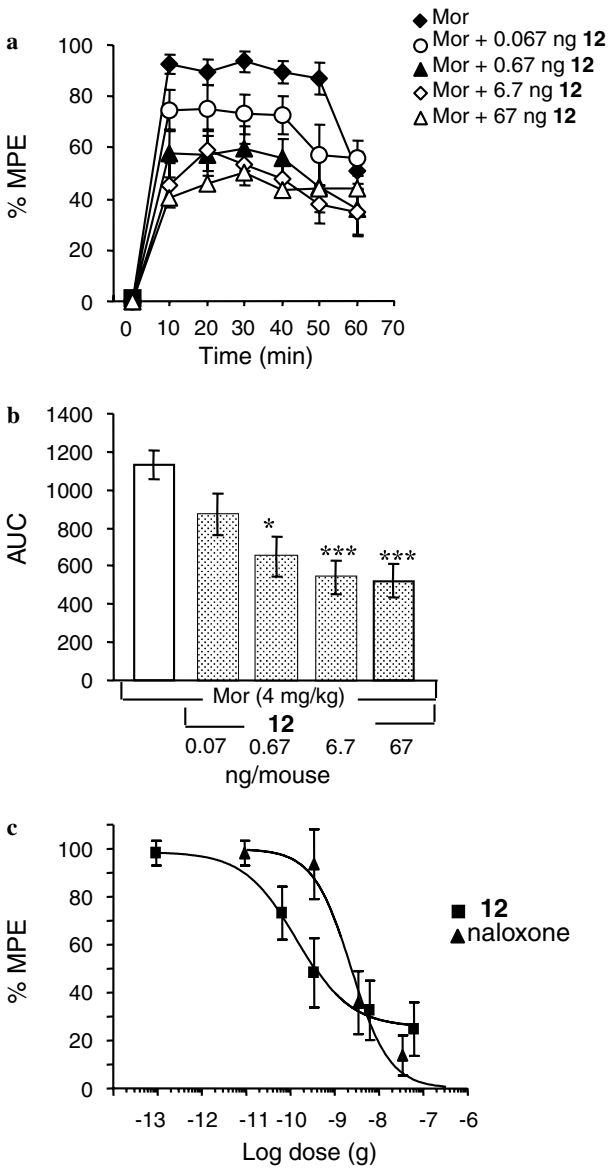


Figure 1. The effect of intracerebroventricularly injected [*N*-allyl-Dmt[1]]EM-2 (**12**) on morphine (Mor)-induced antinociception (4 mg/kg sc) in the hot-plate test in mice (a) Time course. (b) Area under the dose–response curve (AUC). (c) Dose–response curve of **12** and naloxone at 10 min after icv injection. Each value is the mean ± SE (n = 10–17 mice per point). ANOVA and Dunnett's test were used to analyze the data. ***$P < 0.001$ and *$P < 0.05$ represent significant differences in the AUC from morphine-treated mice.

3.4. Changes in spontaneous IPSC frequency

The inhibition of ethanol-induced frequency of spontaneous IPSC (a constituent component in the promotion of $GABA_A$ receptor-mediated neuronal function) in vitro (patch-clamp analysis of isolated neurons in hippocampal slices) verified the direct action of **15** as a μ-opioid antagonist on neuronal activity (Fig. 3). Spontaneous IPSC is one of the most potent CNS actions initiated by ethanol (EtOH) and constitutes an underlying mechanism of the sedative[26] and anxiolytic properties[27] of EtOH. In the presence of glutamatergic receptor blockers 50 μM AVP (D-2-amino-5-phosphonovalerate) and

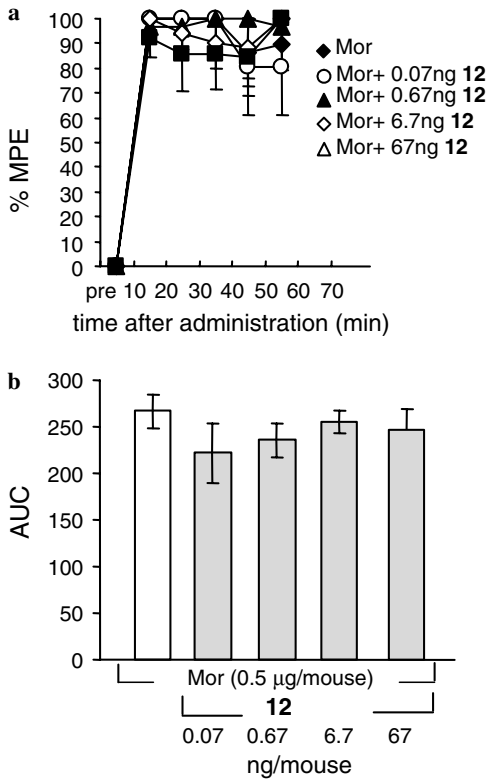


Figure 2. The effect of intracerebroventricularly injected [*N*-allyl-Dmt[1]]EM-2 (**12**) on morphine (Mor)-induced antinociception (0.5 μg/mouse icv) in the tail-flick test in mice. (a) Time course. (b) Area under the dose–response curve (AUC). Each value is the mean ± SE (n = 5–6 mice per point). ANOVA and Dunnett's test were used to analyze the data.

20 μM DNOX (6,7-dinitroquinoxaline-2,3-dione) to block excitory synaptic transmission, spontaneous IPSCs were isolated from hippocampal CA1 pyramidal cells held at −70 mV. Similar to our previous report,[28] after the baseline (control) spontaneous IPSCs were established, addition of 60 mM EtOH to the infusion medium significantly increased average frequency of spontaneous IPSCs of hippocampal CA1 pyramidal cells by 26% of control ($t_{[5]} = 4.40$, $P = 0.007$) and was reversed with 300 nM **15** ($t_{[5]} = 2.87$, $P = 0.0055$) (Fig. 3). Furthermore, **15** did not reduce the baseline recordings of the spontaneous IPSC frequency ($t_{[5]} = 1.05$, $P = 0.341$) indicative that its effect was only on the elevation elicited by EtOH and complements the in vivo antagonism of morphine antinociception that it acted as an antagonist (Fig. 1). Sedative and anxiolytic actions are involved in the reinforcing properties and addictive liability of ethanol, and drugs that attenuate these actions at the μ-opioid receptor[2] could be used therapeutically for the treatment of EtOH dependency. Furthermore, **15** was potent in the nanomolar range in contrast to naloxone and naltrexone, which are well known to inhibit GABA (γ-aminobutyric acid)-mediated chloride uptake into hippocampal synaptoneurosomes at micromolar concentrations.[29] In fact, similar results were also obtained with **12** at a comparable concentration in which naltrexone was active only in the 30–60 μM range (based on data available in the literature), demonstrating that **12** and **15** are at least two orders of magnitude more potent (Li; Swartzwelder, et al., unpublished data, in preparation).

4. Conclusions

The present study validates the conversion of μ-opioid receptor agonists to potent μ-opioid antagonists by N-allylation; and the *N*-allyl-derivatives of [H-Dmt[1]] endomorphins,[17a] bis-[H-Dmt-amino]-alkane,[18] and 3,6-bis-[H-Dmt-aminoalkyl]-2(1*H*)-pyrazinone[19] could generate new drug leads. Moreover, this approach would be applicable to producing analogues on large-scale synthesis with considerable ease relative to the replacement of the C-terminal phenylalanyl in endomorphin that only yielded weak μ-opioid antagonists.[15] Our data provided evidence that [*N*-allyl-Dmt[1]]endomorphin-1 (**15**) and -2 (**12**) are pharmacological probes that could be used to study the role of μ-opioid receptors in mediating physiological and pharmacological processes in the CNS. Their potential value could be to effectively combat alkaloid drug addiction or alleviate alcohol dependency or abuse (Figs. 1–3) due to the involvement of the μ-opioid receptor in the neural reward pathway[2] and the abolishment of spontaneous IPSC (Fig. 3). Further studies are underway to define the biochemical mechanisms of their μ-opioid antagonist action, and the physiological abatement of morphine and alcohol-mediated changes in vitro and in vivo.

5. Experimental

5.1. Chemistry general

Melting points were determined on a Yanagimoto micromelting point apparatus and are uncorrected. TLC was performed on precoated plates of silica gel F254 (Merck, Darmstadt, Germany). R_f values refer to the following solvent systems: (1) AcOEt/hexane = 1:1, (2) AcOEt/hexane = 1:2, (3) AcOEt/hexnane = 10:1, (4) AcOEt, (5) AcOEt/MeOH = 10:1, (6) AcOEt/MeOH = 20:1, (7) AcOEt/hexane = 2:1, (8) *n*-BuOH/H_2O/AcOH = 4:1:5 (upper layer), (9) $CHCl_3$/MeOH/H_2O = 8:3:1 (lower layer). Optical rotations were determined with a DIP-1000 automatic polarimeter (Japan Spectroscopic Co.). Analytical RP-HPLC and semi-preparative RP-HPLC used are Waters Delta 600 with COSMOSIL C18 column (4.6 mm × 250 mm) and COSMOSIL C18 column (20 mm × 250 mm), respectively. The solvent for analytical HPLC was as follows: A, 0.05% TFA in water; B, 0.05% TFA in CH_3CN. The column was eluted at a flow rate of 1 mL/min with a linear gradient of 90% A to 10% A in 30 min; the retention time is reported as t_R (min). Mass spectra were measured with a KRATOS MALDI-TOF (matrix-assisted laser desorption ionization time-of-flight mass spectrometry). ^1H and ^{13}C NMR spectra were measured on a Bruker DPX-400 spectrometer at 25 °C. Chemical shift values are expressed as ppm downfield from tetramethylsilane, used as an internal standard (δ value).

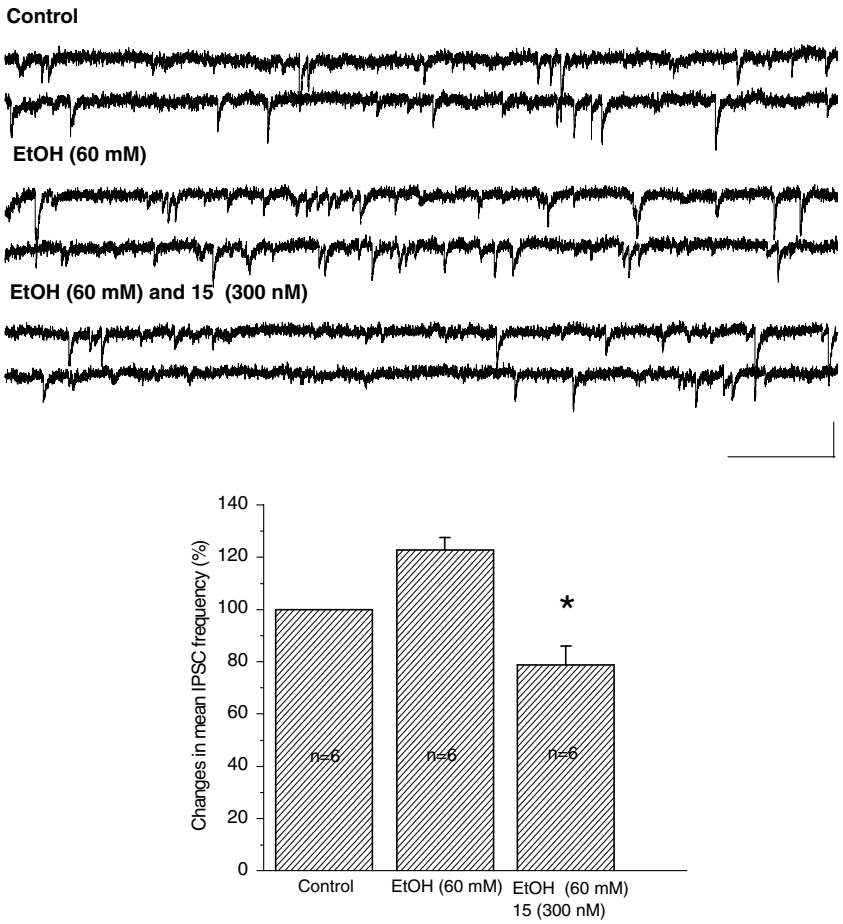


Figure 3. Changes in spontaneous IPSC frequency in the presence of ethanol and ethanol plus [*N*-allyl-Dmt[1]]endomorphin-1 (**15**). All the recordings in this figure were recorded from single neurons in hippocampal slices in vitro using patch-clamp methods. Upper figure: from top to bottom, the recordings refer to baseline spontaneous IPSC recordings, plus 60 mM EtOH, and with 300 nM **15** in 60 mM EtOH. Scale bar = 500 ms/50 pA. Bottom figure: the bars represent the percentage changes in mean spontaneous IPSC frequency (mean ± SE) with $n = 6$ tissue slices per measurement. Left, baseline; middle, plus 60 mM EtOH (26%, $t_{[5]} = 4.40$, $P = 0.007$); and right, the addition of 300 nM **15** in 60 mM EtOH ($t_{[5]} = 2.87$, $P = 0.0055$; ∗ denotes significant difference).

5.2. General methods for peptide synthesis

Gram quantities of H-L-Dmt-OH were prepared according to Dygos et al.,[30] and the syntheses of the endomorphins,[17] 1,6-bis-[H-Dmt-NH]-hexane,[18] and 3,6-bis-[H-Dmt-aminopropyl]-2(1*H*)-pyrazinone[19] followed published methods. The bold numbers in the text refer to the synthetic products (Schemes 1–3).

5.3. Synthesis of [H-Dmt[1]]endomorphin-1 ([H-Dmt[1]]EM-1)

5.3.1. Boc-Dmt-Pro-OCH$_3$. To a solution of HCl·H-Pro-OMe (1.28 g, 7.75 mmol) in *N*,*N*-dimethylformamide (DMF) (50 mL) were added Boc-Dmt-OH (*tert*-butyloxycarbonyl-Dmt-OH, 2.39 g, 7.75 mmol), benztriazol-1-yloxytris-(pyrrolidino)phosphate (PyBop) (4.4 g, 8.52 mmol), and diisopropylethylamine (DIPEA) (3.37 mL, 19.4 mol) at 0 °C. The reaction mixture was stirred at room temperature overnight. After removal of DMF, the residue was dissolved in ethyl acetate (AcOEt) and washed with 10% citric acid solution, 5% Na_2CO_3 solution, and saturated NaCl aqueous solution. The AcOEt layer was dried over Na_2SO_4 and evaporated. Petroleum ether was added to the residue to give crystals, which were collected by filtration. The crude Boc-Dmt-Pro-OMe was purified by flash chromatography (SiO_2, AcOEt/hexane = 1:1). Yield 2.16 g (66.3%), mp 144–145 °C, $R_{f1} = 0.45$, $[\alpha]_D^{20}$ +16.5° (*c* 0.63, MeOH). Anal. Calcd for $C_{22}H_{32}N_2O_6$: C, 62.84; H, 7.67; N, 6.66. Found: C, 62.72; H, 7.68; N, 6.65. ^{1}H NMR ($CDCl_3$)δ: 6.51 (s, 2H), 5.54 (d, 0.4H, $J = 8.4$ Hz), 5.31 (d, 0.6H, $J = 8.9$ Hz), 4.72 (q, 0.6H, $J = 8.7$ Hz), 4.49 (br, 0.6H), 4.40 (q, 0.4H, $J = 7.0$ Hz), 3.73 and 3.69 (s × 2, 3H), 3.65–3.55 (m, 1H), 3.38–3.30 (m, 0.8H), 3.13–2.89 (m, 2.6H), 2.34 and 2.31 (s × 2, 6H), 2.15–1.50 (m, 4H), 1.43 and 1.34 (s × 2, 9H).

5.3.2. Boc-Dmt-Pro-OH. Boc-Dmt-Pro-OCH$_3$ (1.89 g, 4.5 mmol) in methanol (MeOH; 10 mL) was treated with 1.0 N NaOH (11.2 mL, 11.2 mmol) for 2 h at room temperature. MeOH was removed in vacuo, the pH of the residue was adjusted to 3 with 10% citric acid, and the resulting precipitate was extracted with AcOEt (3× 30 mL). The combined organic phase was washed with saturated NaCl (3× 30 mL), dried over Na_2SO_4, and

evaporated down. Petroleum ether was added to the residue to produce crystals, which were filtered and dried in vacuo. Yield 1.79 g (98.0%), mp 84–87 °C, $R_{f2} = 0.25$, $[\alpha]_D^{20}$ −14.8° (*c* 0.54, MeOH). Anal. Calcd for $C_{21}H_{30}N_2O_6$: C, 62.05; H, 7.44; N, 6.89. Found: C, 61.99; H, 7.70; N, 6.76. ^{1}H NMR ($CDCl_3$) δ: 6.52 (s × 2, 2H), 5.80 (d, 0.3H, $J = 8.2$ Hz), 5.45 (d, 0.7H, $J = 8.7$ Hz), 4.72 (br, 0.7H), 4.56 (dd, 0.7H, $J = 8.0$, 3.8 Hz), 4.40 (br, 0.3H), 3.64–3.53 (m, 1H), 3.41–3.29 (m, 0.6H), 3.14–2.85 (m, 2.7H), 2.34 and 2.31 (s × 2, 6H), 2.15–1.50 (m, 4H), 1.42 and 1.37 (s × 2, 9H).

5.3.3. Boc-Dmt-Pro-Trp-Phe-NH_2. To a solution of HCl·H-Trp-Phe-NH_2 [prepared from Boc-Trp-Phe-NH_2 (200 mg, 0.44 mmol), 7.7 N HCl/dioxane (570 μL, 4.4 mmol)] in tetrahydrofuran (THF; 30 mL) containing DIPEA (230 μL, 1.32 mmol), Boc-Dmt-Pro-OH (180 mg, 0.44 mmol) and PyBop (250 mg, 0.48 mmol) were added. The reaction mixture was stirred for 6 h at 35 °C. After removal of the solvent, the residue was extracted with AcOEt (50 mL), and the extract was washed with 10% citric acid, 5% Na_2CO_3, and saturated NaCl, dried over Na_2SO_4, and evaporated. The residue was purified by flash chromatography (SiO_2, AcOEt/MeOH = 10:1). Yield 223 mg (68.6%), mp 150–152 °C, $R_{f3} = 0.57$, $[\alpha]_D^{20}$ −34.9° (*c* 0.49, MeOH). Anal. Calcd for $C_{41}H_{50}N_6O_7 \cdot 1.5H_2O$: C, 64.30; H, 6.97; N, 10.97. Found: C, 64.47; H, 6.89; N, 10.75. ^{1}H NMR (400 MHz, DMSO-d_6)δ: 9.28 (s, 0.63H), 8.85–8.47 (m, 1.2H), 8.22 (s, 0.53H), 8.00–7.87 (m, 0.73H), 7.63–6.82 (m, 11.32H), 6.82–6.60 (m, 0.23H), 6.60–6.33 (m, 1.64H), 6.25 (s, 0.44H), 6.15–5.90 (m, 0.5H), 5.66 (d, 0.16H, $J = 8.03$ Hz), 5.67 (br, 0.48H), 5.26 (br, 0.27H), 4.80–4.59 (m, 1H), 4.52 (br, 1H), 4.46–4.10 (m, 1.24H), 4.10–3.84 (m, 1H), 3.60–3.24 (m, 1.41H), 3.24–2.45 (m, 5.5H), 2.45–2.00 (m, 5H), 1.90–0.90 (m, 13.27H), 0.70–0.40 (m, 1.46H).

5.3.4. H-Dmt-Pro-Trp-Phe-NH_2·TFA (4′). Boc-Dmt-Pro-Trp-Phe-NH_2 (100 mg, 0.136 mmol) was treated with 7.7 N HCl/dioxane (0.18 mL, 1.36 mmol) and anisole (0.15 mL, 1.36 mmol) for 1 h at room temperature. Et_2O was added to the reaction mixture to precipitate the product. The crude product was collected by centrifugation, dried over KOH pellets, and purified by semi-preparative reverse phase high pressure liquid chromatography (RP-HPLC). Yield 86.8 mg (85.1%), t_R 16.8 min, $[\alpha]_D^{20}$ −8.8° (*c* 0.41, H_2O), *m*/*z* $[M+H]^+$ 640. Anal. Calcd for $C_{46}H_{54}N_4O_8 \cdot TFA \cdot 3H_2O$: C, 56.57; H, 6.12; N, 10.42. Found: C, 56.31; H, 5.98; N, 10.28. ^{1}H NMR (400 MHz, DMSO-d_6)δ: 10.83 and 10.81 (s × 2, 1H), 9.15 (br, 1H), 8.36 and 8.25 (br, s × 2, 3H), 8.07–7.97 (m, 1.6H), 7.79 (d, 0.4H, $J = 8.1$ Hz), 7.58 (d, 1H, $J = 7.81$ Hz), 7.46 (s, 0.5H), 7.36–7.30 (m, 1H), 7.27–7.10 (m, 6.5H), 7.10–7.03 (m, 2H), 7.03–6.95 (m, 1H), 6.44 and 6.42 (s × 2, 2H), 4.53–4.32 (m, 2.4H), 4.20–4.10 (m, 0.6H), 3.60–3.50 (m, 0.6H), 3.42–3.30 (m, 1H), 3.21–3.12 (m, 0.7H), 3.08–2.76 (m, 6.7H), 2.34–2.23 (m, 0.4H), 2.16 (s, 2.5H), 1.98 (s, 3.5H), 1.87–1.73 (m, 0.4H), 1.67–1.52 (m, 1.6H), 1.50–1.39 (m, 0.6H), 1.39–1.20 (m, 1H).

5.4. Synthesis of allylated derivatives

5.4.1. *N*,*N*-(Allyl)$_2$-Tyr-OMe (2a) and *N*-allyl-Tyr-OMe (3a). To a solution of HCl·H-Tyr-OMe (2.0 g, 8.6 mmol) in MeOH (20 mL), DIPEA (4.8 mL, 27.5 mmol) and allylbromide (1.86 mL, 21.5 mmol) were added in sequence. After stirred at room temperature for 20 min, the temperature was raised to 50 °C and the reaction mixture kept at this temperature for 5 h. The solvent was removed in vacuo, and the residue was extracted with AcOEt (3× 30 mL). The combined extracts were washed with saturated NaCl, dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was purified by flash chromatography (SiO_2, AcOEt/hexane = 1:1) to give pure **2a** (1.34 g, 59.3%) and **3a** (740 mg, 36.4%). Compound **2a**: mp 127–128 °C, $R_{f1} = 0.79$, $[\alpha]_D^{20}$ −198.0° (*c* 0.63, MeOH). Anal. Calcd for $C_{16}H_{21}NO_3$: C, 69.79; H, 7.69; N, 5.09. Found: C, 69.51; H, 7.81; N, 5.22. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.04 (d, 2H, $J = 8.32$ Hz), 6.73 (d, 2H, $J = 8.32$ Hz), 5.77–5.58 (m, 2H), 5.20–5.00 (m, 4H), 4.68 (br, 1H), 3.70–3.55 (m, 4H), 3.44–3.30 (m, 2H), 3.17–2.93 (m, 3H), 2.90–2.75 (m, 1H). Compound **3a**: mp 117–118 °C, $R_{f4} = 0.74$, $[\alpha]_D^{20}$ +27.0° (*c* 0.51, MeOH). Anal. Calcd for $C_{13}H_{17}NO_3$: C, 66.36; H, 7.28; N, 5.95. Found: C, 66.14; H, 7.13; N, 5.94. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.02 (d, 2H, $J = 8.56$ Hz), 6.71 (d, 2H, $J = 8.56$ Hz), 5.88–5.76 (m, 1H), 5.18–5.07 (m, 2H), 3.67 (s, 3H), 3.57 (t, 1H, $J = 6.6$ Hz), 3.34–3.27 (m, 1H), 3.20–3.12 (m, 1H), 2.94 (d, 2H, $J = 6.6$ Hz).

5.4.2. *N*,*N*-(Allyl)$_2$-Dmt-OMe (2b) and *N*-allyl-Dmt-OMe (3b). To a solution of HCl·H-Dmt-OMe (3.0 g, 11.5 mmol) in MeOH (60 mL), DIPEA (6.0 mL, 34.6 mmol) and allylbromide (2.0 mL, 23.1 mmol) were added in sequence. After stirred at room temperature for 20 min, the temperature was raised to 50 °C and the reaction mixture kept at this temperature for 5 h. The solvent was removed in vacuo, the residue extracted with AcOEt (3× 50 mL). The combined extracts were washed with saturated NaCl, dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was purified by flash chromatography (SiO_2, AcOEt/hexane = 1:1) to give pure **2b** 1.05 g (30%) and **3b** 1.72 g (56.7%). Compound **2b**: oil, $R_{f1} = 0.76$; $[\alpha]_D^{20}$ +2.5° (*c* 0.55, MeOH). Anal. Calcd for $C_{18}H_{25}NO_3$: C, 71.26; H, 8.31; N, 4.62. Found: C, 71.04; H, 8.38; N, 4.65. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 8.93 (s, 1H), 6.37 (s, 2H), 5.74-5.63 (m, 2H), 5.20-5.04 (m, 4H), 3.50 (s, 3H), 3.46 (dd, 1H, $J = 5.41$, 8.74 Hz), 3.93-3.43 (m, 1H), 3.39-3.36 (m, 1H), 3.04-2.95 (m, 3H), 2.70 (dd, 1H, $J = 5.40$, 14.12 Hz), 2.16 (s, 6H). Compound **3b:** oil, $R_{f1} = 0.53$; $[\alpha]_D^{20}$ +42.1° (*c* 0.34, MeOH). Anal. Calcd for $C_{15}H_{21}NO_3 \cdot 0.17H_2O$: C, 67.64; H, 8.07; N, 5.26. Found: C, 67.52; H, 8.08; N, 5.27. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 8.93 (s, 1H), 6.38 (s, 2H), 5.78–5.60 (m, 1H), 5.08–4.95 (m, 2H), 3.53 (m, 3H), 3.29 (t, 1H, $J = 9.28$ Hz), 3.17–3.08 (m, 1H), 2.98–2.90 (m, 1H), 2.83–2.70 (m, 2H), 2.16 (s, 6H).

5.4.3. *N*,*N*-(Allyl)$_2$-Tyr-OH (4a). In an ice bath, 1 N NaOH (11 mL, 11 mmol) was added to a solution of *N*,*N*-(allyl)$_2$-Tyr-OMe (**2a**) (1.31 g, 4.8 mmol) in MeOH (15 mL) and the resulting solution was stirred at room

temperature overnight. The solvent was removed in vacuo, the pH of the residue was adjusted to 3, and the resulting precipitate was filtered, washed with cold water, and dried in vacuo. Yield 866 mg (69%), mp 214–216 °C, R_{f8} = 0.43, $[\alpha]_D^{20}$ −7.5° (*c* 0.48, MeOH). Anal. Calcd for $C_{15}H_{19}NO_3{\cdot}0.5H_2O$: C, 66.65; H, 7.46; N, 5.18. Found: C, 66.72; H, 7.25; N, 5.13. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 12.20 (br, 1H), 9.20 (br, 1H), 6.95 (d, 2H, J = 8.4 Hz), 6.63 (d, 2H, J = 8.4 Hz), 5.73–5.56 (m, 2H), 5.11 (d, 2H, J = 17.24 Hz), 5.04 (d, 2H, J = 10.24 Hz), 3.43 (t, 1H, J = 7.54 Hz), 3.28 (dd, 2H, J = 5.05, 14.8 Hz), 3.06 (dd, 2H, J = 6.92, 14.80 Hz), 2.82 (dd, 1H, J = 7.82, 13.65 Hz), 2.67 (dd, 1H, J = 7.31, 13.65 Hz).

5.4.4. *N*,*N*-(Allyl)$_2$-Dmt-OH (4b). In an ice bath, 1 N NaOH (8 mL, 8 mmol) was added to a solution of *N*,*N*-(allyl)$_2$-Dmt-OMe (**2b**) (967 mg, 3.19 mmol) in MeOH (10 mL) and the resulting solution was stirred at room temperature overnight. The solvent was removed in vacuo, the pH of the residue was adjusted to 3, the resulting precipitate was extracted with AcOEt (3× 50 mL), the combined extracts were washed with saturated NaCl (2× 50 mL), dried over Na_2SO_4, filtered, and purified by flash chromatography (SiO_2, AcOEt/MeOH = 10:1). Yield 428 mg (46.5%), mp 205–207 °C, R_{f5} = 0.50, $[\alpha]_D^{20}$ +25.2° (*c* 0.68, MeOH). Anal. Calcd for $C_{17}H_{23}NO_3{\cdot}0.1H_2O$: C, 70.12; H, 8.03; N, 4.81. Found: C, 69.91; H, 7.94; N, 4.77. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 12.20 (br, 1H), 8.89 (s, 1H), 6.37 (s, 1H), 5.74–5.62 (m, 2H), 5.13 (dd, 2H, J = 1.29, 17.21 Hz), 5.05 (dd, 2H, J = 1.29, 10.23 Hz), 3.42–3.34 (m, 3H), 3.06–2.90 (m, 3H), 2.66 (dd, 1H, J = 5.70, 14.1 Hz).

5.4.5. *N*-Allyl-*N*-Boc-Tyr-OMe (5a). *N*-Allyl-Tyr-OMe (**3a**) (580 mg, 2.5 mmol) was reacted with di-*tert*-butyl dicarbonate (Boc_2O, 600 mg, 2.75 mmol) in dioxane (15 mL) containing triethylamine (Et_3N) (380 μL, 2.75 mmol) at room temperature overnight. After removal of the solvent, the residue was extracted with AcOEt (60 mL), and the extract was washed with 10% citric acid (3× 10 mL), 5% $NaHCO_3$ (3× 10 mL), and saturated NaCl (3× 10 mL), dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was purified by flash chromatography (SiO_2, AcOEt/hexane = 1:3) to give pure **5a**. Yield 580 mg (69.4%), oil, R_{f1} = 0.82, $[\alpha]_D^{20}$ −13.5° (*c* 0.83, MeOH). Anal. Calcd for $C_{18}H_{25}NO_5{\cdot}0.2H_2O$: C, 63.77; H, 7.55; N, 4.13. Found: C, 63.76; H, 7.51; N, 4.00. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.10-6.97 (m, 2H), 6.80–6.70 (m, 2H), 5.65–5.44 (m, 1.3H), 5.01 (d, 2H, J = 14.52 Hz), 4.47–4.37 (m, 0.3H), 4.00–3.85 (m, 1.2H), 3.80-3.62 (m, 3.3H), 3.46–3.36 (m, 0.4H), 3.27–3.03 (m, 2.5H), 1.45 (s, 9H).

5.4.6. *N*-Allyl-*N*-Boc-Dmt-OMe (5b). *N*-Allyl-Dmt-OMe (**3b**) (1.53 g, 5.79 mmol) was reacted with Boc_2O (1.39 g, 6.37 mmol) in dioxane (30 mL) containing Et_3N (890 μL, 6.37 mmol) at room temperature overnight. After removal of the solvent, the residue was extracted with AcOEt (100 mL), and the extract was washed with 10% citric acid (2× 15 mL), 5% $NaHCO_3$ (2× 15 mL), and saturated NaCl (2× 15 mL), dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was purified by flash chromatography (SiO_2, AcOEt/hexane = 1:1) to give pure **5b**. Yield 1.12 g (53%), oil, R_{f1} = 0.65, $[\alpha]_D^{20}$ −174.5° (*c* 0.49, MeOH). Anal. Calcd for $C_{20}H_{29}NO_5{\cdot}0.25H_2O$: C, 65.28; H, 8.08; N, 3.81. Found: C, 65.27; H, 8.10; N, 3.80. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 8.98 (s, 1H), 6.41 (s, 2H), 5.40–5.20 (m, 1H), 5.00–4.83 (m, 2H), 3.96–3.83 (m, 1H), 3.80–3.53 (m, 4H), 3.22–2.96 (m, 3H), 2.12 (s, 6H), 1.36 (s, 9H).

5.4.7. *N*-Allyl-*N*-Boc-Tyr-OH (6a). 1 N NaOH (9.33 mL, 9.33 mmol) was added to a solution of *N*-allyl-*N*-Boc-Tyr-OMe (**5a**) (1.36 g, 4.05 mmol) in MeOH (10 mL) at 0 °C. After stirred at 0 °C for 20 min, the solution was stirred at room temperature overnight. MeOH was removed in vacuo, and the pH of the residue was adjusted to 3 with 10% citric acid. The resulting precipitate was extracted with AcOEt (2× 40 mL), and the combined extracts were washed with saturated NaCl solution (2× 20 mL), dried over Na_2SO_4, filtered, and concentrated in vacuo to give **6a** as a clear oil. Yield 1.09 g (84%), R_{f5} = 0.70, $[\alpha]_D^{20}$ −153.2° (*c* 0.83, MeOH). Anal. Calcd for $C_{17}H_{23}NO_5{\cdot}0.2H_2O$: C, 62.83; H, 7.26; N, 4.31. Found: C, 63.06; H, 7.48; N, 4.06. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 12.55 (s, 1H), 9.17 (s, 1H), 6.95 (d, 2H, J = 8.4 Hz), 6.83–6.58 (m, 2H), 5.54–5.35 (m, 1H), 5.01–4.87 (m, 2H), 4.35–4.24 (m, 0.36H), 4.05–3.95 (m, 0.57H), 3.79–3.60 (m, 1H), 3.35–3.25 (m, 0.5H), 3.18–2.87 (m, 2.57H), 1.35 (s, 9H).

5.4.8. *N*-Allyl-*N*-Boc-Dmt-OH (6b). 1 N NaOH (7.15 mL, 7.15 mmol) was added to a solution of *N*-allyl-*N*-Boc-Dmt-OMe (**5b**) (1.04 g, 2.86 mmol) in MeOH (10 mL) at 0 °C. After stirred at 0 °C for 20 min, the solution was stirred at room temperature overnight. MeOH was removed in vacuo, and the pH of the residue was adjusted to 3 with 10% citric acid. The resulting precipitate was extracted with AcOEt (2× 40 mL), and the combined extracts were washed with saturated NaCl solution (2× 20 mL), dried over Na_2SO_4, filtered, and concentrated in vacuo. The residue was purified by flash chromatography (SiO_2, AcOEt/hexane = 1:1) to give the title compound. Yield 830 mg (83%), mp 150–152 °C, R_{f1} = 0.35, $[\alpha]_D^{20}$ −185.8° (*c* 0.61, MeOH). Anal. Calcd for $C_{19}H_{27}NO_5$: C, 65.31; H, 7.79; N, 4.01. Found: C, 65.20; H, 7.58; N, 3.98. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 6.52 (s, 2H), 5.56–5.35 (m, 1H), 5.00–4.85 (m, 2H), 4.10–3.80 (m, 2H), 3.50–2.80 (m, 3H), 2.24 (s, 6H), 1.48 (s, 9H).

5.4.9. [*N*,*N*-Diallyl-Tyr^1]EM-2·HCl (7). Boc-Pro-Phe-Phe-NH_2 (220 mg, 0.433 mmol) was treated with 6.7 N HCl/dioxane (1.3 mL, 8.66 mmol) at room temperature for 30 min to remove the Boc group. The product was precipitated with ether, filtered, and dried in vacuo. The resulting hydrochloride salt was dissolved in DMF (10 mL) containing DIPEA (190 μL 1.09 mmol), and to this solution *N*,*N*-(allyl)$_2$-Tyr-OH (**4a**) (124 mg, 0.476 mmol) and PyBop (260 mg, 0.5 mmol) were added. The reaction mixture was stirred at 0 °C for 10 min, then at room temperature for 4 h. After removal

of the solvent, the residue was diluted with AcOEt (80 mL). The dilution was washed with cold 10% citric acid (3× 10 mL), 5% Na_2CO_3 (3× 10 mL), and saturated NaCl (3× 10 mL), dried over Na_2SO_4, and evaporated to dryness. The residue was purified by flash chromatography (SiO_2, AcOEt/MeOH = 10:1). The compound was precipitated with hexane, filtered, and dried in vacuo. The solid was dissolved in MeOH, and TFA (36 μL, 0.472 mmol) was added. After concentrated to dryness, the residue was purified by semi-preparative HPLC, and the purified peptide was lyophilized from 1 N HCl (3× 320 μL) to give an amorphous powder. Yield 220 mg (73.8%), R_{f9} = 0.75, t_R 16.40, $[\alpha]_D^{20}$ −30.1° (*c* 0.57, H_2O), *m/z* $[M+1]^+$ 653. Anal. Calcd for $C_{38}H_{45}N_5O_5{\cdot}HCl{\cdot}2H_2O$: C, 63.01; H, 6.96; N, 9.67. Found: C, 63.02; H, 6.68; N, 9.64. ^1H NMR (400 MHz, DMSO-d_6) δ: 11.55 (br, 0.3H, Dmt NH^+), 10.50 (br, 0.2H, Dmt NH^+), 9.63 and 9.42 (br × 2, 0.6H, Dmt OH), 8.40 (d, 0.40H, *J* = 7.95 Hz, Phe *cis*-NH), 8.15 (d, 0.40H, *J* = 8.15 Hz, Phe *cis*-NH), 8.11 (d, 0.54H, *J* = 8.22 Hz, Phe *trans*-NH), 7.99 (d, 0.52H, *J* = 7.76 Hz, Phe *trans*-NH), 7.38–7.04 (m, 13.1H, Phe Ar-H, $CONH_2$, Tyr Ar-H), 6.94 (d, 0.90H, *J* = 8.44 Hz, Tyr Ar-H), 6.76 (d, 0.90H, *J* = 8.44 Hz, Tyr Ar-H), 6.69 (d, 1.1H, *J* = 8.42 Hz, Tyr Ar-H), 6.15–5.97 (m, 1.0H, CH_2=C*H*–CH_2–), 5.86–5.72 (m, 1.0H, CH_2=C*H*–CH_2–), 5.72–5.20 (m, 4.0H, CH_2=CH–CH_2–), 4.50–4.35 (m, 2.0H, Phe αH), 4.35–4.28 (m, 0.57H, Pro *trans*-αH), 4.15–3.57 (m, 5.0H, Tyr αH, CH_2=CH–CH_2–), 3.50–3.20 (m, 2.43H, Pro *cis*-αH, Pro *trans*-δH, Pro *cis*-δH, Tyr *trans*-βH, Tyr *cis*-βH), 3.20–2.73 (m, 5.54H, Pro *cis*-δH, Phe βH, Tyr *trans*-βH, Tyr *cis*-βH), 2.23 (br, 0.46H, Pro *trans*-δH), 1.92–1.78 (m, 0.54H, Pro *trans*-βH), 1.66–1.45 (m, 2.08H, Pro *cis*-βH, Pro *trans*-βH, Pro *trans*-γH), 1.45–1.26 (m, 0.92H, Pro *cis*-γH), 1.27–1.10 (m, 0.46H, Pro *cis*-βH). ^{13}C NMR δ: 172.54, 172.50, 170.56, 170.46, 165.50, 156.84, 156.39, 137.83, 137.72, 137.59 (11q), 130.62, 130.41 (2t, Tyr Ar-C), 129.13, 129.09, 129.01, 128.00, 127.96, 127.94, 127.89, 126.31, 126.13 (9t, Phe Ar-C), 124.40 (s, *C*H_2=CH–CH_2–), 115.41, 115.26 (2t, Tyr Ar-C), 63.46 (t, Tyr *trans*-αC), 62.03 (t, Tyr *cis*-αC), 59.85 (t, Pro *trans*-αC), 59.20 (t, Pro *cis*-αC), 54.82 (t, Phe *cis*-αC), 54.43 (t, Phe *trans*-αC), 53.84 (t, Phe *cis*-αC), 53.74 (t, Phe *trans*-αC), 53.28 (s, CH_2=CH–*C*H_2-), 46.80 (s, Pro *trans*-δC), 46.54 (s, Pro *cis*-δC), 37.38, 37.21, 36.88 (3s, Phe βC), 33.68 (s, Tyr *trans*-βC), 32.74 (s, Tyr *cis*-βC), 30.98 (s, Pro *cis*-βC), 28.63 (s, Pro *trans*-βC), 23.99 (s, Pro *trans*-γC), 21.14 (s, Pro *cis*-γC).

5.4.10. [*N*,*N*-(Allyl)$_2$-Dmt^1]EM-2·HCl (8). The title compound was synthesized using the same method for **7**. Yield 200 mg (71.3%), R_{f9} = 0.73, t_R 17.28, $[\alpha]_D^{20}$ −15.6° (*c* 0.44, H_2O), *m/z* $[M+1]^+$ 681. Anal. Calcd for $C_{40}H_{49}N_5O_5{\cdot}HCl{\cdot}3H_2O$: C, 62.36; H, 7.33; N, 9.09. Found: C, 62.59; H, 7.08; N, 9.13. ^1H NMR (400 MHz, DMSO-d_6) δ: 10.60 (br, 1H, Dmt NH^+), 9.40 and 9.16 (br × 2, 1H, Dmt OH), 8.42 (br, 0.7H, Phe NH), 8.19 (br, 0.7H, Phe NH), 7.99 (br, 0.6H, Phe NH), 7.44 (s, 0.7H, $CONH_2$), 7.37–7.00 (m, 11.3H, Phe Ar-H, $CONH_2$), 6.47 and 6.41 (s × 2, Dmt Ar-H), 6.15 (br, 0.5H, CH_2=C*H*–CH_2–), 5.92–5.28 (m, 5.5H, CH_2=C*H*–CH_2–, CH_2=CH–CH_2–), 4.46–4.35 (m, 2.0H, Phe αH), 4.35–4.28 (m, 0.27H, Pro *trans*-αH), 4.17–3.70 (m, 3.5H, Dmt *trans*-αH, CH_2=CH–CH_2–), 3.41–2.74 (m, 10.23H, Pro *cis*-αH, Dmt *cis*-αH, CH_2=CH–CH_2–, Pro δH, Phe βH, Dmt βH), 2.15 and 2.08 (s × 2, 6.0H, Dmt CH_3), 1.83–1.13 (m, 4.0H, Pro βH, Pro γH). ^{13}C NMR δ: 172.55, 170.66, 170.32, 170.25, 165.88, 156.03, 138.45, 138.42, 138.02, 137.62, 137.54 (11q), 129.11, 129.07, 128.96, 127.97, 127.93 (5t, Phe Ar-C), 127.87 (t, CH_2=*C*H–CH_2–), 126.24, 126.10 (2t, Phe Ar-C), 125.40 (s, *C*H_2=CH–CH_2–), 119.69 (q), 115.18, 115.05 (2t, Dmt Ar-C), 60.77 (t, Pro *cis*-αC), 59.95 (t, Por *trans*-αC), 59.13 (t, Dmt *trans*-αC), 58.87 (t, Dmt *cis*-αC), 55.12 (t, Phe *cis*-αC), 54.20 (s, CH_2=CH–*C*H_2–), 54.04 (t, Phe *trans*-αC), 53.80 (t, Phe *cis*-αC), 53.62 (t, Phe *trans*-αC), 53.22, 51.41 (2s, CH_2=CH–*C*H_2–), 46.99 (s, Pro *cis*-δC), 45.99 (s, Pro *trans*-δC), 37.45, 37.22, 36.69 (3s, Phe βC), 31.33 (s, Pro *cis*-βC), 28.67 (s, Pro *trans*-βC), 27.71 (s, Dmt βC), 23.88 (s, Pro *trans*-γC), 21.17 (s, Pro *cis*-γC), 20.22 (p, Dmt *trans*-*C*H_3), 19.76 (p, Dmt *cis*-*C*H_3).

5.4.11. [*N*-Allyl-*N*-Boc-Tyr]EM-2 (9). Boc-Pro-Phe-Phe-NH_2 (220 mg, 0.433 mmol) was treated with 6.7 N HCl/dioxane (1.3 mL, 8.66 mmol) at room temperature for 30 min to remove the Boc group. The product was precipitated with ether, filtered, and dried in vacuo. The resulting hydrochloride salt was dissolved in DMF (10 mL) containing DIPEA (190 μL, 1.09 mmol), and to this solution *N*-allyl-*N*-Boc-Tyr-OH (**6a**) (153 mg, 0.476 mmol) and PyBop (260 mg, 0.5 mmol) were added. The reaction mixture was stirred at 0 °C for 10 min, then at room temperature for 4 h. After removal of the solvent, the residue was diluted with AcOEt (80 mL). The dilution was washed with cold 10% citric acid (3× 10 mL), 5% Na_2CO_3 (3× 10 mL), and saturated NaCl (3× 10 mL), dried over Na_2SO_4, and evaporated to dryness. The residue was purified by flash chromatography (SiO_2, AcOEt). The compound was precipitated with hexane, filtered, and dried in vacuo. Yield 247 mg (80%), mp 121-122 °C, R_{f4} = 0.42, $[\alpha]_D^{20}$ −75.1° (*c* 0.58, MeOH). Anal. Calcd for $C_{40}H_{49}N_5O_7{\cdot}0.9H_2O$: C, 65.99; H, 7.03; N, 9.62. Found: C, 65.99; H, 6.85; N, 9.61. ^1H NMR (400 MHz, DMSO-d_6) δ: 9.14 and 9.07 (s × 2, 1.0H, Dmt OH), 8.02–7.88 (m, 1.0H, Phe NH), 7.85–7.76 (m, 1.0H, Phe NH), 7.30–7.08 (m, 12.0H, Phe Ar-H, $CONH_2$), 6.98–6.89 (m, 2.0H, Tyr Ar-H), 6.69–6.57 (m, 2.0H, Tyr Ar-H), 5.73–5.59 (m, 1.0H, CH_2=C*H*–CH_2–), 5.07–4.93 (m, 2.4H, CH_2=CH–CH_2–, Tyr *cis*-αH), 4.83–4.71 (m, 0.6H, Tyr *trans*-αH), 4.47–4.35 (m, 2.0H, Phe αH), 4.16–4.06 (m, 1.0H, Pro αH), 3.94–3.82 (m, 0.6H, *trans* CH_2=CH–CH_2–), 3.82–3.71 (m, 0.4H, *cis* CH_2=CH–CH_2–), 3.63–3.37 (m, 2.0H, CH_2=CH–CH_2–, Pro *cis*-δH, Pro *trans*-δH), 3.35–3.18 (m, 1.0H, Pro *cis*-δH, Pro *trans*-δH), 3.14–2.95 (m, 2.0H, Phe βH), 2.95–2.63 (m, 4.0H, Phe βH, Tyr βH), 1.90–1.57 (m, 4.0H, Pro βH, Pro γH), 1.25 and 1.14 (s × 2, 9.0H, Boc). ^{13}C NMR δ: 172.48, 171.31, 170.47, 169.25, 168.72, 155.75, 155.58, 154.36, 153.45, 137.82, 137.72 (11q), 134.70 (t, *cis* CH_2=*C*H–CH_2–), 134.15 (t, *trans* CH_2=*C*H–CH_2–), 130.28, 130.05 (t, Tyr Ar-C), 128.98, 127.99, 127.89 (3t, Phe

Ar-C), 127.64, 127.27 (2q), 126.16, 126.10 (2t, Phe Ar-C), 116.09 (s, *trans* CH_2=CH–CH_2–), 115.98 (s, *cis* CH_2=CH–CH_2–), 114.77, 114.69 (2t, Tyr Ar-C), 79.04, 78.83 (2q, Boc), 60.04 (t, Pro αC), 58.04 (t, Tyr *trans*-αC), 56.28 (t, Tyr *cis*-αC), 54.08, 53.98, 53.74 (3t, Phe αC), 46.31 (s, Pro *cis*-δC), 46.13 (s, Pro *trans*-δC), 45.42 (s, *cis* CH_2=CH–CH_2–), 44.67 (s, *trans* CH_2=CH–CH_2–), 37.27, 36.73, 36.67 (3s, Phe βC), 33.68 (s, Tyr βC), 28.48 (s, Pro βC), 27.73 (p, *cis*-Boc), 27.41 (p, *trans*-Boc), 23.98 (s, Pro γC).

5.4.12. [*N*-Allyl-*N*-Boc-Dmt[1]]EM-2 (10). The title compound was synthesized using the same method for **9**. Yield 225 mg (70%), mp 131–133 °C, R_{f5} = 0.70, $[\alpha]_D^{20}$ −82.9° (*c* 0.51, MeOH). Anal. Calcd for $C_{42}H_{53}N_5O_7{\cdot}0.5H_2O$: C, 67.36; H, 7.27; N, 9.35. Found: C, 67.22; H, 7.33; N, 9.06. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 8.90 and 8.85 (s × 2, 1.0H, Dmt OH), 8.06–7.81 (m, 2.0H, Phe NH), 7.35–7.00 (m, 12.0H, Phe Ar-H, $CONH_2$), 6.40 and 6.36 (s × 2, 2.0H, Dmt Ar-H), 5.64 (br, 1.0H, CH_2=C*H*–CH_2–), 5.10–4.88 (m, 2.27H, CH_2=CH–CH_2–, Dmt *cis*-αH), 4.88–4.73 (m, 0.73H, Dmt *trans*-αH), 4.50–4.30 (m, 2.0H, Phe αH), 4.07–3.93 (m, 0.73H, *trans* CH_2=CH–CH_2–), 3.93–3.82 (m, 0.27H, *cis* CH_2=CH–CH_2–), 3.73–3.60 (m, 0.27H, *cis* CH_2=CH–CH_2–), 3.60–3.48 (m, 0.73H, *trans* CH_2=CH–CH_2–), 3.48–3.18 (m, 2.0H, Pro δH), 3.15–2.62 (m, 6.0H, Phe βH, Dmt βH), 2.16 and 2.12 (s × 2, 6.0H, Dmt CH_3), 1.90–1.55 (m, 4.0H, Pro βH, Pro γH), 1.28 and 1.08 (s × 2, 9.0H, Boc). ^{13}C NMR δ: 172.48, 171.15, 170.41, 168.94, 168.29, 155.10, 153.36, 137.77, 137.62, 137.57 (10q), 134.72 (t, *cis* CH_2=*C*H–CH_2–), 133.85 (t, *trans* CH_2=*C*H–CH_2–), 129.06, 127.95, 127.88, 126.13 (4t, Phe Ar-C), 116.10 (s, *trans* CH_2=CH–CH_2–), 115.79 (s, *cis* CH_2=CH–CH_2–), 114.64 (t, Dmt Ar-C), 79.15, 78.84 (2q, Boc), 59.71 (t, Pro αC), 55.47 (t, Dmt *trans*-αC), 54.74 (t, Dmt, *cis*-αC), 54.21, 53.73 (2t, Phe αC), 46.41 (s, Pro *cis*-δC), 46.15 (s, Pro *trans*-δC), 45.36 (s, CH_2=CH–*C*H_2–), 37.35, 36.96 (2s, Phe βC), 28.55 (s, Pro βC), 28.18 (s, Dmt βC), 27.70 (p, *trans*-Boc), 27.16 (p, *cis*-Boc), 24.00 (s, Pro γC), 19.85 (p, Dmt *C*H_3).

5.4.13. [*N*-Allyl-Tyr[1]]EM-2·HCl (11). [*N*-Allyl-N-Boc-Tyr[1]]EM-2 (**9**) (147 mg, 0.206 mmol) was treated with TFA (320 μL, 4.13 mmol) and anisole (32 μL) for 30 min at room temperature. The reaction solution was diluted with hexane, the solid was collected by filtration, dried over KOH pellets, and purified by semi-preparative RP-HPLC. The purified peptide was lyophilized from 1 N HCl (3× 200 μL) to give amorphous powder. Yield 113 mg (84%), R_{f9} = 0.68, t_R 15.29, $[\alpha]_D^{20}$ −20.7° (*c* 0.46, H_2O), *m*/*z* $[M+1]^+$ 613. Anal. Calcd for $C_{35}H_{41}N_5O_5{\cdot}HCl{\cdot}2H_2O$: C, 61.44; H, 6.78; N, 10.24. Found: C, 61.32; H, 6.56; N, 10.26. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 9.78 (br, 0.82H, –NH_2^+), 9.59 (s, 0.55H, Dmt *cis*-OH), 9.44 (s, 0.43H, Dmt *trans*-OH), 9.12 (br, 0.83H, –NH_2^+), 8.38 (d, 0.5H, *J* = 8.57 Hz, Phe *cis*-NH), 8.19 (d, 0.53H, *J* = 8.26 Hz, Phe *cis*-NH), 8.15 (d, 0.42H, *J* = 8.30 Hz, Phe *trans*-NH), 8.02 (d, 0.42H, *J* = 7.85 Hz, Phe *trans*-NH), 7.44 (s, 0.53H, $CONH_2$), 7.37–7.07 (m, 12.47H, Phe Ar-H, Tyr Ar-H, $CONH_2$), 6.92 (d, 1.0H, *J* = 8.40 Hz, Tyr Ar-H), 6.75 (d, 1.0H, *J* = 8.37 Hz, Tyr Ar-H), 6.69 (d, 1.0H, *J* = 8.42 Hz, Tyr Ar-H), 5.95–5.71 (m, 1.0H, CH_2=C*H*–CH_2–), 5.41–5.22 (m, 2.0H, CH_2=CH–CH_2–), 4.53–4.32 (m, 2.40H, Pro *trans*-αH, Phe αH), 4.13 (br, 0.42H, Tyr *cis*-αH), 3.49 (br, 0.84H, *trans* CH_2=CH–CH_2–), 3.43–3.09 (m, 4.30H, Pro *cis*-αH, Tyr *cis*-αH, *cis* CH_2=CH–CH_2–, Pro *trans*-δH, Pro *cis*-δH, Tyr *cis*-βH, Tyr *trans*-βH), 3.09–2.65 (m, 6.14H, *cis* CH_2=CH–CH_2–, Pro *trans*-δH, Phe βH, Tyr *cis*-βH), 2.65–2.53 (m, 0.46H, Pro *trans*-δH), 1.97–1.84 (m, 0.44H, Pro *trans*-βH), 1.71–1.53 (m, 1.88H, Pro *cis*-βH, Pro *trans*-βH, Pro *trans*-γH), 1.52–1.38 (m, 0.56H, Pro *cis*-γH), 1.38–1.20 (m, 1.12H, Pro *cis*-βH, Pro *cis*-γH). ^{13}C NMR δ: 172.65, 172.57, 170.48, 170.45, 170.40, 169.81, 165.84, 165.81, 156.72, 156.47, 137.92, 137.73, 137.47 (13q), 130.92, 130.84 (2t, Tyr Ar-C), 129.14, 129.11 (2t, Phe Ar-C), 129.09, 128.76 (2t, CH_2=*C*H–CH_2–), 127.99, 127.97, 127.94, 127.88, 126.64, 126.20 (6t, Phe Ar-C), 124.35, 123.42 (2q), 122.39, 121.98 (2s, *C*H_2=CH–CH_2–), 115.41, 115.15 (2t, Tyr Ar-C), 59.59 (s, Pro *trans*-αC), 59.19 (s, Pro *cis*-αC), 58.98 (s, Tyr *cis*-αC), 58.38 (s, Tyr *cis*-αC), 54.52, 54.19 (2s, Phe *trans*-αC), 53.78, 53.75 (2s, Phe *cis*-αC), 48.06 (s, *cis* CH_2=CH–*C*H_2–), 47.84 (s, *trans* CH_2=CH–*C*H_2–), 46.63 (s, Pro *trans*-δC), 46.39 (s, Pro *cis*-δC), 37.45, 37.39, 37.10 (3s, Phe βC), 35.58 (s, Tyr *cis*-βC), 34.84 (s, Tyr *trans*-βC), 31.11 (s, Pro *cis*-βC), 28.85 (s, Pro *trans*-βC), 24.09 (s, Pro *trans*-γC), 21.17 (s, Pro *cis*-γC).

5.4.14. [*N*-Allyl-Dmt[1]]EM-2·HCl (12). The title compound was synthesized using the same method for **11**. Yield 72 mg (71.6%), R_{f9} = 0.68, t_R 15.82, $[\alpha]_D^{20}$ −5.9° (*c* 0.42, H_2O), *m*/*z* $[M+1]^+$ 641. Anal. Calcd for $C_{37}H_{45}N_5O_5{\cdot}HCl{\cdot}3H_2O$: C, 60.85; H, 7.18; N, 9.59. Found: C, 61.19; H, 6.85; N, 9.65. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 10.07 (br, 0.90H, Dmt NH_2^+), 9.40–9.10 (m, 1.96H, Dmt NH_2^+, Dmt OH), 8.36 (d, 0.85H, *J* = 8.60 Hz, Phe *cis*-NH), 8.18 (d, 0.85H, *J* = 8.28 Hz, Phe *cis*-NH), 8.05 (d, 0.15H, *J* = 8.12 Hz, Phe *trans*-NH), 8.01 (d, 0.15H, *J* = 8.28 Hz, Phe *trans*-NH), 7.47 (s, 1.0H, $CONH_2$), 7.35–7.05 (m, 11.0H, Phe Ar-H, $CONH_2$), 6.46 and 6.42 (s × 2, 2.0H, Dmt Ar-H), 6.00–5.87 (m, 0.15H, *trans* CH_2=C*H*–CH_2–), 5.87–5.73 (m, 0.85H, *cis* CH_2=C*H*–CH_2–), 5.43–5.27 (m, 1.15H, *trans* CH_2=CH–CH_2–, *cis* CH_2=CH–CH_2–), 5.23 (d, 0.85H, *J* = 10.39 Hz, *cis* CH_2=CH–CH_2–), 4.48–4.32 (m, 2.22H, Phe αH, Pro *trans*-αH), 4.13–4.04 (m, 0.15H, Dmt *trans*-αH), 3.64–3.56 (m, 0.22H, *trans* CH_2=CH–CH_2–), 3.54–3.45 (m, 0.22H, *trans* CH_2=CH–CH_2–), 3.33–2.73 (m, 11.20H, Pro *cis*-αH, Dmt *cis*-αH, *cis* CH_2=CH–CH_2–, Pro δH, Phe βH, Dmt βH), 2.15 and 2.10 (s × 2, 6.0H, Dmt CH_3), 1.86–1.73 (m, 0.14H, Pro *trans*-βH), 1.67–1.52 (m, 1.21H, Pro *cis*-βH, Pro *trans*-βH, Pro *trans*-γH), 1.52–1.36 (m, 1.0H, Pro *cis*-γH, Pro *trans*-γH), 1.28–1.12 (m, 1.66H, Pro *cis*-βH, Pro *cis*-γH). ^{13}C NMR δ: 172.66, 172.50, 170.58, 170.34, 170.16, 169.91, 166.40, 166.07, 155.88, 155.63, 138.50, 138.20, 137.96, 137.65, 137.61, 137.52 (16q), 129.12, 128.90 (2t, Phe Ar-C), 128.80 (t, CH_2=*C*H–CH_2–), 128.05, 127.96, 127.92, 127.84, 126.44, 126.08 (6t, Phe Ar-C), 122.20 (s, *trans* *C*H_2=CH–CH_2–), 121.87 (s, *cis* *C*H_2=CH–CH_2–),

120.86, 120.47 (2q), 115.03 (t, Dmt Ar-C), 59.76 (t, Pro *trans*-αC), 58.94 (t, Pro *cis*-αC), 56.26 (t, Dmt *cis*-αC), 55.66 (t, Dmt *trans*-αC), 54.84, 53.65 (2t, Phe αC), 47.65 (s, *cis* CH_2=CH–CH_2–), 47.56 (s, *trans* CH_2=CH–CH_2–), 46.63 (s, Pro *cis*-δC), 45.94 (s, Pro *trans*-δC), 37.54 (s, Phe *cis*-βC), 37.44, 37.31 (2s, Phe *trans*-βC), 36.83 (s, Phe *cis*-βC), 31.19 (s, Pro βC), 29.64 (s, Dmt *cis*-βC), 28.78 (s, Dmt *trans*-βC), 23.87 (s, Pro *trans*-γC), 21.09 (s, Pro *cis*-γC), 20.79 (p, Dmt *trans*-CH_3), 19.37 (p, Dmt *cis*-CH_3).

5.4.15. [*N*,*N*-(Allyl)$_2$-Dmt^1]EM-1·HCl (13). The title compound was synthesized using the same method for **7**. Yield 115 mg (71.3%), R_{f9} = 0.69, t_R 18.55, $[\alpha]_D^{20}$ −33.2° (*c* 0.36, H_2O), *m*/*z* $[M+1]^+$ 720. Anal. Calcd for $C_{42}H_{50}N_6O_5$·HCl·3H_2O: C, 62.32; H, 7.10; N, 10.38. Found: C, 62.43; H, 6.99; N, 10.42. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 11.66 (br, 0.16H, Dmt *trans*-NH^+), 11.00 (s, 0.68H, Trp *cis* Ar-NH), 10.89 (s, 0.25H, Trp *trans* Ar-NH), 10.52 (br, 0.56H, Dmt *cis*-NH^+), 9.39 (br, 0.58H, Dmt *cis*-OH), 9.16 (br, 0.15H, Dmt *trans*-OH), 8.39 (d, 0.67H, *J* = 6.90Hz, Trp *cis*-NH), 8.10–8.00 (m, 1.0H, Trp *trans*-NH, Phe *cis*-NH), 7.80 (d, 0.27H, *J* = 8.16 Hz, Phe *trans*-NH), 7.68 (d, 0.74H, *J* = 7.79 Hz, Trp Ar-H), 7.58 (d, 0.26H, *J* = 7.75 Hz, Trp Ar-H), 7.42 (br, 0.73H, *cis*-$CONH_2$), 7.38–7.30 (m, 1.0H, Trp Ar-H), 7.30–7.13 (m, 6.0H, Phe Ar-H, Trp Ar-H), 7.13–6.95 (m, 3.27H, $CONH_2$, Trp Ar-H), 6.47 and 6.41 (s × 2, 2.0H, Dmt Ar-H), 6.16 (br, 0.48H, *trans* CH_2=C*H*–CH_2–), 5.75 (br, 0.76H, *cis* CH_2=C*H*–CH_2–), 5.66–5.36 (m, 2.54H, *cis* CH_2=C*H*–CH_2–, CH_2=CH–CH_2–), 5.23 (d, 2.20H, *J* = 10.23 Hz, CH_2=CH–CH_2–), 4.48–4.36 (m, 2.0H, Phe αH, Trp αH), 4.35–4.28 (m, 0.29H, Pro *trans*-αH), 4.17–3.98 (m, 0.73H, Dmt *trans*-αH, CH_2=CH–CH_2–), 3.98–3.64 (m, 2.81H, CH_2=CH–CH_2–), 3.42–3.16 (m, 3.13H, Pro *cis*-αH, Pro *cis*-δH, Pro *trans*-δH, Dmt *cis*-βH, CH_2=CH–CH_2–), 3.16–2.77 (m, 6.71H, Dmt *cis*-αH, Pro *cis*-δH, Phe βH, Dmt *cis*-βH, Dmt *trans*-βH, Trp βH), 2.20–1.94 (m, 6.29H, Dmt CH_3, Pro *trans*-δH), 1.80–1.72 (m, 0.24H, Pro *trans*-βH), 1.72–1.63 (m, 0.72H, Pro *cis*-βH), 1.60–1.44 (m, 0.72H, Pro *trans*-βH, Pro *trans*-γH), 1.44–1.17 (m, 2.28H, Por *cis*-βH, Pro *cis*-γH). ^{13}C NMR δ: 172.54, 172.49, 171.00, 170.74, 170.23, 165.85, 156.04, 138.47, 137.64, 137.55, 136.07, 135.99 (12q), 129.15, 127.90 (2t, Phe Ar-C), 127.59 (t, CH_2=C*H*–CH_2–), 127.23, 126.96 (2q), 126.13 (t, Phe, Ar-C), 125.16 (t, CH_2=C*H*–CH_2–), 124.25, (s, CH_2=CH–CH_2–), 123.88, 123.48, 118.33, 118.11 (4t, Trp Ar-C), 115.21, 115.05 (t, Dmt Ar-C), 111.32, 115.15 (2t, Trp Ar-C), 109.89, 109.73 (2q), 60.98 (t, Pro *cis*-αC), 60.03 (t, Pro *trans*-αC), 59.34 (t, Dmt *trans*-αC), 58.86 (t, Dmt *cis*-αC), 54.42 (t, Phe αC), 54.19 (s, *cis* CH_2=CH–*C*H_2–), 53.64 (t, Trp *cis*-αC), 53.46 (t, Trp *trans*-αC), 51.48 (s, *trans* CH_2=CH–*C*H_2–), 47.01 (s, Pro *cis*-δC), 46.09 (s, Pro *trans*-δC), 37.39 (s, Phe βC), 31.38 (s, Pro *cis*-βC), 28.63 (s, Pro *trans*-βC), 27.79 (s, Dmt *cis*-βC), 27.49 (s, Dmt *trans*-βC), 27.11 (s, Trp βC), 23.85 (s, Pro *trans*-γC), 21.24 (s, Pro *cis*-γC), 19.78, 19.66 (2p, Dmt *C*H_3).

5.4.16. [*N*-Allyl-*N*-Boc-Dmt^1]EM-1 (14). The title compound was synthesized using the same method for **9**. Yield 246 mg (72.9%), mp 138-140 °C, R_{f6} = 0.69, $[\alpha]_D^{20}$ −71.9° (*c* 0.36, MeOH). Anal. Calcd for $C_{44}H_{54}N_6O_7$·1.25H_2O: C, 65.94; H, 7.11; N, 10.49. Found: C, 66.03; H, 6.80; N, 10.50. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 10.83 (s, 0.91H, Trp Ar-NH), 8.90 (br, 0.70H, Dmt OH), 8.03 (d, 0.65H, *J* = 6.98 Hz, Trp *trans*-NH), 7.96 (d, 0.23H, *J* = 7.46 Hz, Trp *cis*-NH), 7.82–7.71 (m, 0.91H, Phe NH), 7.51 (d, 1.0H, *J* = 7.81 Hz, Trp Ar-H), 7.32 (d, 1.0H, *J* = 8.02 Hz, Trp Ar-H), 7.27–7.14 (m, 6.0H, Phe Ar-H, $CONH_2$), 7.14–7.01 (m, 3.0H, Trp Ar-H, $CONH_2$), 7.01–6.92 (m, 1.0H, Trp Ar-H), 6.39 and 6.35 (s × 2, 2.0H, Dmt Ar-H), 5.71–5.55 (m, 1.0H, CH_2=C*H*–CH_2–), 5.07–4.87 (m, 2.30H, CH_2=CH–CH_2–, Dmt *cis*-αH), 4.79 (d, 0.70H, *J* = 9.29 Hz, Dmt *trans*-αH), 4.47–4.34 (m, 2.0H, Phe αH, Trp αH), 4.05–3.93 (m, 0.73H, *trans* CH_2=CH–CH_2–), 3.93–3.82 (m, 0.27H, *cis* CH_2=CH–CH_2–), 3.70–3.59 (m, 0.27H, *cis* CH_2=CH–CH_2–), 3.53 (dd, 0.73H, *J* = 7.16, 15.26 Hz, *trans* CH_2=CH–CH_2–), 3.47–3.17 (m, 2.0H, Pro δH), 3.13–2.74 (m, 5.27H, Dmt *cis*-βH, Dmt *trans*-βH, Phe βH, Trp βH), 2.72–2.59 (m, 0.73H, Dmt *trans*-βH), 2.15–2.11 (2s, 6.0H, Dmt CH_3), 1.98–1.58 (m, 4.0H, Pro γH, Pro βH), 1.27 and 1.07 (s × 2, 9.0H, Boc). ^{13}C NMR δ: 172.50, 171.41, 170.85, 168.91, 168.27, 155.08, 154.84, 153.38, 137.70, 137.57, 135.92 (11q), 134.76 (t, *cis* CH_2=C*H*–CH_2–), 133.88 (t, *trans* CH_2=C*H*–CH_2–), 129.08, 127.93 (2t, Phe Ar-C), 127.25 (q), 126.13 (t, Phe Ar-C), 124.98 (q), 123.32, 120.75, 118.12 (3t, Trp Ar-C), 116.06 (s, CH_2=CH–CH_2–), 114.61 (t, Dmt Ar-C), 111.17 (t, Trp Ar-C), 109.87 (q), 79.12, 78.82 (2q, Boc), 59.74 (t, Pro αC), 55.56 (t, Dmt αC), 53.90 (t, Phe αC), 53.57 (t, Trp αC), 46.44 (s, Pro *cis*-αC), 46.19 (s, Pro *trans*-δC), 45.37 (s, CH_2=CH–*C*H_2–), 37.27 (s, Phe βC), 28.73 (s, Pro βC), 28.16 (s, Dmt βC), 27.71, 27.15 (2p, Boc), 26.95 (s, Trp βC), 24.05 (s, Pro γC), 19.83 (p, Dmt *C*H_3).

5.4.17. [*N*-Allyl-Dmt^1]EM-1·HCl (15). The title compound was synthesized using the same method for **11**. Yield 131 mg (77.0%), R_{f9} = 0.63, t_R 17.10, $[\alpha]_D^{20}$ −12.3° (*c* 0.34, H_2O), *m*/*z* $[M+1]^+$ 680. Anal. Calcd for $C_{39}H_{46}N_6O_5$·HCl·2.5H_2O: C, 61.61; H, 6.89; N, 11.05. Found: C, 61.59; H, 6.59; N, 11.09. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 11.06 (s, 0.77H, Trp *cis* Ar-NH), 10.85 (s, 0.13H, Trp *trans* Ar-H), 10.08 and 9.88 (br × 2, 0.82H, Dmt OH), 9.27 (s, 1.60H, Dmt *cis*-NH_2^+), 9.14 (s, 0.28H, Dmt *trans*-NH_2^+), 8.30 (d, 0.81H, *J* = 8.20 Hz, Trp *cis*-NH), 8.04 (d, 0.14H, *J* = 8.47 Hz, trp *trans*-NH), 8.00 (d, 0.81H, *J* = 8.09 Hz, Phe *cis*-NH), 7.80 (d, 0.16H, *J* = 8.10 Hz, Phe *trans*-NH), 7.63–7.46 (m, 1.0H, Trp Ar-H), 7.43 (br, 0.84H, *cis*-$CONH_2$), 7.38–7.30 (m, 1.0H, Trp Ar-H), 7.27–7.13 (m, 5.19H, Phe Ar-H, *trans*-$CONH_2$), 7.13–7.03 (m, 3.0H, $CONH_2$, Trp Ar-H), 7.03–6.94 (m, 1.0H, Trp Ar-H), 6.43, 6.42 (2s, 2.0H, Dmt Ar-H), 5.97–5.86 (m, 0.16H, trasn CH_2=C*H*–CH_2–), 5.81–5.76 (m, 0.84H, *cis* CH_2=C*H*–CH_2–), 5.44–5.30 (m, 0.32H, *trans* CH_2=CH–CH_2–), 5.21 (d, 0.84H *J* = 17.06 Hz, *cis* CH_2=CH–CH_2–), 5.09 (d, 0.84H, *J* = 10.36 Hz, *cis* CH_2=CH–CH_2–), 4.50–4.13 (m, 2.18H, Phe αH, Trp αH, Pro *trans*-αH), 4.15–4.04 (m, 0.15H, Dmt *trans*-αH), 3.65–3.55 (m, 0.18H, *trans*

CH_2=CH–CH_2–), 3.55–3.45 (m, 0.18H, *trans* CH_2=CH–CH_2–), 3.45–2.87 (m, 11.41H, *cis* CH_2=CH–CH_2–, Pro *cis*-αH, Dmt *cis*-αH, Pro δH, Phe βH, Dmt βH, Trp βH), 2.15, 2.06 (2s, Dmt CH_3), 1.87–1.76 (m, 0.16H, Pro *trans*-βH), 1.68–1.48 (m, 1.32H, Pro *cis*-βH, Pro *trans*-βH, Pro *trans*-γH), 1.48–1.33 (m, 0.84H, Pro *cis*-γH), 1.33–1.77 (m, 1.68H, Pro *cis*-βH, Pro *cis*-γH). ^{13}C NMR δ: 172.57, 172.42, 170.86, 170.67, 170.19, 169.96, 166.31, 155.81, 155.62, 138.52, 138.27, 137.62, 137.53, 135.98, 135.89 (15q), 129.11 (t, Phe Ar-C), 128.69 (s, CH_2=CH–CH$_2$–), 127.83 (t, Phe Ar-C), 127.19, 126.94 (2q), 126.08 (t, Phe Ar-C), 123.43, 122.89 (t, Trp Ar-C), 121.79 (t, CH_2=CH–CH$_2$–), 120.83 (t, Trp Ar-C), 120.47 (q), 118.18, 118.12 (2t, Trp Ar-C), 114.96 (t, Dmt Ar-C), 111.35 (t, Trp Ar-C), 110.18, 109.67 (2q), 59.82 (t, Pro *trans*-αC), 58.88 (t, Pro *trans*-αC), 56.29 (t, Dmt *cis*-αC), 55.78 (t, Dmt, *trans*-αC), 53.49 (t, Phe αC), 53.49 (t, Trp αC), 47.64 (s, Pro *trans*-αC), 46.67 (s, Pro *cis*-αC), 45.91 (s, CH_2=CH–CH_2–), 37.45 (s, Phe βC), 31.27 (s, Pro βC), 29.73 (s, Dmt βC), 27.10 (s, Trp βC), 24.01 (s, Pro *trans*-γC), 21.20 (s, Pro *cis*-γC), 19.34, 19.28 (2p, Dmt CH_3).

5.4.18. 1,6-Bis-(*N*-allyl-*N*-Boc-Dmt-NH)-hexane (16). To a solution of 1,6-diaminohexane (33 mg, 0.286 mmol) in DMF (10 mL), *N*-allyl-*N*-Boc-Dmt-OH (**6b**) (200 mg, 0.572 mmol), DIPEA (119 μL, 0.687 mmol), and PyBop (313 mg, 0.60 mmol) were added. The reaction mixture was stirred at 0 °C for 10 min, then at room temperature for 4 h. After removal of the solvent, the residue was extracted with AcOEt (50 mL). The organic phase was washed with cold 10% citric acid (3× 10 mL), 5% Na_2CO_3 (3× 10 mL), and saturated aqueous NaCl (3× 10 mL), dried over Na_2SO_4, and evaporated to dryness. The residue was purified by flash chromatography (SiO_2, AcOEt/hexane = 2:1), precipitated with hexane, filtered, and dried in vacuo. Yield 132 mg (59.2%), mp 90–92 °C, R_{f7} = 0.65, $[\alpha]_D^{20}$ −57.6° (*c* 0.32, MeOH). Anal. Calcd for $C_{44}H_{66}N_4O_8{\cdot}0.5H_2O$: C, 67.06; H, 8.57; N, 7.11. Found: C, 67.09; H, 8.57; N, 7.13. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.04 (s, 1.3H, Dmt OH), 6.53 (s, 4.0H, Dmt Ar-H), 5.96–5.52 (m, 4.0H, NH, CH_2=CH–CH$_2$–), 5.26–4.95 (m, 4.0H, CH_2=CH–CH$_2$–), 4.55–4.23 (m, 2.0H, Dmt αH), 4.23–3.85 (m, 4.0H, CH_2=CH–CH_2–), 3.53–3.10 (m, 4.0H, Dmt βH), 3.10–2.55 (m, 4.0H, NHCH_2), 2.28 (s, 12.0H, Dmt CH_3), 1.50 and 1.46 (s × 2, 18.0H, Boc), 1.40–0.98 (m, 4.0H, $NHCH_2CH_2$), 0.98–0.50 (m, 4.0H, $NHCH_2CH_2CH_2$). ^{13}C NMR δ: 170.75, 156.43, 154.73, 154.10, 138.44 (5q), 135.63 (t, CH_2=CH–CH$_2$–), 126.34 (q), 115.64 (s, CH_2=CH–CH$_2$–), 115.39, 115.04 (2t, Dmt Ar-C), 81.50 (q, Boc), 59.25 (t, Dmt αC), 46.87 (s, CH_2=CH–CH$_2$–), 39.15 (s, NHCH_2), 29.89 (s, Dmt βC), 28.81 (s, $NHCH_2CH_2$), 28.38 (p, Boc), 26.29 (s, $NHCH_2CH_2CH_2$), 20.31 (p, Dmt CH_3).

5.4.19. 1,6-Bis-(*N*-allyl-Dmt)-hexane·2HCl (17). The title compound was synthesized using the same method for **11**. Yield 36.4 mg (57.3%), R_{f9} = 0.66, t_R 15.43, $[\alpha]_D^{20}$ +55.0° (*c* 0.36, H_2O), *m*/*z* $[M+1]^+$ 680. Anal. Calcd for $C_{34}H_{50}N_4O_4{\cdot}2HCl{\cdot}2.5H_2O$: C, 58.21; H, 8.25; N, 8.04. Found: C, 58.30; H, 7.90; N, 8.03. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 9.07 (s, 2H, Dmt OH), 8.02 (br, 2H, NHCH$_2$) 6.39 (s, 4H, Dmt Ar-H), 5.95–5.84 (m, 2H, CH_2=CH–CH$_2$–), 5.46–5.30 (m, 4H, CH_2=CH–CH$_2$–), 3.72–3.60 (m, 2H, Dmt αH), 3.48–3.35 (m, 4H, CH_2=CH–CH_2–), 3.05–2.93 (m, 6H, Dmt βH, $NHCH_2CH_2$), 2.92–2.80 (m, 2H, Dmt βH), 2.17 (s, 12H, Dmt CH_3), 1.20–1.02 (m, 4H, $NHCH_2CH_2CH_2$), 0.96–0.82 (m, 4H, $NHCH_2CH_2CH_2$). ^{13}C NMR δ: 165.40, 155.46, 140.33, 138.11 (4q), 129.50 (t, CH_2=CH–CH$_2$–), 122.11 (s, CH_2=CH–CH$_2$–), 114.76 (t, Dmt Ar-C), 58.27 (t, Dmt αC), 47.54 (s, CH_2=CH–CH_2–), 38.52 (s, NHCH_2), 28.29 (s, $NHCH_2CH_2$), 25.71 (s, $NHCH_2CH_2CH_2$), 19.93 (p, Dmt CH_3).

5.4.20. 3,6-Bis-(*N*-allyl-*N*-Boc-Dmt-aminopropyl)-2(1*H*)-pyrazinone (18). 3,6-Bis- (Z-aminopropyl)-2(1*H*)-pyrazinone (141 mg, 0.286 mmol) was stirred in 25% HBr/HOAc (960 μL, 4 mmol) with an ice bath for 10 min and then at room temperature for 3 h. The resulting amine was precipitated with ether, collected by filtration, and dried in vacuo. The amine hydrobromide was dissolved in DMF (10 mL) containing DIPEA (240 μL, 1.37 mmol), to which *N*-allyl-*N*-Boc-Dmt-OH (**6b**) (200 mg, 0.572 mmol) and PyBop (312 mg, 0.60 mmol) were added. The solution was first stirred in an ice bath for 10 min, then at room temperature for 4 h. After removal of the solvent in vacuo, the residue was extracted with AcOEt (30 mL), which was washed with 10% citric acid (2× 10 mL), 5% $NaHCO_3$ (2× 10 mL), and saturated aqueous NaCl solution (2× 10 mL), dried over Na_2SO_4, filtered, and evaporated in vacuo. The resulting residue was purified by flash chromatography (SiO_2, AcOEt/MeOH = 10:1) and the compound was precipitated with ether. Yield 109 mg (43%), mp 122–124 °C, R_{f5} = 0.59, $[\alpha]_D^{20}$ −57.0° (*c* 0.31, MeOH). Anal. Calcd for $C_{49}H_{70}N_6O_9{\cdot}0.5H_2O$: C, 65.67; H, 7.99; N, 9.38. Found: C, 65.49; H, 7.91; N, 9.33. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 11.89 (br, 1H, Pyrazinone NH), 8.89 (br, 2H, Dmt OH), 7.66 (s, 2H, $NHCH_2CH_2CH_2$), 6.37 (s, 4H, Dmt Ar-H), 5.62 (br, 2H, CH_2=CH–CH$_2$–), 5.10–4.70 (m, 4H, CH_2=CH–CH$_2$–), 4.45–3.94 (m, 2H, Dmt αH), 3.94–3.20 (m, 4H, CH_2=CH–CH_2–), 3.20–2.60 (m, 8H, Dmt βH, $NHCH_2CH_2CH_2$), 2.60–2.00 (m, 19H, Dmt CH_3, pyrazinone CH_3, $NHCH_2CH_2CH_2$), 1.80–1.05 (m, 22H, Boc, $NHCH_2CH_2CH_2$). ^{13}C NMR δ: 170.01, 169.77, 155.54, 154.97, 153.94, 137.60 (6q), 135.72, 134.81 (2t, CH_2=CH–CH$_2$–), 125.51 (q), 116.45, 115.61 (2s, CH_2=CH–CH$_2$–), 114.71 (t, Dmt Ar-C), 79.03 (q, Boc), 58.48 (t, Dmt αC), 48.03 (s, CH_2=CH–CH_2–), 38.52, 38.01 (2s, $NHCH_2CH_2CH_2$), 29.21 (s, $NHCH_2CH_2CH_2$), 28.51 (s, Dmt βC), 26.09 (s, $NHCH_2CH_2CH_2$), 19.80 (p, Dmt CH_3), 18.02 (p, pyrazinone CH_3).

5.4.21. 3,6-Bis-(*N*-allyl-Dmt-aminopropyl)-2(1*H*)-pyrazinone·2HCl (19). The title compound was synthesized using the same method for **11**. Yield 31.4 mg (64.6%), R_{f9} = 0.60, t_R 16.8, $[\alpha]_D^{20}$ +32.1° (*c* 0.36, H_2O), *m*/*z* $[M+1]^+$ 688. Anal. Calcd for $C_{39}H_{54}N_6O_5{\cdot}2HCl{\cdot}5.5H_2O$: C, 54.54; H, 7.86; N, 9.78. Found: C, 54.66; H, 7.50; N, 9.84. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 9.99 (br, 2H, Dmt NH_2^+), 9.33 (br, 2H, Dmt NH_2^+), 9.07 (br, 2H, Dmt OH), 8.23 (t, 1H, *J* = 4.77 Hz, $NHCH_2CH_2CH_2$), 8.15 (t, 1H, *J* = 4.52 Hz, $NHCH_2CH_2CH_2$), 6.38

(s, 4H, Dmt Ar-H), 5.98–5.85 (m, 2H, CH_2=C*H*–CH_2–), 5.50–5.32 (m, 4H, CH_2=CH–CH_2–), 3.80–3.30 (m, 6H, Dmt αH, CH_2=CH–CH_2–), 3.13–2.85 (m, 8H, Dmt βH, NHC$H_2$$CH_2$$CH_2$), 2.47–2.42 (m, 2H, NHC$H_2$ CH_2CH_2), 2.30–2.05 (m, 17H, Dmt CH_3, Pyrazinone CH_3, NHC$H_2$$CH_2CH_2$), 1.70–1.38 (m, 4H, NHC$H_2CH_2$$CH_2$). ^{13}C NMR δ: 166.81, 166.70, 155.57, 155.52, 155.40, 138.20 (6q), 128.54, 128.45 (2t, CH_2=*C*H–CH_2–), 122.78, 122.68 (2s, *C*H_2=CH–CH_2–), 121.69, 121.64 (2q), 114.82 (t, Dmt Ar-C), 57.90, 57.86 (2t, Dmt αC), 47.28 (s, CH_2=CH–*C*H_2–), 38.59, 38.20 (2s, NH*C*$H_2$$CH_2$$CH_2$), 29.27, 28.76 (2s, NHC$H_2$ CH_2*C*H_2), 27.17 (s, Dmt βC), 25.42 (s, NHCH_2 *C*$H_2$$CH_2$), 19.90, 19.87 (2p, Dmt *C*H_3), 17.97 (p, pyrazinone *C*H_3).

5.5. Competitive receptor binding

Rat brain P_2 synaptosomal preparations were used in competitive displacement assays for μ- and δ-opioid receptors after removal of endogenous opioids as published elsewhere in considerable detail,[11,17–19] and using 3.5 nM [^{3}H]DAMGO [(D-Ala^2,*N*-Me-Phe^4,Gly-ol^5)enkephalin] (50.0 Ci/mmol, Amersham Biosciences, Buckinghamshire, UK) and 1.9 nM [^{3}H]deltorphin II (45.0 Ci/mmol, Perkin-Elmer, Boston, MA) to radiolabel μ- and δ-opioid receptors, respectively. After a 2.5-h incubation at room temperature (22–23 °C), samples were rapidly rinsed on Whatman GF/C glass fiber filters, presoaked in 0.1% polyethyleneimine to enhance the response : background ratio, washed with ice-cold buffer, dried, and the radioactivity determined using EcoLume scintillation fluid.[17–19]

5.6. Functional opioid bioassays

Myenteric plexus longitudinal muscle from the small intestine of male Hartley strain guinea pigs (GPI) measured μ-opioid agonism and a single mouse vas deferens (MVD) determined δ-opioid agonism. The isolated tissues were suspended in organ baths containing balanced salt solutions in a physiological buffer, pH 7.5.[17–19] Agonists inhibited electrically evoked contractions; the IC_{50} (nM) was obtained from dose–response curves (mean ± SE five to six separate tissue preparations). Antagonism was determined against the δ-opioid agonist deltorphin II or μ-opioid agonist endomorphin-2 and expressed as the pA_2 (Table 1), which was determined using the Schild Plot.[31] The Schild slopes of all antagonists were 0.917–1.189 in the GPI and 0.883–1.088 in the MVD assays.

5.7. Inhibition of morphine antinociception

Male mice were used according to protocols approved by the Animal Care and Use Committee at the NIEHS. Intracerebroventricular (icv) administration was performed as described, and antinociception measured using a hot-plate (55.0 ± 0.1 °C) and by tail-flick tests.[18,19] Five minutes after subcutaneous administration of morphine (4 mg/kg), **12** or naloxone was injected icv. As a control to determine the effect of the compound on mice, only **12** was injected icv. The hot-plate latency is the interval between placement of mice on the hot plate and movement consisting of either licking or shaking their hind paws with a baseline latency of 15 s and maximal cut off time of 30 s. Tail-flick latency is the latency for removal of the tail from the onset of radiant heat with a baseline latency of 2–3 s, and with a cut-off time of 8 s. The duration time after icv administration was 10 min and the test was terminated when hot-plate and tail-flick latencies were close to the pre-response times. Statistical significance applied one-way analysis of variance (ANOVA) followed by Dunnett's test (significant at $P < 0.05$). The area under the time–response curve (AUC) combines the response time and time after administration.[18,19] The percent of maximal possible effect (%MPE) = $(T_1 - T_0)/(T_2 - T_0) \times 100$, where the T_0 and T_2 are pre-response and cut off times, respectively. AD_{50} presents the dose of **12** or naloxone to inhibit 50% of the morphine-induced antinociception at 10 min post-injection.

5.8. Brain slice preparation and whole cell recording

Vibratome-prepared hippocampal slices (300 μm) of rat brains were incubated in a holding chamber containing artificial cerebrospinal fluid (ACSF) bubbled with O_2/CO_2 (95:5%) at room temperature for 60 min. Spontaneous $GABA_A$ receptor-mediated IPSCs were pharmacologically isolated by adding 50 μM D-(−)-2-amino-5-phosphonovaleric acid and 20 μM 6,7-dinitroquinoxaline-2,3-dione.[28] Patch-clamp pipettes were prepared as detailed previously.[28] Individual CA1 pyramidal cells were visualized using an infrared differential interference contrast Zeiss Axioskop microscope (40-X water immersion objective) and spontaneous IPSCs were recorded at −70 mV for 10 min periods consistently of baseline conditions, plus 60 mM EtOH, and with 300 nM **15** in EtOH. Series resistance was monitored throughout the recording and cells were discarded if they changed significantly, and the data were analyzed using paired Student's *t*-tests. More comprehensive controls, dose–response curves, and analyses were conducted using **12** with comparable data (Li; Swartzwelder, et al. unpublished data).

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