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Conformationally Restricted TRH Analogues: Constraining the Pyroglutamate Region

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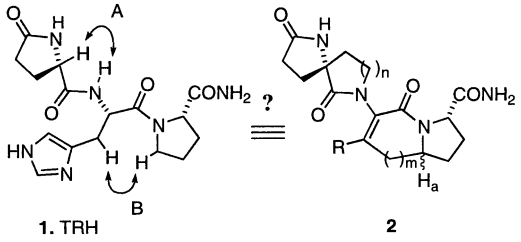
Abstract—A modified synthetic route has been developed so that the steric size of constraints added to the pyroglutamate region of TRH (pGluHisProNH$_2$) can be varied. Both an analogue with a smaller ethylene bridge and a larger, more flexible propane bridge in this region have been synthesized. These analogues were synthesized in order to probe why the initial incorporation of an ethane bridge into this region of the molecule had led to an analogue with a binding constant and potency three times lower than that of an directly analogous unconstrained analogue. The data for both analogues indicated that the fall off in activity caused by the ethane bridge in the initial analogue was not caused by the size of the bridge.

Introduction

Lactam based peptidomimetics can be valuable tools for probing the activity of peptide conformations.[1] These analogues, which exhibit increased hydrolytic stability and preserve both backbone and sidechain functionality, can be designed by simply replacing spatially close hydrogens within a conformation of interest with a carbon bridge. For example, the family of constrained peptidomimetics (**2**) illustrated in Scheme 1 was designed to mimic a proposed endocrine receptor bound conformation (**1**) of thyrotropin-releasing hormone (thyroliberin, TRH).[2–4] However, while lactam based peptidomimetics offer many advantages, their utility is often limited by the difficulty associated with their syntheses. Work to evaluate the effectiveness of TRH analogues like **2** and probe the biological relevance of the conformation represented by **1** stalled when the constrained analogues could not be made.[3b]

In order to address this issue, we began to develop convenient synthetic routes to lactam based peptidomimetics.[5] This work led to the synthesis of a TRH analogue with a constraint added to the pyroglutamate region (bridge A),[6] several analogues with constraints added to the His-Pro region (bridge B),[7] and a pair of analogues with constraints added to both regions.[8] While the initial synthetic routes developed allowed us to start answering questions about the nature of TRH binding to its endocrine receptor (TRH-R$_1$), they were limited in scope. This was especially true of the strategy employed to constrain the pyroglutamate region of the molecule. One one hand, the chemistry utilized to build **6** (Scheme 2) allowed for the synthesis in just six steps from pyroglutamic acid. On the other, it was hampered by both the use of an intermolecular reductive amination reaction that required a large excess of the NH$_2$–



Scheme 1.

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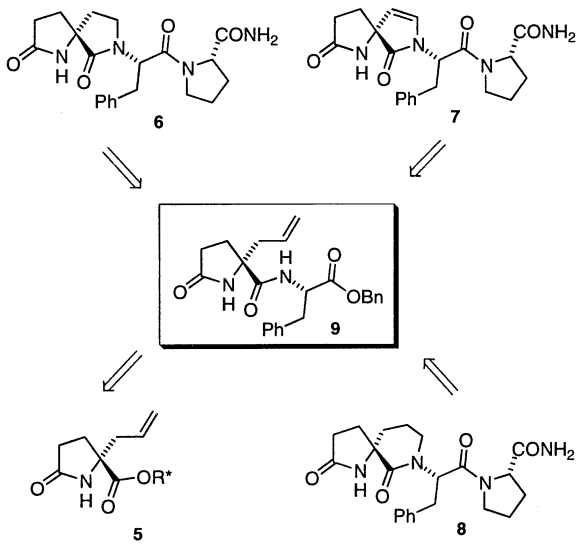
PhePro–NH_2 dipeptide, inconsistent yields and the competitive formation of diketopiperazide products during the last two steps of the synthesis. Because of these problems the synthesis was difficult to scale up, impractical for the synthesis of rings with larger, more flexible constraints, and incompatible with the synthesis of analogues containing a smaller ethylene bridge constraint.

This lack of synthetic flexibility was particularly bothersome because analogue **6** showed both an affinity and a level of potency for TRH-R_1 that was approximately three times lower than the corresponding unconstrained Phe^2-TRH.[6] While **6** was still a full agonist for the receptor, we were curious as to whether the reduction in its activity was due to the bridge in **6** constraining the analogue into a conformation that was not quite correct or whether the reduction in its activity was due to the steric size of the bridge. Adding to this interest was the initial observation that the fully constrained TRH analogue **2a** ($n=1$, $m=1$, Ha = *R*-configuration) might be a partial agonist for TRH-R_1.[8,9] One suggestion for explaining this observation was that analogue **2a** might be too rigid to allow for the conformational changes in the ligand–receptor complex needed for initiating second messenger release. If this were the case, then increasing the flexibility of the bridges might restore potency to the analogue. But would a larger constraint in the spirocyclic portion of **2a** be tolerated by the receptor? It was clear that a more versatile route to building the spirocyclic ring skeleton was needed if questions of this nature were to be answered.

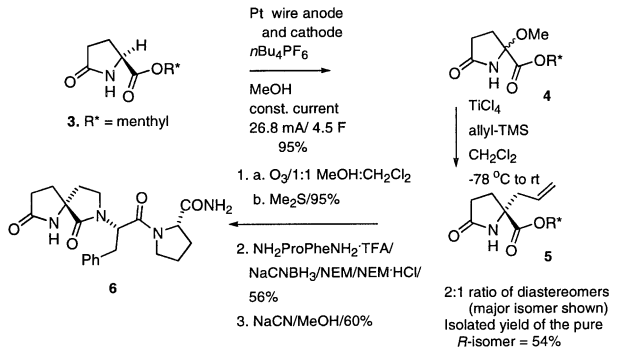
Synthesis

With this in mind, a revised strategy for synthesizing the spirocyclic lactam ring skeletons was proposed (Scheme 3). The key to this plan was the conversion of the 5-allylpyroglutamate derivative (**5**) into an advanced intermediate (**9**) that could be used to construct all of the desired analogues. This would be accomplished by using the olefin of the allyl group in **9** to set up either an intramolecular reductive amination reaction or an intramolecular Mitsunobu reaction. Once the spirocyclic ring was in place, the benzyl ester would be deprotected and the resulting acid coupled to prolinamide. In this way, both the intermolecular reductive amination and the diketopiperzide formation that hindered the original route used for **6** (Scheme 2) would be avoided.

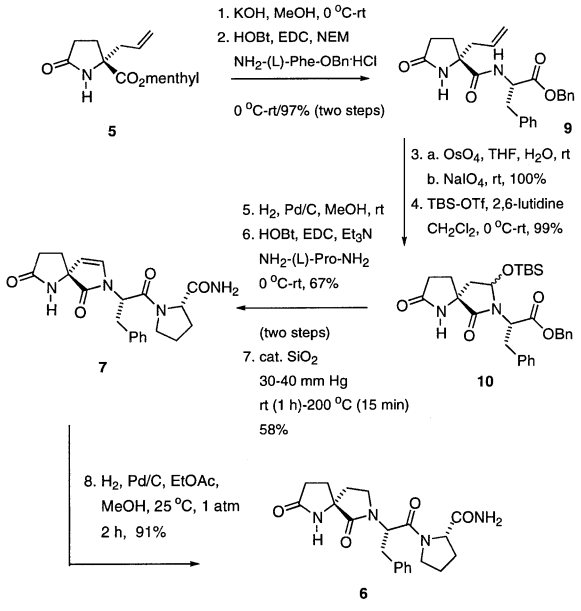
This approach to analogues **6–8** proved very useful. The synthesis of the TRH analogue constrained with an ethylene bridge (**7**) is outlined in Scheme 4. As in the earlier synthesis of **6**, the allyl-substituted pyroglutamate building block **5** was made by first functionalizing menthyl pyroglutamic acid with the use of an anodic amide oxidation reaction (Scheme 2),[6a,10] and then treating the resulting methoxylated product with allylsilane and $TiCl_4$ to afford a 2:1 mixture of stereoisomeris allylpyroglutamate derivatives. A separation by fractional crystallization led to **5** in a 54% isolated yield. Once in hand, the building block was saponified with KOH in methanol to form the carboxylic acid and the acid coupled to phenylalanine benzyl ester using



Scheme 3.



Scheme 2.

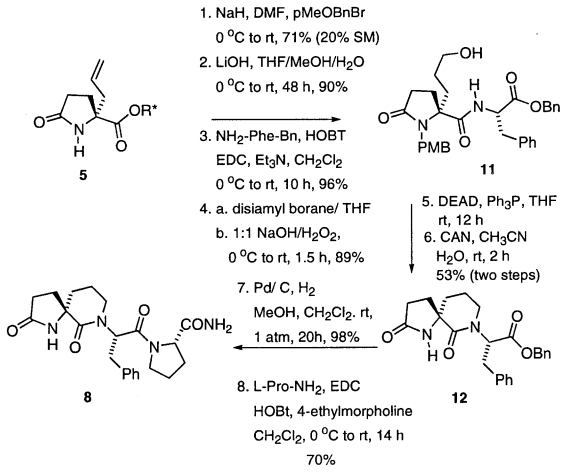


Scheme 4.

HOBt/EDCI conditions in order to form **9**. Oxidation and cleavage of the olefin with OsO_4 and $NaIO_4$ led directly to the cyclized α-hydroxyalkylamide which was then protected as the *t*-butyldimethylsilyl ether (**10**). The benzyl ester was deprotected using hydrogenolysis conditions, the resulting acid coupled to prolinamide, and the synthesis completed by eliminating the *t*-butyldimethylsilyl ether group with the use of reduced pressure and silica gel as a catalyst.[11]

Several items about this synthesis deserve comment. First, the use of the benzyl protecting group for the phenylalanine carboxylic acid was essential. Both acid and base cleavage of alternative ester groups led to competitive hydrolysis of the tertiary amide. Second, protection of the *N*-α-hydroxyamide group was important. Elimination prior to the final coupling steps was problematic because of the incompatibility of the enamide moiety with the reaction conditions required for deprotection and coupling steps. Alternatively, attempts to carry the alcohol through the final steps of the synthesis were complicated by lactone formation. Third, a number of elimination reactions were attempted using both the silyl ether and the corresponding deprotected alcohol. Among the reagents tried were TFA with heat, $POCl_3$, $SOCl_2$, Burgess reagent, and methyltriphenylphosphonium iodide. None of these alternatives was competitive with the silica gel based strategy. Fourth, enamide analogue **7** was a very stable compound. It could be stirred in methanol for 24 h, chromatographed through silica gel, and recrystallized without any decomposition. The purity of the analogue used for biological testing was established by C, H, and N analysis. Finally, the constrained analogue **7** could be readily hydrogenated to form the previously synthesized TRH analogue **6** having an ethane bridge in the pyroglutamate region. In this way, both of the five-membered ring lactam pyroglutamate derivatives could be made using the same synthetic route.

The synthesis of the six-membered ring analogue followed a similar path (Scheme 5). In this synthesis, the pyroglutamate nitrogen was first protected with a *para*-methoxybenzyl ether. This protection step was essential for avoiding competitive intramolecular cyclization reactions involving this nitrogen later in the synthesis. The PMB group was selected after several other groups (Bzl, Cbz, and MOM) proved difficult to remove following formation of the spirocyclic ring skeleton. With the pyroglutamate nitrogen protected, the menthyl ester was hydrolyzed using lithium hydroxide, and the resulting carboxylic acid coupled to the phenylalanine benzyl ester. A hydroboration/oxidation sequence using disiamylborane and hydrogen peroxide was employed to convert the olefin into primary alcohol **11**. The desired spirocyclic ring was then generated with the use of a Mitsunobu reaction. At this point, the PMB group was removed with the use of ceric ammonium nitrate to form **12**. The revoval of the protecting group at this stage of the synthesis was critical. Removal of the PMB group during the final step of the synthesis failed when the final CAN deprotection step led to an over-oxidation of the PMB benzylic carbon prior to the desired collapse of the hemiaminal intermediate. The result was the formation of a PMB ester that could not be cleaved from the product tripeptide. This problem did not interfere with the deprotection reaction leading to **12**, and the synthesis was completed by deprotecting the benzyl ester using a catalytic hydrogenolysis and coupling the resulting acid to prolinamide with the use of HOBt/EDCI conditions. As with the earlier analogues, the purity of the analogue used for testing was established by C, H, and N analysis.



Scheme 5.

Biological Testing and Discussion

Analogues **7** and **8** were examined for both their affinitiy for TRH-R_1 and their ability to serve as agonists for the receptor. Affinities, reported as K_i (μM) in Table 1, were determined by measuring the concentration of the analogue required to completely displace [N$^\tau$Me-His]-TRH from the receptor. [N$^\tau$Me-His]-TRH is an unrestricted TRH analogue in which the imidazole side chain of His is methylated. [N$^\tau$Me-His]-TRH is known to bind to TRH-R_1 with an affinity about 10 times as great as that of the natural hormone. The agonist behavior of the analogues was tested in HEK 293EM cells stably expressing mTRH-R_1 by incubating the cells with various doses of the analogues being examined.[12] The extent of agonist behaviour was then determined by measuring the amount of second messenger (IP_3) released. The data are reported as EC_{50} (μM) values (Table 1).

From the data in Table 1, two things become readily apparent. First, the analogue having the ethylene bridge (**7**) did not bind TRH-R_1 with as high an affinity as did the analogue with the ethane bridge (**6**). The same conclusion was reached about the potency of the analogues. Second, the use of the larger propane bridge (**8**) in place of the ethane bridge (**6**) did not significantly alter either the affinity or the potency of the analogue for TRH-R_1.

Initially, it was not clear why changing the ethane bridge in **6** to the ethylene bridge found in **7** would decrease the potency of the analogue. A direct compar-

Table 1. Biological data

Analogue	K_i (μM)	EC_{50} (μM)
TRH (*p*-GluHisPro-NH_2)	0.0038 (0.0025–0.0055)[a]	0.0013 (0.00099–0.0018)
Phe^2TRH (*p*-GluPhePro-NH_2)	0.45 (0.25–0.75)	0.11 (0.080–0.150)
Analogue **6**[b]	1.6 (0.95–2.7)	0.10 (0.057–0.19)
Analogue **7**	11 (7.1–15)	0.78 (0.55–1.1)
Analogue **8**	1.5 (0.95–2.4)	0.16 (0.10–0.26)

[a]The numbers in parentheses represent the 95% confidence intervals for the data given.
[b]Analogue **6** was re-examined in this study so that the activity of **6** could be directly compared to that of **7** and **8**.

ison of analogues **6** and **7** suggested that the two analogues were very similar. For example, Figure 1 compares the minimized structures of the two analogues.[13] In this drawing, the molecules were aligned by overlaying the key pharmacophoric groups (the pyroglutamate carbonyl, the phenyl ring of the second residue, and the primary amide of the prolinamide) in the two molecules. When this was done, the only difference between the analogues appeared to be a slight twist of the pyroglutamate group relative to the rest of the molecule. The conclusion could be drawn for the overlap between analogues **8** and **6** (Fig. 2). Again, the only difference between these analogues was a slight twist of the pyroglutamate group, although the twist in **8** was in the opposite direction of the one observed for **7**.

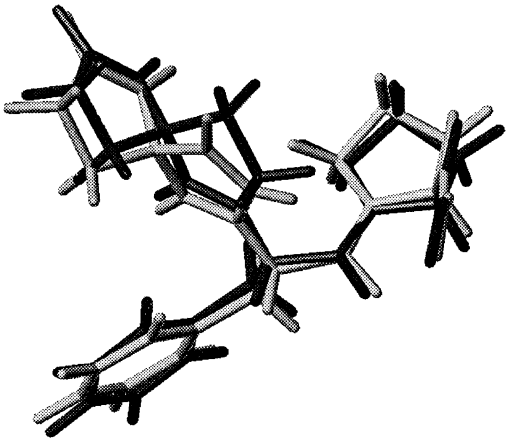


Figure 1. Compound **6** = black; compound **7** = grey.

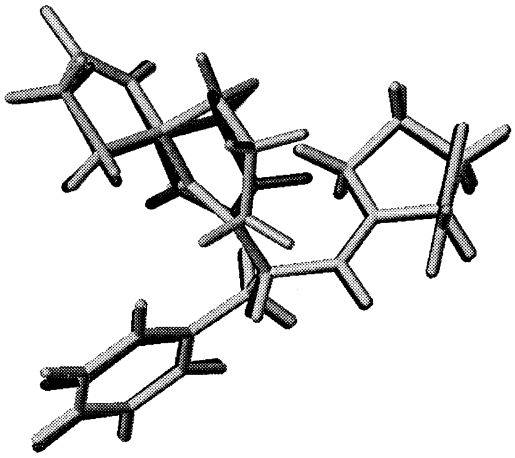


Figure 2. Compound **6** = black; Compound **8** = grey.

A more significant difference between the analogues could be seen when their overlap with the receptor model developed by Marshall and coworkers was taken into account.[2–4] This model was derived by determining a common conformation for all of the active analogues of TRH.[14] Once the common conformation was found, the analogues were aligned and a set of average distances between the pharmacophoric groups within the molecules determined. The compatibility of analogues **6**–**8** with this model was then evaluated by using the average distances as constraints for the distances between the pharmacophoric groups in **6**–**8**. The energy for each analogue was then minimized and compared to the minimized energy of the analogue without the distance constraints. In this way, a measure for what it 'costs' each analogue to overlap with the receptor was obtained. Imposing the distance constraints associated with the model for TRH-R_1 onto analogue **6** led to an increase in energy of 5.66 kcal/mol. For the analogue having the ethylene bridge in the pyroglutamate region (**7**), this increase in energy jumped to 10.85 kcal/mol. For the analogue having the six-membered ring constraint (**8**), the increase in energy was only 1.87 kcal/mol. This data suggested that while both **7** and **8** could overlap with the previously active analogue **6** to roughly the same extent, the two analogues were significantly different with respect to their ability to overlap with the receptor. It was more difficult for analogue **7** to obtain the conformation necessary for binding the receptor; a suggestion that was consistent with the lower level of activity obtained for this analogue relative to that obtained for analogue **8**.

The energy calculations were also consistent with the relative affinities and potencies of **6** and **7**; the largest increase in energy corresponding to the less active analogue (Table 2). Clearly, it was not beneficial to decrease the steric size of the bridge in **6** at the expense of further constraining the analogue. However, the analysis of **8** relative to the original **6** was not so straightforward. The energy calculations using the model for TRH-R_1 failed to predict the nearly identical activity of these two analogues. In this case, the lower increase in energy calculated for **8** (1.87 kcal/mol relative to 5.66 kcal/mol) suggested that **8** should have had a higher affinity and potency for TRH-R_1 than **6**. The most reasonable explanation for this observation was that the increase in the steric size of the propane bridge in **8** relative to the ethane bridge undermined the ability of **8** to bind to TRH-R_1. When **8** was overlapped with the model for TRH/TRH-R_1 binding developed using all of the pre-

Table 2. Energy calculations

Analogue	Minimized Energy (no distance constraints) (kcal/mol)	Minimized Energy (with distance constraints) (kcal/mol)	Difference (kcal/mol)
6	28.56	34.22	+5.66
7	29.50	40.35	+10.85
8	28.90	30.77	+1.87

viously active analogues, it was found that the propane bridge in **8** occupied a position in space that was unique to **8**. While the active analogue approach does not involve a model for the receptor and can not directly identify interactions between the receptor and elements of a particular ligand, the observation that the bridge in **8** occupied space not utilized by the other analogues was consistent with the suggestion that the bridge might interfere with the receptor in ways not available to the smaller ethane bridge in **6**.

Like the biological results obtained using **5**, the results obtained for analogues **6** and **8** supported the idea that the loss of affinity and potency for **6** relative to its acyclic counterpart was due to its being constrained in a conformation that was not quite correct and not sterics. If the ethane bridge in **6** had decreased the activity of the analogue through steric interactions, then the use of a larger ring should have increased these interactions and led to an even less active analogue; a result that was not observed.

Conclusions

We have found that a revised strategy for adding constraints to the pyroglutamate region of TRH provides a significantly improved method for varying the nature of the constraint. The development of this approach allowed for the synthesis of analogues having an ethylene, an ethane, and a propane bridge in this region. From the biological data obtained, it was determined that altering the original bridge used to constrain the pyroglutamate region of TRH in **6** did not lead to an improvement in the affinity or potency of the analogue for TRH-R_1. The use of a smaller, more highly constrained ethylene bridge (**7**) proved to be detrimental to the affinity and potency of the analogue while the use of a larger, more flexible propane bridge (**8**) made no siginificant change in the biological activity of the analogue. The decrease in activity for the molecule containing the ethylene bridge was consistent with the model for TRH-R_1 generated using the active analogue approach. This model showed that it cost more energy for the analogue with the ethylene bridge to overlap with the receptor than it did for the corresponding analogue with the ethane bridge. This model also suggested that the use of the propane bridge should improve the potency of the analogue. While this turned out not to be the case, the fact that the use of the larger bridge led to a level of affinity and potency for the analogue equal to that of the smaller analogue **6** suggested that the observation that **6** was less potent than its unrestricted counterpart was most likely not due to the size of the ethane bridge.

Experimental[15]

(2*R*,*S*)-2-Methoxy-5-oxoproline-(1*S*,2*R*,5*S*)-5-methyl-2-(1-methylethyl)cyclohexyl ester (4). An oven-dried 250 mL three-neck flask with stir bar was cooled under N_2. To the flask was added 11.5 g (43 mmol) of (+)-menthylpyroglutamate and 8.31 g (21.5 mmol, 0.5 equiv) of *n*BuNPF$_6$. The solids were dissolved in 86 mL of anhydrous MeOH, two platinum wire (0.8 mm diameter by 1.5 cm length) were inserted, and the entire reaction setup was degassed by sonication for 10 min. The solution was then oxidized at a constant current of 26.8 mA until 18,630 C (4.5 F/mol) had passed. The completed reaction solution was transferred to a 500 mL flask (single-neck), the MeOH was removed in vacuo, and 100 mL of Et_2O was added. The electrolyte was then filtered, and the filtrate was dried over anhydrous $MgSO_4$, filtered, and concentrated completely in vacuo. The crude product was then purified by gravity-flow chromatography through 350 g of slurry packed silica gel using a 1:1 Et_2O/hexane to 100% Et_2O gradient solvent system. Fractions containing **4** (TLC solvent Et_2O, R_f 0.74, yellow/blue spot with *p*-anisaldehyde stain) were combined, dried over anhydrous $MgSO_4$, filtered, and concentrated in vacuo to yield 12.2 g (41.0 mmol, 95%) of pure product as a clear, viscous liquid. ^{1}H NMR (300 MHz, $CDCl_3$) two isomers, δ 8.63 (br s, 0.6H), (8.53 (br s, 0.4H), 4.76 (tt, 1H, J = 8.6, 2.4 Hz), 3.26 (s, 3H), 2.63–2.56 (m, 1H), 2.40–2.30 (m, 3H), 2.04–2.00 (m, 1H), 1.92–1.87 (m, 1H), 1.70 (dm, 2H, J_d = 11 Hz), 1.53–1.45 (m, 2H), 1.15–0.80 (unresolved m, 3H), 0.92–0.89 (2 unresolved d, 6H), 0.77–0.74 (2 unresolved d, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) two isomers, δ 178.3, 178.2, 168.3, 168.2, 91.8, 91.7, 76.1, 51.2, 51.0, 46.4, 40.1, 33.8, 31.3, 31.1, 29.0, 28.0, 25.8, 22.8, 21.6, 20.4, 20.4, 15.7, 15.6. IR (thin film) 3205 (bs), 2945 (s), 2877 (s), 1716 (s), 1460 (m), 1076 (s), 737 (m) cm^{-1}. LRFAB MS (3-NBA/gly/TFA matrix) *m*/*e* (relative intensity) identity of ion if known; 298 (28) MH^+; 266 (52) MH^+–$HOCH_3$; 160 (35) 2-OMe-oxoprolineH$^+$, 128 (100) MH^+–OCH_3–$C_{10}H_{19}$; 114 (90) M–$C_{11}H_9O_2$. HRFAB MS (3-NBA/gly/TFA matrix): *m*/*e* calcd for $C_{16}H_{28}NO_4$ (MH^+) 298.2018; found 298.2017 (dev −0.4 ppm).

(2*r*)-2-Prop-2-enyl-5-oxoproline (1*S*,2*R*,5*S*)-5-methyl-2-(1-methylethyl)cyclohexyl ester (5). To a dry 250 mL flask under an argon atmosphere containing compound **4** and a stir bar was added 40 mL of CH_2Cl_2. The resulting solution was cooled to −78 °C in an acetone/dry ice bath. When cool and while vigorously stirring under argon, 21.1 mL of a 1 M solution of $TiCl_4$ in CH_2Cl_2 (21.1 mmol, 1.1 equiv) was added dropwise via syringe. The dark-brown solution was stirred for 5 min, and then 14 mL (9.8 g, 86 mmol, 4.5 equiv) of allyl-TMS was added via syringe. The reaction solution was allowed to stir while warming slowly to room temperature over

16 h, after which the solution was transferred to a 1 L Erlenmeyer flask and diluted with 200 mL of CH_2Cl_2. While stirring vigorously, 200 mL of saturated aqueous $NaHCO_3$ solution was slowly added to neutralize any excess $TiCl_4$. The layers were separated, and the aq layer was extracted twice more with 200 mL portions of CH_2Cl_2. All organic layers were combined, concentrated in vacuo to approximately 75 mL, dried over $MgSO_4$, filtered, and concentrated completely in vacuo. The crude product was then purified by flash chromatography through 175 g of slurry packed silica gel using Et_2O as eluent. Fractions containing product (TLC solvent Et_2O, R_f 0.30, blue spot with *p*-anisaldehyde stain) were combined and concentrated in vacuo to a yellow oil which was dissolved in 25 mL of hexane in a 250 mL Erlenmeyer flask The hexane solution was cooled to 0 °C at which time a seed crystal of **5** was added, and the flask was placed in the freezer overnight to complete the selective crystallization of the desired *R* isomer. Careful filtration of the crystals with cold hexane yielded 3.30 g (10.7 mmol, 56%) of pure diastereomer **5**. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.03 (br s, 1H), 5.74–5.60 (m, 1H), 5.18–5.16 (m, 1H), 5.21 (s, 1H), 4.72 (dt, 1H, $J_t = 11.0$ Hz, $J_d = 4.4$ Hz), 2.69 (dd, 1H, $J = 13.7$, 6.3 Hz), 2.45–2.34 (m, 4H), 2.20–2.04 (m, 1H), 1.96 (dm, 1H, $J_d = 12.0$ Hz), 1.92 (septet of d, 1H, $J_{sep} = 6.8$ Hz, $J_d = 2.7$ Hz), 1.74–1.66 (m, 2H), 1.46–1.39 (m, 2H), 1.14–0.80 (m, 3H), 0.92–0.89 (2 unresolved d, 6H), 0.75 (d, 3H, $J = 7.1$ Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 176.6, 172.6, 131.0, 120.6, 76.0, 65.2, 46.8, 43.4, 40.6, 34.1, 31.4, 30.5, 29.7, 26.2, 23.05, 21.9, 20.8, 15.9. IR (thin film) 3343 (bm), 2952 (s), 2931 (s), 2870 (m), 1730 (s), 1699 (s), 1455 (m), 1370 (m), 1182 (m), 1065 (m), 930 (m), 915 (m), 731 (s) cm^{-1}. LRFAB MS (3-NBA/gly/TFA matrix) *m*/*e* (relative intensity) identity of ion if known; 308 (86) MH^+; 170 (100) 2-allyloxoprolineH$^+$; 128 (12) 2-oxoprolineH$^+$; 124 (64) M-$C_{11}H_{18}O$. HRFAB MS (3-NBA/gly/TFA matrix): *m*/*e* calcd for $C_{18}H_{29}NO_3$ (MH^+) 308.2226; found 308.2225 (dev +0.1 ppm).

(2*R*)-2-Prop-2-enyl-5-oxoproline. To a 100 mL flask with stir bar was added 4.74 g of (15.4 mmol) menthyl ester **5** and 15 mL of anhydrous MeOH. The mixture was then stirred to dissolve under N_2 and then was cooled to 0 °C. Meanwhile, 0.95 g (16.9 mmol, 1.1 equiv) of finely ground KOH was dissolved in 20 mL of MeOH. This KOH/MeOH solution was then added to the cold, stirring ester/MeOH solution by quantitative transfer via cannula. An additional 5 mL of MeOH was used for rinsing. The reaction flask was allowed to slowly warm to 25 °C over several hours and then stirred 18 h more, checking periodically by TLC (Et_2O eluent) for completion as evidenced by disappearance of **5**. Approximately 90 mL of H_2O was added and the MeOh was then removed in vacuo. The cloudy aq solution was extracted with 50 mL of Et_2O three times to remove the liberated menthol and then acidified to a pH of 2 by the addition of 150 mL of 2 N aq HCl. The solution was concentrated in vacuo completely. To the resulting product/salt mixture was added 100 mL of CH_2Cl_2, and then the KCl salt was removed by filtration. The filtrate, containing product, was dried over anhyd $MgSO_4$, filtered, and concentrated in vacuo to yield 1.71 g (10.1 mmol, 97%) of the acid as a white solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 11.11 (br s, 1H), 7,85 (br s, 1H), 5.74 (ddt, 1H, $J = 15.1$, 11.8, 6.6 Hz), 5.205 (d, 1H, $J = 11.8$ Hz), 5.199 (d, 1H, $J = 15.1$ Hz), 2.69 (A of ABX, 1H, $J_{AB} = 13.8$ Hz, $J_{AX} = 6.6$ Hz), 2.53–2.41 (unresolved m, 4H), 2.19–2.06 (m, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 179.5, 176.1, 130.9, 120.6, 66.1, 42.8, 30.1, 29.5. IR (thin film) 3010 (bm), 3271 (bm), 3078 (m), 2557 (m), 1715 (s), 1644 (s), 1416 (m), 1234 (s), 995 (m), 924 (m), 728 (m) cm^{-1}. LRFAB MS (3-NBA/gly/TFA matrix) *m*/*e* (relative intensity) identity of ion if known; 170 (94) MH^+; 128 (16) MH^+–propene; 124 (100) MH^+–CO_2. HRFAB MS (3-NBA/gly/TFA matrix): *m*/*e* calcd for $C_8H_{12}NO_3$ (MH^+) 170.0817; found 170.0813 (dev −2.4 ppm).

(2*R*)-2-Prop-2-enyl-5-oxoprolyl-L-phenylalanine benzyl ester (9). Into a dry 25 mL flask with stir bar was dissolved 0.505 g (2.98 mmol) of the allylated pyroglutamic acid in 6 mL of CH_2Cl_2. The mixture was cooled to 0 °C, and then 0.44 g (3.3 mmol, 1.1 equiv) of HOBt and 0.42 mL (0.38 g, 3.3 mmol, 1.1 equiv) of NEM was added while stirring vigorously to completely dissolve under N_2. When all was dissolved, 0.63 g (3.3 mmol, 1.1 equiv) of EDC was added. After 5 min, 0.84 g (3.3 mmol, 1.1 equiv) of H_2N-L-Phe-Obn•HCl was added. The mixture was allowed to warm to 25 °C and stirred for 2 h. The reaction was then diluted with 10 mL of EtOAc and the CH_2Cl_2 was distilled in vacuo. The remainder was diluted further by the addition of 40 mL of EtOAc and then washed with 30 mL of 5% aq $NaHCO_3$ solution, 30 mL of 5% aq citric acid solution, and 30 mL of saturated aq NaCl solution, extracting each aq wash twice with additional EtOAc before proceeding. The EtOAc extracts were combined, dried over $MgSO_4$, filtered, and concentrated in vacuo to a sticky brown oil. The crude material was then gravity-flow chromatographed through 125 g of slurry packed silica gel using a 5:95 MeOH/EtOAc solvent mixture. Fractions containing product (TLC solvent same as column, R_f 0.54, UV-active spot or faint yellow/white spot with ninhydrin stain) were combined and concentrated in vacuo to afford 1.2 g (2.98 mmol, 100%) of pure **9** as a white solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.37–7.02 (m, 10H), 6.48 (br s, 1H), 5.68–5.54 (m, 1H), 5.21–5.08 (unresolved m, 4H), 4.96–4.88 (m, 1H), 3.24 (dd, A of ABX, 1H, $J_{AB} = 13.9$ Hz, $J_{AX} = 5.5$ Hz), 2.99 (dd, B of ABX, 1H, $J_{AB} = 13.9$ Hz, $J_{BX} = 8.5$ Hz), 2.80 (dd, A of ABX, 1H, $J_{AB} = 13.8$ Hz, $J_{AX} = 5.6$ Hz), 2.48–1.91 (unresolved m, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 178.2, 173.3, 171.8, 136.9, 135.1, 131.8, 129.1, 128.6, 128.6, 128.5, 127.2, 120.4, 67.4, 65.4, 53.1, 42.7, 37.7, 31.8, 29.6. IR (thin film) 3308 (bm), 3062 (m), 3027 (m), 2931 (m), 1743 (s), 1702 (s), 1671 (s), 1500 (m), 1455 (m), 1271 (m), 1100 (w), 997 (w), 922 (w), 737 (s), 699 (s) cm^{-1}. LRFAB MS (3-NBA/gly/TFA matrix) *m*/*e* (relative intensity) identity of ion if known; 407 (100) MH^+; 365 (2) MH^+–propene; 256 (6.5); 214 (8.5). HRFAB MS (3-NBA/gly/TFA matrix): *m*/*e* calcd for $C_{24}H_{27}N_2O_4$ (MH^+) 407.1971; found 407.1977 (dev +0.1 ppm).

N^{α}-[(5*R*,8*RS*)-8-Hydroxy-2,6-dioxo-1-azaspiro[4.4]-octyl]-L-phenylalanine benzyl ester. Compound **9** (3.3 g, 8.12 mmol) was placed in a 500 mL flask with stir bar and dissolved in 80 mL of distilled THF and 40 mL of distilled/deionized H_2O while stirring under N_2 at 25 °C. Next, 1.75 mL of a 0.05 M solution of OSO_4 in H_2O (1 mol%) was added via syringe. The solution was allowed to stir for 5 min over which time the reaction solution slowly turned brown. Then, 4.34 g (20.3 mmol, 2.5 equiv) of solid $NaIO_4$ was added. Almost immediately a white precipitate began to form, and the reaction was left to stir under N_2 for 5 h more to ensure reaction completion. The THF was removed in vacuo and the remaining aq reaction solution was poured into a 250 mL separatory funnel and extracted eight times with 100 mL portions of CH_2Cl_2, checking by TLC for complete transfer of product to organic phase (15:85 MeOH/EtOAc as eluent, R_f 0.46, UV-active spot or pink spot with ninhydrin stain). The CH_2Cl_2 extracts were combined, dried over $MgSO_4$, filtered, and concentrated in vacuo to afford a yellow viscous oil. This crude product was then flash chromatographed through 100 g of slurry-packed silica gel using a 5:95 MeOH/EtOAc solvent mixture. Fractions containing product were combined, concentrated in vacuo, redissolved in 80 mL of CH_2Cl_2, dried over $MgSO_4$, filtered, and concentrated in vacuo completely to give 3.29 g (8.12 mmol, 100%) of pure cyclized *N*-α-hydroxyamide as a white solid. ^{1}H NMR (300 MHz, $CDCl_3$) approx 1:1 mixture of isomers, δ 7.83 (br s, 0.5H), 7.38–7.11 (m, 10H), 6.23 (br s, 0.5H), 5.20 (dd, 1H, J = 10.0, 6.8 Hz), 5.16–5.10 (m, 2H), 4.58 (dd, J = 9.5, 6.8 Hz), 4.38 (dd, 0.5H, J = 6.1, 2.4 Hz), 4.6 (very br unresolved s, 1H), 3.48–3.32 (m, 1.5H), 3.10 (A of ABX, 0.5H, J_{AB} = 14.4, J_{AX} = 10.0), 2.93–2.02 (unresolved m, 5H), 1.92 (dd, 0.5H, J = 13.3, 5.7 Hz), 1.83–1.76 (m, 0.5 H); ^{13}C NMR (75 MHz, $CDCl_3$) two isomers, δ 178.6, 178.4, 175.7, 173.7, 172.7, 169.7, 137.2, 135.3, 135.2, 134.3, 128.9, 128.7, 128.7, 128.6, 128.5, 128.4, 128.3, 128.1, 127.3, 126.8, 81.2, 77.7, 68.0, 67.3, 62.8, 62.2, 56.8, 55.4, 42.5, 42.0, 35.2, 34.8, 33.1, 31.5, 30.1, 29.8. IR (thin film) 3314 (bm), 3055 (m), 2986 (w), 1710 (s), 1440 (m), 1400 (m), 1265 (s), 880 (w), 720 (s), 690 (s) cm^{-1}. LRFAB MS (3-NBA/gly/TFA matrix) *m*/*e* (relative intensity) identity of ion if known; 409 (12) MH^+; 391 (100) MH^+–H_2O; 363 (10) MH^+–H_2O–CO; 322 (68); 273 (9) MH^+–CO–HOC_7H_7; 250 (20); 120 (68). HRFAB MS (3-NBA/gly/TFA matrix): *m*/*e* calcd for $C_{23}H_{25}N_2O_5$ (MH^+) 409.1763; found 409.1760 (dev −0.8 ppm).

N^{α}-[(5*R*,8*RS*)-8-*tert*-Butyldimethylsilyloxy-2,6-dioxo-1-azaspiro[4.4]-octyl]-L-phenylalanine benzyl ester (10). In a dry 50 mL flask with stir bar under N_2 was placed 3.73 g (9.13 mmol) of the *N*-a-hydroxy amide synthesized above, 20 mL of CH_2Cl_2, and 2.13 mL (1.96 g, 18.3 mmol, 2 equiv) of 2,6-lutidine. The mixture was stirred to dissolve and then cooled to 0 °C. When cool, 4.5 mL (5.65 g, 21.4 mmol, 2.3 equiv) of TBS-OTf was added slowly via syringe. The reaction was then allowed to warm to 25 °C and stirred for 6 h, monitoring by TLC for completion (5:95 MeOH/EtOAc, product R_f 0.63 and 0.51, two diastereomers, UV-active or pink spot with ninhydrin stain). When complete, the reaction solution was diluted to approximately 100 mL with CH_2Cl_2 and washed with 50 mL of 5% aq citric acid solution and 50 mL of 5% aq $NaHCO_3$ solution, re-extracting each aq wash once with additional CH_2Cl_2 before proceeding. All organic extracts were combined, dried over $MgSO_4$, filtered, and concentrated in vacuo. The crude material was then flash chromatographed through 250 g of slurry packed silica gel using a 5:94:1 MeOH/EtOAc/Et_3N solvent mixture. Fractions containing product (two diastereomers) were combined, concentrated in vacuo, redissolved in 50 mL of CH_2Cl_2, dried over $MgSO_4$, filtered, and concentrated in vacuo to give 4.70 g (8.99 mmol, 99%) of the silated product **10** as a viscous oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.37–7.14 (m, 10H), 5.75 (br s, 1H), 6.16 (d, A of AB, 1H, J = 12.0 Hz), 5.11 (d, B of AB, 1H, J = 12.0 Hz), 4.39 (dd, X of ABX, 1H, J = 1.8, 5.0 Hz), 4.23 (dd, X of ABX, 1H, J = 5.8, 10.3 Hz), 3.40–3.22 (m, 2H), 2.70–2.60 (m, 1H), 2.45–2.28 (m, 2H), 2.03–1.78 (unresolved m, 3H), 0.76 (s, 9H), −0.05 (s, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 176.8, 173.3, 169.0, 137.5, 129.1, 129.0, 128.9, 128.8, 128.7, 128.5, 128.4, 126.9, 81.6, 67.5, 61.7, 57.1, 43.5, 35.5, 29.5, 25.6, 17.5, −4.7, −5.2. IR (thin film) 3418 (m), 3375 (bm), 2952 (m), 2890 (w), 2856 (m), 1740 (s), 1709 (s), 1456 (m), 1387 (m), 1261 (s), 1223 (m), 1110 (m), 877 (m), 836 (s), 781 (m), 737 (s), 699 (s) cm^{-1}. LRFAB MS (3-NBA/gly/TFA matrix): *m*/*e* (relative intensity) identity of ion if known; 523 (2) MH^+; 391 (92) MH^+–$HOSiR_3$; 91 (100) $C_7H_7^+$. HRFAB MS (3-NBA/gly/TFA matrix): *m*/*e* calcd for $C_{23}H_{23}N_2O_4$ (MH^+–$HOSiR_3$) 391.1658; found 391.1668 (dev +2.6 ppm).

N^{α}-[(5*R*,8*RS*)-8-*tert*-Butyldimethylsilyloxy-2,6-dioxo-1-azaspiro[4.4]-octyl]-L-phenylalanyl-L-prolinamide. Benzyl ester compound **11** (1.26 g, 2.41 mmol) was placed in a dry 100 mL flask with stir bar. Approximately 1 mL of 5% Pd/C and 5 mL of CH_2Cl_2 were added, and the flask was filled with N_2 and stoppered. A 2.5 L H_2 balloon fitted with a 0.5 mm syringe needle was placed on the reaction, and the N_2 was forced out of the flask. The reaction mixture was then left to stir at 25 °C under H_2 for 18 h, after which the balloon was removed and the flask flushed with N_2. The reaction mixture was diluted with 10 mL of CH_2Cl_2 and 10 mL of EtOAc and then filtered through 250 g of well-packed Celite to remove the Pd/C. The filtrate was dried over $MgSO_4$, filtered, and concentrated completely in vacuo to give 1.01 g (97% by NMR) of crude carboxylic acid as a sticky white solid which was not further purified.[16] A 0.48 g portion (1.11 mmol, 48% of the total) of the crude carboxylic acid made above was placed in a dry 25 mL flask with 0.18 g (1.33 mmol, 1.2 equiv) of HOBt and 2.5 mL of CH_2Cl_2. The mixture was stirred under N_2 and cooled to 0 °C, at which time 0.16 mL (0.112 g, 1.11 mmol, 1 equiv) of Et_3N and 0.23 g (1.22 mmol, 1.1 equiv) of EDC were added. After 5 min, 0.15 g (1.33 mmol, 1.2 equiv) of HN-L-Pro-NH_2 was added, and the reaction solution was allowed to slowly warm to 25 °C over 2.5 h. Then, the reaction solution was diluted with 10 mL of EtOAc and the CH_2Cl_2 was distilled in vacuo. The remaining EtOAc mixture was washed with 30 mL of 5% aq citric acid solution, 30 mL of 5% aq $NaHCO_3$

solution, and 30 mL of saturated aq NaCl solution, extracting each aq wash twice with 60 mL of additional EtOAc. The combined EtOAc extracts were dried over $MgSO_4$, filtered, and concentrated completely in vacuo. The crude product was then purified by gravity-flow chromatography through 35 g of slurry packed silica gel using a 10:90:2 MeOH/EtOAc/Et_3N solvent mixture. Fractions containing product (TLC solvent same as column, R_f 0.47 and 0.38 of two diastereomers, UV-active spot or faint pink/brown spot with ninhydrin stain) were combined, concentrated in vacuo, redissolved in 25 mL of CH_2Cl_2, dried over $MgSO_4$, filtered, and concentrated completely in vacuo to afford 0.406 g (0.77 mmol, 67% over two steps) of the pure proline amide derivative as a white solid. ^{1}H NMR (300 MHz, $CDCl_3$) multiple indistinguishable isomers δ 7.80 (br s, 0.1H), 7.34–7.22 (m, 5H), 6.90 (d, 0.1H, $J=7.8$ Hz), 6.72 (br s, 0.2H), 6.51 (br s, 0.2H), 6.25–6.10 (2 unresolved br s, 0.9H), 5.96 (br s, 0.1H), 5.92 (br s, 0.1H), 5.75 (d, 0.8H, $J=4.4$ Hz), 5.60 (br s, 0.8H), 5.22–5.20 (m 0.2H), 5.12 (m, 0.1H), 5.05 (dd, X of ABX, 0.8H, $J=5.4$ Hz, 16.1 Hz), 4.62 (m, 0.2H), 4.52 (d, 0.2H, $J=4.4$ Hz), 4.44 (t, 0.9H, $J=5.6$ Hz), 4.05 (d, 0.3H, $J=7.8$ Hz), 3.80–3.70 (m, 0.3H), 3.61–3.43 (m, 1.6H), 3.40–3.36 (unresolved m 0.5H), 3.30 (dd, A of ABX, 1H, $J=11.0$, 12.9 Hz), 3.21–3.00 (m, 1H), 2.97 (0.7H, dd, $J=4.9$, 6.6 Hz), 2.78–1.65 (unresolved m, 13H), 1.62–1.48 (m, 0.2H), 0.97–0.84 (unresolved m, 9H), 0.28–0.09 (unresolved m, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 177.5, 177.0, 174.4, 173.3, 173.0, 169.8, 151.9, 136.3, 136.0, 129.5, 129.3, 128.9, 127.4, 80.6, 78.2, 61.7, 60.4, 59.8, 54.0, 47.5, 43.6, 43.4, 37.3, 33.8, 29.6, 29.1, 27.8, 25.7, 24.5, 20.0, 17.6, −4.2, −4.7. IR (thin film) 3466 (m), 3418 (m), 3329 (bm), 3205 (bm), 3055 (m), 2959 (m), 2931 (m), 2884 (mw), 2863 (m), 1705 (s), 1647 (s), 1647 (s), 1422 (m), 1363 (w), 1343 (w), 1267 (s), 1188 (w), 1113 (m), 1079 (w), 1035 (w), 898 (w), 870 (w), 836 (m), 781 (mw), 733 (s), 703 (s) cm^{-1}. LRFAB MS (3-NBA/gly/TFA matrix): *m*/*e* (relative intensity) identity of ion if known; 471 (14) MH^+–*t*BuH; 415 (13) MH^+–$SiR_3^+H^+$; 398 (25) MH^+–SiR_3–NH_2; 397 (100) MH^+–$HOSiR_3$; 387 (9) M_{415}–C_2H_4 (or CO); 370 (13) M_{398}–CO; 369 (47) M_{397}–C_2H_4 (or CO); 352 (12) M_{369}–NH_3 or M_{397} H^+–$CONH_2$; 324 (3) M_{352}–C_4H_7N; 255 (27) M_{238}–CO; 240 (17); 227 (26); 130 (20); 115 (8); $Sir3^+$; 91 (30) $C_7H_7^+$. HRFAB MS (3-NBA/gly/TFA matrix): *m*/*e* calcd for $C_{21}H_{25}N_4O_4$ (MH^+–$HOSiR_3$) 397.1876; found 397.1864 (dev −2.9 ppm).

N^{α}-[(5*R*)-2,6-Dioxo-1-azaspiro[4.4]-oct-8-enyl]-L-phenylalanyl-L-prolinamide (7). The *N*-α-siloxyamide synthesized above (0.250 g, 0.47 mmol) was placed in a 25 mL flask with about 2 mL of CH_2Cl_2 and 3 mL of silica gel (200 mesh). The CH_2Cl_2 was then heat distilled to leave the starting material adsorbed directly onto the silica gel. The flask was fitted with a reflux condenser and attached to a variable vacuum pump. The reaction mixture was then heated slowly over 1 h to 200 °C under a 30–40 mm Hg argon atmosphere, then left at 200 °C for 15 min. The reaction mixture was allowed to cool to 25 °C at 1 atm (Ar) and then filtered to remove the silica gel, rinsing well with EtOAc and MeOH. The filtrate was dried over $MgSO_4$, filtered, and concentrated completely in vacuo to a brown viscous oil. The crude product was purified by gravity-flow chromatography through 20 g of slurry packed silica gel using a 20:80 MeOH/EtOAc solvent mixture. Fractions containing product (TLC solvent same as column, R_f 0.43, UV-active spot or pink spot with *p*-anisaldehyde stain) were combined, concentrated in vacuo, redissolved in 20 mL of CH_2Cl_2, dried over $MgSO_4$, filtered, and concentrated again in vacuo to afford 0.11 g (58%) of pure **7**. The purified product could then be recrsytallized as the monohydrate from hexane for use in biological testing. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.34–7.18 (m, 5H), 7.03 (d, 1H, $J=4$ Hz), 6.96 (d, minor isomer, $J=5$ Hz), 6.28 (br s, 1H), 5.98 (br s, minor isomer), 5.32 (d, 1H, $J=5$ Hz), 5.42 (d, minor isomer, $J=5$ Hz), 5.25 (br s, 1H), 5.18 (br s, minor isomer), 5.17 (t, 1H, $J=8.1$ Hz), 4.88 (br s, 1H), 4.51 (m, 1H), 3.64 (dd, A of ABX, 1H, $J_{AB}=16$ Hz, $J_{AX}=8.9$ Hz), 3.46–3.38 (m, 1H), 3.25 (dd, A of ABX, 1H, $J_{AB}=13.7$ Hz, $J_{AX}=7.5$ Hz), 3.03 (dd, B of ABX, 1H, $J_{AB}=13.7$ Hz, $J_{BX}=8.7$ Hz), 2.72–2.61 (m, 1H), 2.57–1.75 (unresolved m, 7H), 1.55 (br s due to H_2O, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 178.0, 173.9, 169.2, 135.2, 132.0, 129.3, 128.9, 128.7, 127.6, 110.8, 65.0, 59.7, 52.9, 47.6, 37.1, 29.6, 29.3, 27.7, 12.2; minor isomer peaks visible at δ 130.2, 60.0, 54.5, 42.0, 35.3. IR (diffusion reflectance) 3310 (bm), 3197 (s), 2962 (m), 2879 (m), 1705 (s), 1656 (s), 1547 (w), 1445 (s), 1385 (s), 1343 (s), 1294 (s), 1268 (s), 1196 (m), 1154 (m), 1120 (m), 1086 (m), 1037 (w), 973 (w), 920 (mw), 871 (mw), 840 (mw), 792 (m), 750 (m), 697 (ms), 648 (m), 501 (m), 471 (m) cm^{-1}. LRFAB MS (3-NBA/gly/TFA matrix): *m*/*e* (relative intensity) identity of ion if known; 397 (100) MH^+; 371 (4) MH^+–C_2H_2; 352 (3) MH^+–$CONH_3$; 283 (8) MH^+–prolinamide. HRFAB MS (3-NBA/gly/TFA matrix): *m*/*e* calcd for $C_{21}H_{25}N_4O_4$ (MH^+) 397.1876; found 397.1876 (dev 0.0 ppm). Elemental analysis calcd for $C_{21}H_{24}N_4O_4$&z_rad;H_2O: C, 60.86; H, 6.08; N, 13.52. Found: C, 60.93, H, 6.10, N, 13.05.

N^{α}-[(5*R*)-2,6-Dioxo-1-azaspiro[4.4]-octyl]-L-phenylalanyl-L-prolinamide (6). To a 25 mL flask with stir bar was added 90 mg (0.23 mmol) of **7** and excess 5% Pd/C (approximately 1 mL). The flask was filled with N_2 and 2 mL each of MeOH and EtOAc were added, then a 2.5 L H_2 balloon fitted with a 0.2 mm syringe needle was placed on the reaction flask. The N_2 was forced out of the flask, and the reaction mixture was then left to stir under H_2 at 25 °C for 2 h after which the balloon was removed and the flask flushed with N_2. The reaction mixture was filtered through 125 g of well-packed Celite to remove the Pd/C using MeOH to rinse well, then the filtrate was dried over $MgSO_4$, filtered, and concentrated completely in vacuo. The crude product was purified by gravity-flow chromatography through 3 g of slurry-packed silica gel using a 20:80 MeOH/EtOAc solvent mixture. Fractions containing product (TLC solvent same as column, R_f 0.14, UV-active spot or pink spot with *p*-anisaldehyde stain) were combined, concentrated in vacuo, redissolved in CH_2Cl_2, dried over $MgSO_4$, filtered, and concentrated in vacuo to give 82 mg (91%) of a white solid whose ^{1}H NMR was identical to the previously synthesized **6**.[6b]

(2*R*)-1-(4-Methoxybenzyl)-2-prop-2-enyl-5-oxoproline (1*S*,2*R*,5*S*)-5-methyl-2-(1-methylethyl)cyclohexyl ester. Preparation of pMeOBnBr. To a 10 mL flask containing 1.75 mL (1.93 g, 14 mmol) of *p*MeOBnOH was added 4 mL of concentrated HBr while vigorously stirring. After 15 min, 80 mL of Et_2O was added, and the entire mixture was poured into a 125 mL separatory funnel. The product *p*MeOBnBr was extracted into the Et_2O by shaking, and then the layers were separated. The Et_2O phase was washed with 50 mL of saturated aq $NaHCO_3$ solution and 50 mL of saturated aq NaCl solution, then dried over $CaCl_2$, filtered, and concentrated in vacuo to a pale yellow oil which was kept under an argon atmosphere until used in the following protection step. A flame-dried 250 mL flask with stir bar was cooled under argon, then 0.41 g of a 60% NaH dispersion of NaH in mineral oil (0.25 g NaH, 10.2 mmol, 1.1 equiv) was weighed into the flask under an argon atmosphere. The dispersion was washed several times with dry cyclohexane, decanting the oil/solvent mixture each time. To the flask was then added 35 mL of anhyd DMF and the mixture was cooled to 0 °C. Next, 2.85 g (9.3 mmol) of compound **5** was added as a DMF solution (15 mL) by transferring via cannula. The reaction mixture was stirred for 15 min, and then the *p*MeOBnBr prepared above was added by transferring via cannula as a solution in 20 mL of anhydrous DMF. The mixture was stirred at 0 °C for 1.5 h and then allowed to warm to 25 °C and stirred for 14 h. The reaction was neutralized by the addition of 10 mL of glacial HOAc, then poured into a 250 mL separatory funnel containing 80 mL of ice water. The water was extracted three times with 100 mL portions of Et_2O, and the combined organic extracts were dried over $MgSO_4$, filtered, and concentrated in vacuo to a brown oil. The crude product was then partially purified by gravity-flow chromatography through 200 g of slurry-packed silica gel using a 3:1 to 1:1 hexane/Et_2O gradient solvent mixture. Fractions containing product (TLC solvent 3:1 hexane/Et_2O, R_f 0.3, UV-active spot or blue spot with *p*-anisaldehyde stain) were combined, dried over $MgSO_4$, filtered, and concentrated in vacuo to afford an 80:20 mixture (mol ratio) of the protected product (2.81 g, 6.5 mmol, 71%) and PMB-Br (0.33g, 1.6 mmol). In addition, 0.58 g (1.9 mmol, 20%) of the unprotected starting material **5** was isolated from the reaction product mixture. ^{1}H NMR of the mixture (300 MHz, $CDCl_3$) two rotamers visible plus PMB-Br, δ 7.27–7.21 (m, 2H), 6.83–6.79 (m, 2H), 5.42–5.29 (m, 1H), 5.08–4.98 (m, 2H), 4.80 (d, A of AB, 0.7H, J = 15.1 Hz), 4.67 (td, 1H, J_d = 4.4 Hz, J_t = 11 Hz), ~4.67 (buried d, A of AB 0.3H), 4.13 (d, B of AB, 0.3H, J = 15.1 Hz), 4.02 (d, B of AB, 0.7H, J = 15.1 Hz), 3.77 (s, 3H), 2.66–2.34 (m, 4H), 2.25–2.15 (m, 1H), 2.07–1.95 (m, 1H), 1.88–1.70 (m, 2H), 1.69–1.65 (m, 2H), 1.49–1.26 (m, 3H), 1.12–0.75 (unresolved m, 2H), 0.91–0.82 (3 unresolved d, 6H), 0.79–0.72 (3 unresolved d, 3H); PMB-Br peaks visible at δ 7.30–7.27 (m, 2H), 6.89–6.84 (m, 2H), 4.61 (s, 2H), 2.79 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) two rotamers visible, δ 176.2, 172.2, 158.7, 158.7, 131.3, 129.9, 129.7, 129.4, 128.4, 120.2, 120.0, 113.7, 113.6, 75.9, 75.7, 69.3, 68.9, 64.7, 55.1, 55.0, 46.7, 44.3, 40.3, 40.0, 39.7, 39.3, 33.9, 31.2, 29.5, 28.0, 27.8, 26.1, 26.0, 22.8, 21.8, 21.7, 20.8, 15.8, 15.7. IR (thin film) 3438 (w), 3075 (w), 2959 (s), 2918 (s), 2875 (s), 1730 (s), 1695 (s), 1606 (ms), 1586 (w), 1514 (s), 1432 (m), 1391 (s), 1446 (m), 1245 (s), 1172 (s), 1035 (s), 966 (m), 898 (m), 812 (m), 737 (s) cm^{-1}. LRFAB MS (3-NBA matrix): *m*/*e* (relative intensity) identity of ion if known; 428 (30) MH^+; 386 (1) MH^+–propene; 308 (<1) MH^+–$PMB^+ + H^+$; 244 (23) M–CO_2menthyl; 216 (1) M_{244}–CO (or C_2H_4); 182 (12); 124 (6) M_{308}–CO_2menthyl–H^+; 122 (18); 121 (100) PMB^+; 91 (5) $C_7H_7^+$. HRFAB MS (3-NBA matrix): *m*/*e* calcd for $C_{26}H_{38}NO_4$ (MH^+) 428.2801; found 428.2780 (dev −4.8 ppm).

(2*R*)-1-(4-Methoxybenzyl)-2-prop-2-enyl-5-oxoproline. In a 25 mL flask with stir bar was dissolved 1.47 g (3.44 mmol) of the PMB protected, allylated pyroglutamate menthyl ester synthesized above in 4.5 mL of MeOH, 2.5 mL of THF, and 2.5 mL of H_2O. The reaction solution was cooled to 0 °C while stirring under N_2, and 0.36 g (8.6 mmol, 2.5 equiv) of $LiOH \cdot H_2O$ was added. The reaction solution was allowed to warm to 25 °C and then was stirred for 48 h, at which time it was diluted with 10 mL of H_2O and 40 mL of Et_2O. The menthol and unreacted starting material were extracted into the Et_2O phase by shaking, the layers were separated, and then the aq phase was acidified to a pH of 2 by the addition of 0.5 mL of 2 N aq HCl. The product was then extracted with Et_2O (two 50 mL portions). The combined Et_2O extracts were dried over $MgSO_4$, filtered, and concentrated in vacuo to afford 0.89 g (3.1 mmol, 90%) of the crude acid. The product acid was not further purified. ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.60 (br s, 1H), 7.27–7.22 (m, 2H), 6.82–6.78 (m, 2H), 5.46–5.35 (m, 1H), 5.11–5.05 (m, 2H), 4.60 (d, 1H, J = 15.2 Hz), 4.33 (d, 1H, J = 15.2 Hz), 3.76 (s, 3H), 2.69–2.55 (m, 2H), 2.50–2.40 (m, 2H), 2.28–2.19 (m, 1H), 2.09–1.98 (m, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 177.4, 176.1, 158.9, 131.2, 129.9, 129.1, 120.4, 113.7, 68.9, 55.2, 44.5, 39.0, 29.4, 27.8. IR (thin film) 2800 (v bm), 3053 (ms), 2962 (ms), 2841 (ms), 2773 (bm), 2545 (bm), 2311 (w), 1724 (s), 1690 (s), 1641 (s), 1513 (s), 1449 (m), 1415 (ms), 1362 (mw), 1335 (mw), 1268 (s), 1241 (s), 1173 (s), 1116 (mw), 1033 (m), 928 (m), 890 (mw), 814 (mw), 739 (s), 709 (s) cm^{-1}. LRFAB MS (3-NBA/gly/TFA matrix) *m*/*e* (relative intensity) identity of ion if known; 290 (39) MH^+; 244 (7) MH^+–H_2O–CO; 182 (6) M–H_3COPh; 121 (100) $H_3COPhCH_2^+$; 107 (12) H_3COPh^+. HRFAB MS (3-NBA/gly/TFA matrix): *m*/*e* calcd for $C_{16}H_{20}NO_4$ (MH^+) 290.1392; found 290.1392 (dev −0.1 ppm).

(2*R*)-1-(4-Methoxybenzyl)-2-prop-2-enyl-5-oxoprolyl-L-phenylalanine benzyl ester. The carboxylic acid made in the previous experiment (1.41 g, 4.9 mmol) and 0.79 g (5.8 mmol, 1.2 equiv) of HOBt were placed in a dry 50 mL flask with stir bar and 10 mL of CH_2Cl_2. While stirring the mixture under N_2, 1.4 mL (1.04 g, 10.3 mmol, 2.1 equiv) of Et_3N was added and all was dissolved. The reaction solution was then cooled to 0 °C, and then 1.57 g (5.4 mmol, 1.1 equiv) of H_2N-L-Phe-Obn·HCl and 1.03 g (5.4 mmol, 1.1 equiv) of EDC were added. The reaction mixture was allowed to warm to 25 °C and was stirred vigorously under N_2 for 10 h. The

CH_2Cl_2 was then removed in vacuo and the crude residue was dissolved in 20 mL of EtOAc and 20 mL of Et_2O. The organic solution was washed with 25 mL of 5% aq citric acid solution followed by 25 mL of 5% aq $NaHCO_3$ solution. Both aq extracts were re-extracted twice with 40 mL portions of Et_2O before proceeding with the next wash. The combined organic extracts were dried over $MgSO_4$, filtered, and concentrated in vacuo to a thick brown oil. The crude product was then purified by gravity-flow chromatography through 125 g of slurry packed silica gel using a 1:1 hexane/Et_2O to 100% Et_2O gradient solvent mixture. Fractions containing product (TLC solvent Et_2O, R_f 0.16, UV-active spot or pale-yellow spot with *p*-anisaldehyde stain) were combined, dried over $MgSO_4$, filtered, and concentrated in vacuo to afford 2.50 g (4.75 mmol, 96%) of the pure coupled product as a white solid. ^{1}H NMR (300 MHz, $CDCl_3$) two rotamers visible, δ 7.39–7.15 (m, 11H), 7.03–6.96 (m 2H), 6.80–6.74 (m, 2H), 6.22 and 6.20 (2 unresolved br s, 1H), 5.33–4.94 (unresolved m, 4H), 4.72–4.65 (m, 1H), 4.49 (d, 0.7H, J = 15.0 Hz), 4.48 (unresolved m, 0.3H), 4.07 (d, 0.7H, J = 15.0 Hz), 3.82–3.70 (unresolved m, 0.3H), 3.74 (s, 3H), 3.15 (dd, A of ABX, 0.7H, J_{AB} = 14.1 Hz, J_{AX} = 5.6 Hz), 3.05–2.94 (m, 0.3H), 2.91–2.79 (m, 1H), 2.59 (dd, A of ABX, 1H, J_{AB} = 14.9 Hz, J_{AX} = 8.5 Hz), 2.44 (dd, B of ABX, 1H, J_{AB} = 14.9 Hz, J_{BX} = 5.4 Hz), 2.35–2.10 (unresolved m, 2.7H), 2.03–1.93 (unresolved m, 1.3H), 1.84–1.73 (m, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) two rotamers visible, δ 176.8, 173.6, 170.8, 159.8, 136.0, 135.6, 131.9, 130.2, 130.1, 130.0, 129.9, 128.8, 128.7, 128.6, 128.5, 128.4, 126.7, 120.4, 114.0, 169.9, 169.8, 167.7, 167.6, 54.4, 53.8, 53.7, 44.4, 39.0, 38.9, 37.2, 29.8, 29.3, 29.0. IR (thin film) 3322 (m), 3205 (w), 3055 (m), 3027 (m), 2925 (m), 1740 (s), 1671 (bs), 1613 (m), 1582 (m), 1514 (ms), 1456 (ms), 1404 (m), 1353 (mw), 1264 (m), 1247 (m), 1113 (mw), 1083 (w), 1035 (m), 1004 (mw), 925 (w), 819 (w), 733 (s), 703 (s) cm^{-1}. LRFAB MS (3-NBA/gly/TFA matrix) *m*/*e* (relative intensity) identity of ion if known; 527 (17) MH^+; 244 (52) MH^+–H_2N-Phe-OBn–CO; 211 (11); 124 (13) MH^+–H_2N-Phe-OBn–CO–$H_3COPhCH_2^+ + H^+$; 121 (100) $H_3COPhCH_2^+$; 107 (7) H_3COPh^+; 91 (90) $C_7H_7^+$. HRFAB MS (3-NBA/gly/TFA matrix): *m*/*e* calcd for $C_{32}H_{35}N_2O_5$ (MH^+) 527.2546; found 527.2553 (dev +1.3 ppm).

(2*R*)-1-(4-Methoxybenzyl)-2-(3-hydroxypropyl)-5-oxoprolyl-L-phenylalanine benzyl ester (11). A 50 mL flask with stir bar was flame dried and cooled under argon, and then 4.5 mL of THF and 0.93 mL of a 10 M solution of BH_3•Me_2S in Me_2S (9.3 mmol, 2 equiv) were added to the flask. The solution was cool to 0 °C under argon and then 2.0 mL (1.3 g, 18.6 mmol, 4 equiv) of 2-methyl-2-butene was added slowly via syringe. The solution was stirred while slowly warming to 10 °C over 45 min and then re-cooled to 0 °C at which time 2.45 g (4.65 mmol) of the olefinic product from the previous step was added by quantitative transfer via *cannula* as a solution in 19 mL of THF. The reaction solution was then allowed to slowly warm to 25 °C over 1.5 h and then re-cooled to 0 °C and diluted with 25 mL of Et_2O. Next, 23 mL of a 2:1 mixture of 3 N aq NaOH and 30% aq H_2O_2 was added very slowly. The solution was slowly warmed to 25 °C while stirring, and then the layers were separated. The aq phase was extracted twice with 50 mL portions of Et_2O. The Et_2O extracts were combined, dried over $MgSO_4$, filtered, and concentrated in vacuo. The crude product was then purified by flash chromatography through 125 g of slurry-packed silica gel using a 1:1 Et_2O/EtOAc solvent mixture. Fractions containing product (TLC solvent same as column, R_f 0.20, UV-active spot or blue spot with *p*-anisaldehyde stain) were combined, dried over $MgSO_4$, filtered, and concentrated in vacuo to afford 2.26 g (4.15 mmol, 89%) of pure **11** as a white solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.39–7.14 (m, 10H), 7.03–6.98 (m, 2H), 6.79–6.74 (m, 2H), 6.19 and 6.17 (2 br s, 1H), 5.19 (d, A of AB, 1H, J = 12.1 Hz), 5.08 (d, B of AB, 1H, J = 12.1 Hz), 4.76–4.68 (m, 1H), 4.53 (d, A of AB, 1H, J = 15.1 Hz), 3.93 (d, B of AB, 1H, J = 15.1 Hz), 3.74 (s, 3H), 3.32–3.27 (m, 2H), 3.14 (dd, A of ABX, 1H, J_{AB} = 14.0 Hz, J_{AX} = 5.9 Hz), 2.84 (dd, B of ABX, 1H, J_{AB} = 14.0 Hz, J_{AX} = 8.1 Hz), 2.36–2.22 (m, 2H), 2.04–1.87 (unresolved m, 3H), 1.67–1.57 (m, 1H), 1.43–1.35 (m, 1H), 1.28–1.23 (m, 1H), 1.10–1.01 (m, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 176.2, 173.0, 170.8, 158.8, 135.6, 134.8, 130.0, 129.6, 128.9, 128.6, 128.55, 128.5, 127.2, 113.6, 70.4, 67.4, 62.1, 55.3, 53.2, 44.3, 37.3, 30.5, 29.4, 29.0, 26.3. IR (diffusion reflectance) 3462 (s), 3280 (s), 3200 (bm), 3076 (m), 2992 (m), 2939 (m), 1732 (s), 1668 (s), 1555 (s), 1453 (m), 1407 (m), 1249 (m), 1116 (m), 1071 (m), 1037 (m), 965 (m), 845 (m), 814 (m), 746 (s), 705 (s), 584 (m), 524 (m) cm^{-1}. LRFAB MS (3-NBA/gly/TFA matrix) *m*/*e* (relative intensity) identity of ion if known; 545 (100) MH^+; 527 (5) MH^+–H_2O; 437 (14) MH^+–C_7H_7OH or MH^+–CH_3OPh^+; 407 (18). HRFAB MS (3-NBA/gly/TFA matrix): *m*/*e* calcd for $C_{32}H_{37}N_2O_6$ (MH^+) 545.2651; found 545.2640 (dev −1.0 ppm).

N^{α}-[(5*R*)-2,6-Dioxo-1-(4-methoxybenzyl)-1-azaspiro[5.4]-nonyl]-L-phenylalanine benzyl ester. In a dry 10 mL flask with stir bar were placed 0.37 g (0.8 mmol) of **11** and 0.36 g (1.4 mmol, 2 equiv) of Ph_3P. The mixture was dissolved in 3.4 mL of THF, and then 0.21 mL (0.24 g, 1.4 mmol, 2 equiv) of DEAD was added slowly via syringe while vigorously stirring under an atmosphere of argon. The reaction solution was then allowed to stir at 25 °C for 12 h, after which the solution was diluted with 5 mL of EtOAc and washed with 10 mL of saturated aq $NaHCO_3$ solution. The organic phase was then dried over $MgSO_4$, filtered, and concentrated in vacuo. The crude product was partially purified by flash chromatography through 40 g of slurry packed silica gel using a 1:1 Et_2O/hexane to 100% Et_2O to 1:1 Et_2O/EtOAc gradient solvent mixture. Fractions containing product (TLC solvent Et_2O, R_f 0.17, UV-active spot or blue spot with *p*-anisaldehyde stain) along with Ph_3PO byproduct were combined, dried over $MgSO_4$, filtered, and concentrated in vacuo to afford approximately 0.35 g (0.66 mmol, 98%) of the cyclized product in an 85:15 (by NMR) mixture with Ph_3PO. The product was not further purified. ^{1}H NMR of mixture (300 MHz, $CDCl_3$) δ 7.71–6.71 (m, 14H + Ph_3PO contaminant), 5.31–5.13 (unresolved m, 3H), 4.93 (d, 0.5H, J = 15.6 Hz), 4.76 (d, 0.5H, J = 15.9 Hz), 3.76 and 3.74 (2 s, 3H),

3.74–3.42 (unresolved m, 1.5H), 3.16–3.07 (m, 2H), 2.97 (dm, 1H, $J_d = 4.4$ Hz), 2.85 (d, 0.5H, $J = 15.9$ Hz), 2.68–2.55 (m, 0.5H), 2.68–2.55 (m, 0.5H), 2.43–2.18 (m, 2H), 1.80–1.33 (unresolved m, 5.5H). LRFAB MS of mixture (3-NBA/gly/TFA matrix) *m*/*e* (relative intensity) identity of ion if known; 527 (14) MH^+; 419 (8) MH^+–C_7H_7OH; 391 (12) MH^+–C_7H_7OH–CO; 279 (70) Ph_3POH^+; 121 (100) $H_3COPhCH_2^+$; 91 (60) $C_7H_7^+$. HRFAB MS (3-NBA/gly/TFA matrix): *m*/*e* calcd for $C_{32}H_{35}N_2O_5$ (MH^+) 527.2546; found 527.2789 (dev −1.6 ppm).

N^{α}-[(5*R*)-2,6-Dioxo-1-azaspiro[5.4]-nonyl]-L-phenylalanine benzyl ester (12). The spirocyclic building block made in the previous experiment (ca. 0.40 g, 0.77 mmol) contaminated with approx 0.07 g (0.26 mmol) of Ph_3PO was dissolved in 3 mL of CH_3CN and 3 mL of H_2O in a 25 mL flask with stir bar. When all was dissolved, 1.05 g (1.9 mmol, 2.5 equiv) of CAN was added, and the reaction solution was left to stir at 25 °C for 2 h. The solution was diluted with 5 mL of H_2O, poured into a 125 mL separatory funnel, and extracted twice with 30 mL portions of EtOAc. The EtOAc extract was then washed with 15 mL of 5% aq $NaHCO_3$ solution, and then was dried over $MgSO_4$, filtered, and concentrated in vacuo. The crude product was purified by flash chromatography through 20 g of slurry-packed silica gel using EtOAC as eluent. Fractions containing product (TLC solvent same as column, R_f 0.14, UV-active spot or blue spot with *p*-anisaldehyde stain) were combined, concentrated in vacuo, dried over $MgSO_4$, filtered, and concentrated again in vacuo to afford 0.20 g (0.49 mmol, 53% over two steps) of pure **12** as a white solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.40–7.15 (m, 10H), 5.35 (d, A of AB, 1H, $J = 12.1$ Hz), 5.10 (d, B of AB, 1H, $J = 12.1$ Hz), 4.86 (dd, X of ABX, 1H, $J = 5.8$, 10.7 Hz), 3.39 (dd, A of ABX, 1H, $J_{AB} = 14.3$ Hz, $J_{AX} = 5.8$ Hz), 3.28–3.14 (unresolved m, 2H), 2.93–2.85 (m, 1H), 2.65–2.56 (m, 1H), 2.39–2.22 (m, 2H), 1.82–1.58 (unresolved m, 5H), 1.68 (s due to H_2O); ^{13}C NMR (75 MHz, $CDCl_3$) δ 178.1, 171.7, 169.8, 136.8, 135.3, 128.8, 128.6, 128.4, 128.3, 128.1, 126.7, 66.9, 61.3, 59.9, 47.3, 34.7, 34.1, 33.4, 30.0, 19.5. IR (thin film) 3233 (bm), 3062 (m), 3072 (m), 2945 (s), 2877 (m), 1740 (s), 1702 (s), 1647 (s), 1493 (ms), 1452 (s), 1346 (m), 1326 (s), 1267 (m), 1209 (ms), 1083 (w), 1028 (mw), 980 (w) cm^{-1}. LRFAB MS (3-NBA/gly/TFA matrix) *m*/*e* (relative intensity) identity of ion if known; 407 (63) MH^+; 299 (2) MH^+–C_7H_7OH; 271 (2) MH^+–C_7H_7OH–CO; 243 (8) MH^+–C_7H_7OH–CO–CO (or C_2H_4); 154 (32); 136 (26); 124 (16); 107 (11); 91 (100) $C_7H_7^+$ HRFAB MS (3-NBA/gly/TFA matrix): *m*/*e* calcd for $C_{24}H_{27}N_2O_4$ (MH^+) 407.1971; found 407.1963 (dev −1.9 ppm).

N^{α}-[(5*R*)-2,6-Dioxo-1-azaspiro[5.4]nonyl]-L-phenylalanine. Compound **12** (0.48 g, 1.2 mmol) and approx 1 g of Pd on activated carbon (Pd 5% by weight) were placed in a dry 25 mL flask with stir bar. The flask was flushed with argon, and then the contents were dissolved in 2.5 mL of CH_2Cl_2. A 2.0 L H_2 balloon was then placed on the reaction flask and the argon was forced out, filling the flask with H_2. The reaction was then left to stir at 25 °C and 1 atm for 20 h, after which the H_2 balloon was removed and the flask flushed with argon once more. The reaction mixture was then filtered through 200 g of well-packed Celite to remove the catalyst, and the filtrate was concentrated in vacuo to afford 0.36 g (1.1 mmol, 98%) of the deprotected acid as a white solid that was >98% pure by NMR. The product was not purified further. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.99 (br s, 1H), 7.67 (br s, 1H), 7.33–7.17 (m, 5H), 3.93 (dd, 1H, $J = 6.1$, 9.8 Hz), 3.45–3.30 (unresolved m, 3H), 2.72–2.60 (m, 2H), 2.44–2.32 (m, 2H), 2.04–1.96 (m, 1H), 1.88–1.46 (unresolved m, 4H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 180.4, 172.5, 172.0, 138.1, 129.0, 128.6, 128.4, 126.6, 64.2, 62.2, 50.9, 34.2, 34.1, 32.9, 30.0, 19.5. IR (diffusion reflectance) 3250 (bs), 3000 (very bs), 2939 (s), 2864 (s), 2576 (bm), 1705 (s), 1649 (s), 1490 (m), 1449 (ms), 1354 (ms), 1328 (ms), 1283 (m), 1207 (s), 1139 (m), 1086 (mw), 1033 (w), 939 (w), 894 (w), 822 (w), 750 (m), 701 (m), 671 (mw), 644 (mw), 569 (w), 508 (w) cm^{-1}. LRFAB MS (3-NBA/Li^+ matrix) *m*/*e* (relative intensity) identity of ion if known; 323 (20) MLi^+; 249 (5) MLi^+–H_2O–2CO; 202 (9); 160 (100); 136 (30); 91 (25) $C_7H_7^+$. LREI MS: *m*/*e* (relative intensity) identity of ion if known; 316 (12) M^+; 298 (34) M^+–CHBn–CO_2; 124 (93); 110 (100); 91 (74) $C_7H_7^+$. HRFAB MS (3-NBA/Li^+ matrix): *m*/*e* calcd for $C_{17}H_{20}N_2O_4Li$ (MLi^+) 323.1583; found 323.1582 (dev-0.3 ppm).

N^{α}-[(5*R*)-2,6-Dioxo-1-azaspiro[5.4]-nonyl]-L-phenylalanyl-L-prolinamide (8). The carboxylic acid made in the previous step (0.23 g, 0.71 mmol) and 0.12 g (0.85 mmol, 1.2 equiv) of HOBt were placed in a dry 50 mL flask with stir bar and 1.5 mL of CH_2Cl_2, and the mixture was cooled to 0 °C. While stirring the mixture under N_2, 0.14 mL (0.12 g, 1.1 mmol, 1.5 equiv) of NEM and 0.09 g (0.8 mmol, 1.1 equiv) of HN-L-Pro-NH_2 were added. When all was dissolved, 0.16 g (0.85 mmol, 1.2 equiv) of EDC was added. The reaction mixture was allowed to slowly warm to 25 °C and stirred 14 h under N_2. The reaction solution was then diluted with 5 mL of EtOAc washed with 3 mL of 5% aq citric acid solution followed by 3 mL of 5% aq $NaHCO_3$ solution. Both aq extracts were re-extracted twice with 5 mL portions of EtOAc before proceeding with the next wash. The combined EtOAc extracts were dried over $MgSO_4$, filtered, and concentrated in vacuo. The crude product was then purified by flash chromatography through 15 g of slurry packed silica gel using a 20:80 MeOH/EtOAc solvent mixture. Fractions containing product (TLC solvent same as column, R_f 0.40, faint UV-active spot or pale brown spot with *p*-anisaldehyde stain) were combined, concentrated in vacuo, re-dissolved in 10 mL of CH_2Cl_2, dried over $MgSO_4$, filtered, and concentrated in vacuo to afford 0.21 g (0.51 mmol, 70%) of pure product **8** as a white solid that could be recrystallized as the monohydrate from hexane for biological testing. ^{1}H NMR (300 MHz, $CDCl_3$) two rotamers, δ 7.36–7.22 (m, 5H), 7.09 (br s, 0.25H), 6.71 (br s, 0.75H), 6.68 (br s, 0.25H), 6.55 (br s, 0.75H), 6.12 (br s, 0.25H), 5.47 (br s, 0.75H), 5.20–5.15 (m, 0.75H), 4.60–4.50 (m, 0.75H), 4.50–4.45 (m, 0.25H), 4.20–4.16 (m, 0.25H), 3.82–3.79 (m, 0.25H), 3.67–3.56 (m, 1H), 3.43–3.22 (unresolved m, 3.5H), 3.09 (dd, A of ABX,

0.75H, $J_{AB}=13.7$ Hz, $J_{AX}=8.5$ Hz), 2.87–2.82 (m, 0.25H), 2.67–2.28 (unresolved m, 3H), 2.15–2.07 (unresolved m, 1.5H), 1.98–1.56 (unresolved m, 7.5H), 1.30–1.20 (m, 0.25H); ^{13}C NMR (75 MHz, $CDCl_3$) two rotamers, δ 178.7, 178.5, 174.0, 173.8, 172.4, 169.5, 169.2, 137.6, 129.4, 129.2, 128.6, 128.5, 127.2, 62.1, 61.7, 61.6, 59.8, 47.4, 45.5, 34.9, 34.8, 33.5, 32.2, 30.0, 28.0, 25.0, 21.9, 19.6, 19.2. IR (diffusion reflectance) 3196 (m), 3025 (w), 2960 (m), 2871 (m), 1687 (s), 1640 (s), 1625 (s), 1439 (m), 1350 (m), 1289 (m), 1187 (m), 915 (w), 704 (m), 500 (m) cm^{-1}. LRFAB MS (3-NBA/Li^+ matrix) *m*/*e* (relative intensity) identity of ion if known; 419 (32) MLi^+; 313 (52); 160 (100) MLi^+–$C_{14}H_{17}N_2O_2$–N; 127 (47); 119 (38). HRFAB MS (matrix): *m*/*e* calcd for $C_{22}H_{28}N_4O_4Li$ (MLi^+) 419.2270; found 419.2253 (dev 5 ppm). Elemental analysis calcd for $C_{22}H_{28}N_4O_4$&z_-rad;H_2O: C, 61.38; H, 7.02; N, 13.01. Found: C, 61.05, H, 7.08, N, 13.33.

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