

## Brief Articles

### Synthesis of Stable and Potent $\delta/\mu$ Opioid Peptides: Analogues of H-Tyr-*c*[D-Cys-Gly-Phe-D-Cys]-OH by Ring-Closing Metathesis

Adriano Mollica,<sup>†</sup> Giovanni Guardiani,<sup>‡</sup> Peg Davis,<sup>§</sup> Shou-Wu Ma,<sup>§</sup> Frank Porreca,<sup>§</sup> Josephine Lai,<sup>§</sup> Luisa Mannina,<sup>||,⊥</sup> Anatoli P. Sobolev,<sup>⊥</sup> and Victor J. Hruby\*<sup>#,§</sup>

Università degli Studi "G. D'Annunzio", Via dei Vestini 31, I-66013 Chieti, Italy, Dipartimento di Studi Farmaceutici, Università degli Studi di Roma "La Sapienza", P.le A. Moro 5, I-00185, Rome, Italy, Departments of Pharmacology and Chemistry, University of Arizona, Tucson, Arizona 85721, Dipartimento di S.T.A.A.M, Università degli Studi del Molise, Via De Sanctis, I-86100, Campobasso, Italy, and Istituto di Metodologie Chimiche, CNR, Area della Ricerca di Roma, I-00016, Monterotondo Stazione, Rome, Italy

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Ring-closing metathesis has emerged as a powerful tool in organic synthesis for generating cyclic structures via C–C double bond formation. Recently, it has been successfully used in peptide chemistry for obtaining cyclic molecules bridged through an olefin unit in place of the usual disulfide bond. Here, we describe this approach for obtaining cyclic olefin bridged analogues of H-Tyr-*c*[D-Cys-Gly-Phe-Cys]-OH. The synthesis of the new ligands was performed using the second generation Grubbs' catalyst. The resulting *cis*-**8** (*c*DADAE) and *trans*-**9** (*t*DADAE) were fully characterized and tested at  $\delta$ ,  $\mu$ , and  $\kappa$  opioid receptors. Also the linear precursor **13** (*D*DADAE) and the hydrogenated derivative **11** (*r*DADAE) also were tested. All the cyclic products containing a olefinic bond are slightly selective but highly active and potent for the  $\delta$  and  $\mu$  opioid receptors. Activity toward the  $\kappa$  opioid receptors was absent or very low.

#### Introduction

Cysteine-based disulfide bridges are a common feature in proteins and natural peptides where they play essential roles in a variety of structural and biochemical functions. Among the different functions, the limitation of the conformational flexibility with stabilization of secondary structures and the consequent lowering of the unfavorable entropy loss upon binding is extremely important.<sup>1,2</sup> Disulfide-bridge cyclization is also one of the most common tools in the design of synthetic models starting from their linear counterparts.<sup>3</sup> The 14-membered cyclic enkephalin analogues H-Tyr-*c*[D-Cys-Gly-Phe-D-Cys]-OH<sup>4</sup> and the related [D-Pen<sup>2</sup>, D-Pen<sup>5</sup>] model (DPDPE)<sup>5</sup> are the most relevant cyclic opioid peptides. The side-chain thiol groups at the 2- and 5-position residues of these molecules are oxidized to form a disulfide bridge that imposes a global constraint to the structure. In particular, the cystine bridge

containing analogue shows an exceptionally good activity on  $\delta$  and  $\mu$  receptors, whereas due to the presence of the *gem*-dimethyl groups of the penicillamine residues, DPDPE is highly selective toward  $\delta$  receptors and is used in the opioid binding assays as the radio-labeled  $\delta$  receptor full agonist.<sup>5</sup> Furthermore, while the cystine analogues possessing a C-terminal carboxamide function are essentially nonselective, the corresponding free acids show a certain preference for  $\delta$  over  $\mu$  receptors.<sup>4a</sup>

Although widely used with success, the disulfide-based cyclization approach still maintains some limitations. Due to its redox properties, the S–S bond is exposed to the enzymatic attack by reductases and can be cleaved by sulfhydryl groups or other soft nucleophiles. Moreover, the geometry of the bond is limited by the stereoelectronic effects exerted by the sulfur atoms' lone pairs, which fix the dihedral angle C–S–S–C at about  $\pm 90^\circ$ .<sup>6</sup>

Because the stabilization of well-ordered secondary structures is a major issue in peptide chemistry, several cyclization strategies, not based on the disulfide bridge formation, have been applied to obtain more stable and versatile products.<sup>7</sup> Very attractive in this context appears the replacement of the critical S–S junction with a noncleavable C–C bond. An early application performed on a bioactive peptide, reported by Keller and Rudinger<sup>8</sup> concerns the synthesis of the dicarba-analogue of oxytocin: it is based on the incorporation of a 1,6- $\alpha,\alpha'$ -diamino suberic acid residue in place of the native cystine fragment. More recently, Stymiest et al.<sup>9</sup> reported that the replacement of cysteines with allylglycine residues, followed by ruthenium-catalyzed ring-closing olefin metathesis (RCM), gives an easy access to *cis* and *trans* olefinic analogs of oxytocin as well as to the previously reported<sup>8</sup> corresponding bis-methylene derivative.

RCM is an emerging powerful tool in the peptide chemistry for generating cyclic structures via C–C double bond formation.

\* To whom correspondence should be addressed. Tel.: +1 520 621 6332. Fax: +1 520 621 8407. E-mail: hruby@u.arizona.edu.

<sup>†</sup> Università degli Studi "G. D'Annunzio".

<sup>‡</sup> Università degli Studi di Roma "La Sapienza".

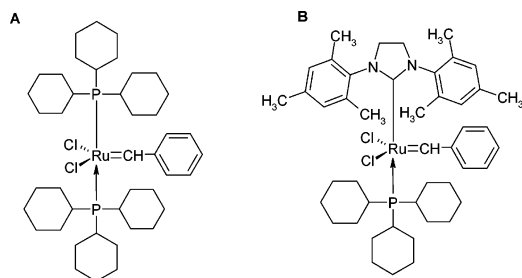
<sup>§</sup> Department of Pharmacology, University of Arizona.

<sup>||</sup> Università degli Studi del Molise.

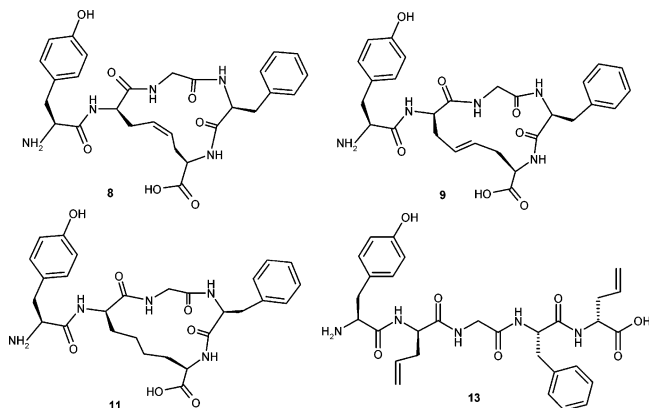
<sup>⊥</sup> Istituto di Metodologie Chimiche, CNR.

<sup>#</sup> Department of Chemistry, University of Arizona.

<sup>a</sup> Abbreviations: D-Allylgly, D-Allylglycine; *c*DADAE, *cis*-*c*[2-D-allylglycine, 5-D-allylglycine]enkephalin; <sup>3</sup>H-DAMGO, [<sup>3</sup>H]-[D-Ala(2),N-Me-Phe(4),Gly-ol(5)]enkephalin; <sup>3</sup>H-DPDPE, [<sup>3</sup>H]-*c*[2-D-penicillamine,5-D-penicillamine]enkephalin; <sup>3</sup>H-U69593, [<sup>3</sup>H]-(+)-5 $\alpha$ ,7 $\alpha$ ,8 $\beta$ )-N-methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro[4,5]dec-8-yl]-benzeneacetamide; DCM, dichloromethane; DMF, *N,N*-dimethyl formamide; DMSO, dimethylsulfoxide; EDC, 1-ethyl-(3-dimethylaminopropyl)carbodiimide; GPI/LMMP, guinea pig ileum/longitudinal muscle myenteric plexus ( $\mu$  opioid receptors); GTP, guanosine triphosphate; hMOR, human  $\mu$  opioid receptor; HOBT, 1-hydroxybenzotriazole; KOR,  $\kappa$  opioid receptor; *D*DADAE, [2-D-allylglycine, 5-D-allylglycine]enkephalin; MVD, mouse vas deferens ( $\delta$  opioid receptors); *r*DADAE, reduced bond-*c*[2-D-allylglycine, 5-D-allylglycine]enkephalin; *r*DOR, rat  $\delta$  opioid receptor; *t*DADAE, *trans*-*c*[2-D-allylglycine, 5-D-allylglycine]enkephalin; TEA, triethylamine; TFA, trifluoroacetic acid.



**Figure 1.** First (A) and second (B) generation Grubbs' catalysts.



**Figure 2.** Structures of *c*DADAE (**8**), *t*DADAE (**9**), *r*DADAE (**11**), and *t*DADAE (**13**).

The reaction is catalyzed by ruthenium complexes, first- or second-generation Grubbs catalysts (Figure 1), and can directly be used for the synthesis of a variety of peptide diene systems.<sup>10</sup> Thus, by adopting the RCM methodology, a "third" generation of cyclic opioid analogues, characterized by metabolic stability and rigid conformation, becomes available. However, although many studies are dedicated to the application of the RCM methodology in peptide chemistry<sup>11</sup> and the perspective of a fine control of the conformations, based on the incorporation of *cis* or *trans* olefin junctions into the cyclic backbone is extremely important and promising, only the synthesis and properties of carboxamides of the dicarba-analogues of the cyclic 14-membered disulfide enkephalin analogues<sup>12</sup> are reported in a symposium record by Schiller et al.

On the basis of the above observations, here we report the synthetic protocols and *in vitro* biological tests of cystine-containing enkephalin dicarba analogues **8**, **9**, **11**, and **13** (Figure 2). All these derivatives possess a C-terminal free carboxyl group: this feature, in analogy with the behavior exhibited by the corresponding disulfide analogues, should improve the  $\delta/\mu$  selectivity as compared with the previously reported C-terminal carboxamide derivatives.<sup>12</sup>

## Results and Discussion

The ability of the synthesized enkephalin analogues to inhibit electrically evoked contractions of the myenteric plexus of the guinea pig ileum (GPI) and the mouse vas deferens (MVD) proves to be very high: IC<sub>50</sub> values for **8**, **9**, **11**, and **13** are 0.59, 0.76, 3.4, and 5.7 nM in the predominant DOR containing MVD and 5.3, 5.2, 59, and 34 nM in the MOR-rich GPI (Table 1). Tests performed by using nor-binaltorphimine (nor-BNI), an antagonist of  $\kappa$  Kappa opioid receptors (10nM nor-BNI), show little effect on  $\kappa$  receptor activity (data not reported).

To test the affinity of **8**, **9**, **11**, and **13** for opioid receptors, binding tests with radiolabeled standards (<sup>3</sup>H-DPDPE for hDOR, <sup>3</sup>H-DAMGO for MOR, and <sup>3</sup>H-U69593 for KOR) were performed<sup>19</sup> (see Table 1). Literature data for DPDPE and H-Tyr-*c*[D-Cys-Gly-Phe-D-Cys]-OH are also reported for comparison.

The results show that compounds **8**, **9**, **11**, and **13** have a different binding affinity for  $\delta$ ,  $\mu$ , and  $\kappa$  receptors: *K<sub>i</sub>* values for **8** and **9** are 1.35 and 1.30 nM, respectively, for the MOR and 0.43 and 0.57 nM for the DOR; *K<sub>i</sub>* values for **11** and **13** are 56.9 and 64.5 nM, respectively, for the MOR and 7.9 and 8.1 nM, respectively, for the DOR. All compounds show very small affinity for KOR (Table 1). Therefore, the binding affinity of **8** and **9** for the  $\mu$  receptor is higher than that of **11** and **13** and much higher than that of DPDPE, which is similar to H-Tyr-*c*[D-Cys-Gly-Phe-D-Cys]-X (X = NH<sub>2</sub>).

The above-reported *in vitro* bioassays show that both **8** and **9** are highly potent agonists at  $\mu$  and  $\delta$  receptors, but only slightly selective ( $\delta/\mu$  ratio is in the range of about 7–9): they are 10 times more potent than DPDPE for the  $\delta$  receptors and more than 1000 times more potent for  $\mu$  receptors. These results suggest that the novel H-Tyr-*c*[D-Cys-Gly-Phe-D-Cys]-OH analogues show high potency but a low selectivity as previously found for the related peptides family of H-Tyr-*c*[D-Cys-Gly-Phe-D-Cys]-X (X = OH or NH<sub>2</sub>). However, the two analogues **8** and **9**, characterized by a different geometrical isomerism, do not show any significant difference in their biological profile.

It is well-established that the spatial orientation of the Tyr<sup>1</sup> and Phe<sup>4</sup> aromatic moieties<sup>19</sup> is a key conformational feature for the selectivity of the ligands of opioid receptors. The results reported here suggest that the olefin bridge, present in the H-Tyr-*c*[D-Cys-Gly-Phe-D-Cys]-OH dicarba analogues, do not significantly affect the orientation of the aromatic moieties when compared to the disulfide bond. The lack of selectivity obtained clearly confirms that the selectivity of DPDPE is principally attributable to the *gem*-dimethyl moiety of the penicillamine residues, which generates an enhancement of the affinity for the  $\delta$  receptor. It is important to notice that the different configuration of the double bond in the two new ligands does not influence their biological profile, but the increased structural

**Table 1.** Binding Affinity and *In Vitro* Activity for Compounds **8**, **9**, **11**, and **13** and Reference Compounds

cmpds	bioassay IC <sub>50</sub> <sup>a</sup> (nM)		binding <i>K<sub>i</sub></i> <sup>a,b</sup> (nM)		
	MVD	GPI/LMMP	hDOR	hMOR	KOR
<i>c</i> DADAE ( <b>8</b> )	0.59 ± 0.19	5.3 ± 0.21	0.43 ± 0.16	1.35 ± 0.45	576 ± 23
<i>t</i> DADAE ( <b>9</b> )	0.76 ± 0.070	5.2 ± 0.61	0.57 ± 0.21	1.30 ± 1.2	74.8 ± 5.1
<i>r</i> DADAE ( <b>11</b> )	3.4 ± 0.60	59 ± 24	7.9 ± 0.61	56.9 ± 3.5	3960 ± 210
<i>t</i> DADAE ( <b>13</b> )	5.7 ± 1.1	34 ± 11	8.1 ± 0.22	64.5 ± 4.1	3870 ± 185
H-Tyr- <i>c</i> [D-Cys-Gly-Phe-D-Cys]-X <sup>c</sup>	0.12	1.48	0.822	0.550	44.9
DPDPE <sup>d</sup>	4.1 ± 0.46	7300 ± 1700	1.6 ± 0.2	609 ± 70	

<sup>a</sup> Displacement of [<sup>3</sup>H]DAMGO ( $\mu$ -selective) and [<sup>3</sup>H]DPDPE ( $\delta$ -selective) from rat brain membrane binding sites; displacement of [<sup>3</sup>H]U69593 ( $\kappa$ -selective) from guinea pig brain membrane binding sites. <sup>b</sup> ±S.E.M. <sup>c</sup> MVD and GPI data refers to H-Tyr-*c*[D-Cys-Gly-Phe-D-Cys]-X<sup>c</sup> (X = OH) according to ref 4a and hDor, hMor, and Kor data refers to H-Tyr-*c*[D-Cys-Gly-Phe-D-Cys]-X<sup>c</sup> (X = NH<sub>2</sub>) according to ref 12a. <sup>d</sup> Data according to ref 19b.

**Table 2.** GTP Binding Assay

	[ <sup>35</sup> S]GTPγS binding <sup>a</sup>			
	EC <sub>50</sub> (nM) hDOR	E <sub>max</sub> (%) <sup>b</sup>	EC <sub>50</sub> (nM) hMOR	E <sub>max</sub> (%) <sup>b</sup>
cDADAE ( <b>8</b> )	0.83 ± 0.05	121	2.35 ± 0.20	65
tDADAE ( <b>9</b> )	0.88 ± 0.28	128	2.65 ± 0.45	70

<sup>a</sup> Reference compound: [<sup>35</sup>S]GTP-γ-S. <sup>b</sup> Net total bound/basal binding × 100 ± SEM for compounds **8** and **9**.

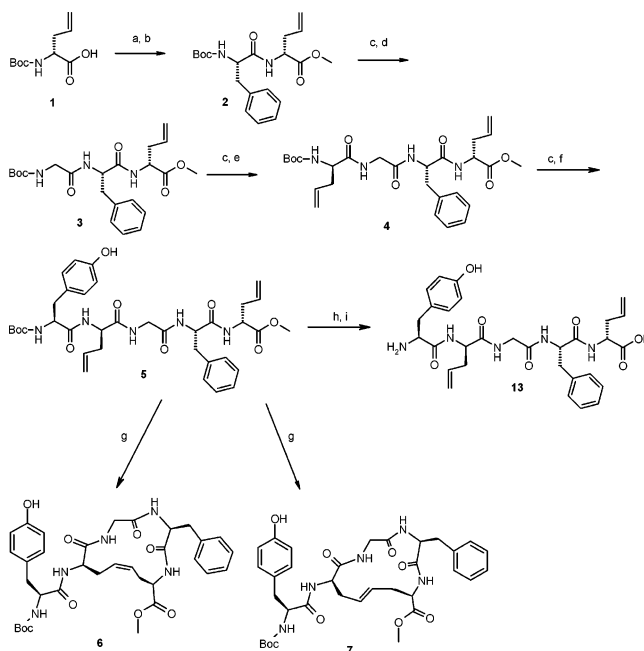
flexibility of the ring scaffold obtained by the reduction of the double bond of compounds **8** and **9** leads to a decreased binding affinity and biological activity for both the DOR and the MOR. Therefore, the higher activity of cyclic products **8** and **9** compared to that of linear compound **13** can be attributed to their rather rigid conformations.

A final consideration concerns the results of the [<sup>35</sup>S]GTP-γ-S binding. EC<sub>50</sub> values exhibited by **8** and **9** for GPTγ[S<sup>35</sup>] binding to δ and μ receptor. The results (Table 2) show that the ligands have a high ability to activate the transduction of the μ and δ receptors. Also the E<sub>max</sub>% value expresses the high efficacy of all the tested compounds.

In conclusion, the reported modification of the disulfide bridge in H-Tyr-c[D-Cys-Gly-Phe-D-Cys]-X (X = OH or NH<sub>2</sub>) leads to analogue products that show a significant improvement of both chemical and biological stability with respect to their disulfide analogues. This property is accompanied by a slightly enhanced selectivity toward the δ opioid receptor and by the same efficacy and potency of D-cysteine-containing peptides. The choice to synthesize derivatives with a C-terminal free carboxylic function, instead of the C-terminal amide groups aimed at verifying if this feature could improve the δ versus μ selectivity, as observed in the case of the disulfide counterparts. The present results show that the δ/μ ratio is not significantly altered by the carboxy terminus: this observed lack of selectivity may be useful in clinical trials to evaluate the effects of the synergistic stimulation of both δ and μ opioid receptors.<sup>20</sup> Therefore, the presented results may be considered a further step in understanding the opioid system and the design of more stable, potent, and efficacious drugs.

## Experimental Section

**General Information.** All solvents, reagents, and starting materials were obtained from commercial sources unless otherwise indicated. All reactions were performed under N<sub>2</sub> unless otherwise noted. Intermediate products **2**, **3**, **4**, and **5** were purified by silica gel chromatography. The fully protected products **6**, **7**, and **10** were purified by RP-HPLC using a semipreparative Vydac (C<sub>18</sub>-bonded, 300 Å) column and an isocratic elution at a flow rate of 10 mL/min monitored at 254 and 270 nm. The mobile phase used was 30% acetonitrile in 0.1% aqueous TFA over 40 min. Approximately 20 mg of crude peptide was injected each time, and the fractions containing the purified peptide were collected and lyophilized to dryness. In the case of products **6** and **7**, this method was unable to separate the two geometric isomers that were collected, and their mixture was purified by HPLC. Products **8**, **9**, **11**, and **13** used for the biological assay were further purified by RP-HPLC using a semipreparative Vydac (C<sub>18</sub>-bonded, 300 Å) column and a gradient elution at a flow rate of 10 mL/min. The gradient used was 10–90% acetonitrile in 0.1% aqueous TFA over 40 min. Approximately 10 mg of crude peptide was injected each time, and the fractions containing the purified peptide were collected and lyophilized to dryness. The purity of the final products, determined by NMR analysis and by analytical RP-HPLC (C<sub>18</sub>-bonded 4.6 × 150 mm) at a flow rate of 1 mL/min on a Waters Binary pump 1525 using an isocratic elution of 20% CH<sub>3</sub>CN/H<sub>2</sub>O 0.1% TFA, monitored with a Waters 2996 Photodiode Array Detector, was found to be >95%.

**Scheme 1.** Synthesis of Fully Protected H-Tyr-c[D-Cys-Gly-Phe-D-Cys]-OH Analogues **5**, **6**, and **7** and Linear Deprotected Product **13**<sup>a</sup>

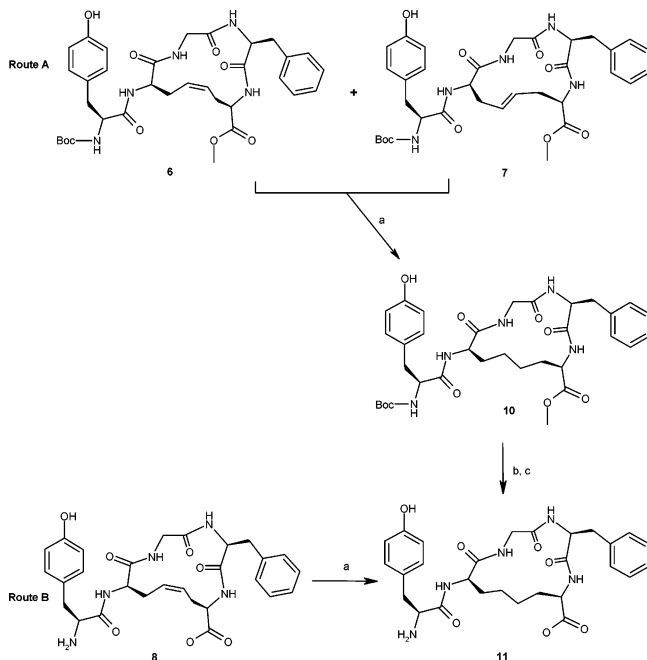
<sup>a</sup> Reagents and conditions: (a) SOCl<sub>2</sub>/MeOH 10 min at 0 °C, then 6 h at rt; (b) Boc-Phe-OH/EDC/HOBT·H<sub>2</sub>O/DMF/NMM, 16 h at rt; (c) TFA/CH<sub>2</sub>Cl<sub>2</sub> (1:2) 1 h, rt; (d) Boc-Gly-OH/EDC/HOBT·H<sub>2</sub>O/NMM/DMF, 16 h at rt; (e) Boc-D-Allyl-Gly-OH/EDC/HOBT·H<sub>2</sub>O/NMM/DMF, 16 h at rt; (f) Boc-Tyr-OH/EDC/HOBT·H<sub>2</sub>O/NMM/DMF, 16 h at rt; (g) Grubbs' catalyst second generation (20%)/CH<sub>2</sub>Cl<sub>2</sub> at rt; (h) TFA/CH<sub>2</sub>Cl<sub>2</sub> (1:2) 1 h at rt; (i) NaOH 1 N 6 equiv/MeOH, 4 h at rt.

Peptide structures were determined by NMR spectroscopy and confirmed by high resolution-mass spectra (HR-MS). For the final products, **8** (cDADAE), **9** (tDADAE), **11** (rDADAE), and **13** (lDADAE), elemental analyses (within ±0.4% of the theoretical values) were performed (for detailed experimental and analytical data, refer to the Supporting Information).

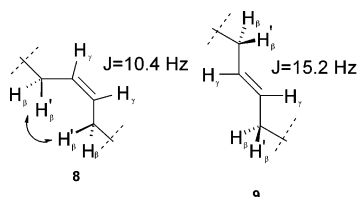
**Chemistry.** The linear intermediate pentapeptide **5** (lDADAE; Scheme 1) was synthesized in the solution phase by using the N<sup>α</sup>-Boc strategy. Amino acid couplings were performed by using either EDC or BOP-Cl/HOBT·H<sub>2</sub>O/NMM in DMF,<sup>13</sup> both resulting in high yields. N-Terminus N<sup>α</sup>-Boc deprotection of compound **1** was accomplished with SOCl<sub>2</sub>/MeOH, thus realizing contemporary C-terminal esterification; in all the other cases, Boc cleavage was performed by using excess of TFA/CH<sub>2</sub>Cl<sub>2</sub> (1:2) at rt for 1 h. All the intermediate products, unless otherwise specified, were purified by RP-HPLC and then characterized by Fab<sup>+</sup> MS and NMR spectroscopy. All new final compounds gave correct elemental analysis. For the RCM reaction, the second generation of Grubbs' catalyst (Figure 1B), which is rated to be more stable and reactive than the first generation (Figure 1A), was used.<sup>14</sup> The ring closure was accomplished by adding a catalytic amount of the catalyst to a 10 mM solution of **5** in CHCl<sub>3</sub> at rt until the reaction was complete. The cyclization progress was monitored by TLC (EtOAc). The two isomeric peptides *cis*-**8** (cDADAE) and *trans*-**9** (tDADAE; Figure 2) were obtained in about 1:3 ratio, respectively (Scheme 1). While the two products were clearly distinguishable by TLC (EtOAc), they showed very close RP-HPLC retention times in the condition of the analysis. The *cis* and *trans* isomers were then separated by PLC, charging a maximum of 10 mg on each 20 × 20 cm/0.25 mm silica gel plate.

To synthesize the analogue **11** (rDADAE), containing a –CH<sub>2</sub>–CH<sub>2</sub>– junction, a portion of the RP-HPLC purified mixture, containing both the geometrical isomers **6** and **7** (1:3 ratio), was catalytically hydrogenated at atmospheric pressure in MeOH (Scheme 2; route A). The reaction was monitored every 6 h by TLC. After about 48 h, all the starting material disappeared, giving

**Scheme 2.** Synthesis of the Bismethylene Analogue **11**: Route A, Catalytic Hydrogenation of (**6** + **7**) Mixture; Route B, Catalytic Hydrogenation of *cis*-**8** (cDADAe)<sup>a</sup>



<sup>a</sup> Reagents and conditions: (a) H<sub>2</sub>/Pt/C 10%/MeOH, 48 h, rt; (b) NaOH (1 N, 3 equiv), 6 h, rt; (c) TFA/CH<sub>2</sub>Cl<sub>2</sub> 1:2, 1 h, rt.



**Figure 3.** Double bond geometry in **8** (*cis* conformer) and **9** (*trans* conformer).

rise to a single spot. The catalyst was filtered and the solvent was evaporated under vacuum. After purification by silica gel chromatography, the fully protected methyl ester **10** was obtained in almost quantitative yield. Hydrolysis followed by Boc deprotection under standard conditions and purification by RP-HPLC gave the desired free acid **11**. This compound was also obtained in one step starting from the *cis* analogue **8** (Scheme 2; route B) by applying the same catalytic hydrogenation conditions described before.

The configuration of the double bond of compounds **8** and **9** was accurately established by the NMR analysis.<sup>15</sup> The value of the  $J$  coupling constants between  $\gamma$ -protons of D-All-Gly<sup>2</sup> and d-All-Gly<sup>5</sup> (10.4 Hz for **8** and 15.2 Hz for **9**) corresponds to a *cis* and a *trans* configuration of the double bond in **8** and **9**, respectively. This assignment is confirmed by the presence of a correlation peak between H- $\beta$  of D-All-Gly<sup>5</sup> and H- $\beta$  of D-All-Gly<sup>2</sup> in the ROESY map of **8**. In the case of **9**,  $\beta$ -protons of D-All-Gly<sup>5</sup> and D-All-Gly<sup>2</sup> do not give any observable NOE correlation, confirming the *trans* configuration of the double bond (Figure 3).

**Biological Testing.** In vitro biological assays were performed on **8**, **9**, **11**, and **13** as TFA salts.

**GPI and MVD In Vitro Bioassays.** The in vitro tissue bioassays were performed as described previously.<sup>16</sup> Electrically induced smooth muscle contractions of mouse vas deferens and guinea pig ileum longitudinal muscle-myenteric plexus were used as bioassays. Tissue came from male ICR mice weighing 25–30 g and from male Hartley guinea pigs weighing 150–400 g. The tissues were tied to gold chains with suture silk, suspended in 20 mL baths containing 37 °C oxygenated (95% O<sub>2</sub>, 5% CO<sub>2</sub>) Krebs bicarbonate solution (magnesium-free for MVD), and allowed to equilibrate

for 15 min. The tissues were then stretched to optimal length previously determined to be 1 g tension (0.5 g for MVD) and allowed to equilibrate for 15 min. The tissues were stimulated transmurally between platinum plate electrodes at 0.1 Hz for 0.4 ms pulses (2.0 ms pulses for MVD) and supramaximal voltage. Grugs were added to the baths in 20–60  $\mu$ L volumes. The agonists remained in contact with the tissue for 3 min and the baths were then rinsed several times with fresh Krebs solution. Tissues were given 8 min to re-equilibrate and regain predrug contraction height. IC<sub>50</sub> values represent the mean of not less than four tissues. IC<sub>50</sub> estimates and relative potency estimates were determined by fitting the mean data to the Hill equation by using a computerized nonlinear least-squares method. All biological data are summarized in Table 1.

**Radioligand-Labeled Binding Assays.** Receptor binding affinities to the  $\delta$ ,  $\mu$ , and  $\kappa$  opioid receptors were performed using cell membrane preparations from transfected cells that stably express the respective receptor type and were evaluated as previously described.<sup>17</sup> The ligands used were [<sup>3</sup>H]DPDPE, [<sup>3</sup>H]DAMGO, and [<sup>3</sup>H]U69593 for  $\delta$ ,  $\mu$ , and  $\kappa$  opioid receptors, respectively.

**GTP Binding and E<sub>max</sub>% (Agonist Stimulated [<sup>35</sup>S]GTP $\gamma$ S Binding).**<sup>18</sup> We used [<sup>35</sup>S]GTP- $\gamma$ -S binding to examine opioid agonist efficacy for functional characterization of the ligands at the  $\delta$  and  $\mu$  opioid receptors, which are members of the seven transmembrane G-protein-coupled receptor super family. Agonist efficacy can be determined at the level of receptor G-protein interaction by measuring agonist-stimulated binding with a non-hydrolyzable GTP analogue. The ability of  $\mu$  and  $\delta$  opioid agonists to activate G-proteins has been demonstrated by studying the binding of the GTP analogue guanosine-5'-O-(3-[<sup>35</sup>S]thio)triphosphate ([<sup>35</sup>S]GTP- $\gamma$ -S). The opioid receptor mediated assay was performed as previously described.<sup>19</sup> Cells expressing hDOR for  $\delta$  receptor (or rMOR for  $\mu$  receptor) were incubated with increasing concentrations of the test compounds in the presence of 0.1 nM [<sup>35</sup>S]GTP- $\gamma$ -S (1000–1500 Ci/mmol, MEN, Boston, MA) in assay buffer (total volume of 1 mL, duplicate samples) as a measure of agonist-mediated G-protein activation. After incubation (90 min, 30 °C), the reaction was terminated by rapid filtration under vacuum through Whatman GF/B glass fiber filters, followed by four washes with ice-cold 15 mM Tris/120 mM NaCl, pH 7.4. Filters were pretreated with assay buffer prior to filtration to reduce nonspecific binding. Bound reactivity was measured by liquid scintillation spectrophotometry after an overnight extraction with EcoLite (ICN, Biomedicals, Costa Mesa, CA) scintillation cocktail. The data was analyzed using GraphPad Prism Software (San Diego, CA).

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**Supporting Information Available:** Detailed experimental procedures and representative scheme of the synthesis of compounds **1–13**, elemental analyses for products **6–11** and **13**, HPLC and HR-MS for products **5–11** and **13**, NMR analyses of products **1–13**, and Roesy spectra of **8** and **9**. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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