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Synthesis and Biological Activity of Novel Neuroprotective Diketopiperazines

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Abstract—The cyclic dipeptide cyclo[His-Pro] (CHP) is synthesized endogenously de novo and as a breakdown product of thyrotropin-releasing hormone (TRH), a tripeptide with known neuroprotective activity. We synthesized two isomeric compounds based on the structure of CHP, in which the histidine residue was replaced by 3,5-di-*tert*-butyltyrosine (DBT), a phenolic amino acid that traps reactive oxygen species. These novel diketopiperazines prevented neuronal death in an in vitro model of traumatic injury. In addition, they dose-dependently prevented death caused by the direct induction of free radicals, and by calcium mobilization through an agent that evokes rapid, necrotic death. The drugs showed activity in the latter system at picomolar concentrations. The neuroprotective profile of these compounds suggests that they may be useful as treatments for neuronal degeneration in vivo, potentially through several different mechanisms.

Introduction

Thyrotropin-releasing hormone (TRH)[1] is a tripeptide present in the central nervous system (CNS) as well as in the periphery. Its best-described role is that of a hypothalamic neuroendocrine hormone. Exogenous administration of TRH elicits a variety of behavioral responses. Among these are changes in sleep/arousal,[2] temperature,[3] autonomic outflow,[4] and interactions with many physiological effects of opioids/opiates.[5]

TRH and its analogues have been investigated as potential treatments for a variety of disorders, including CNS trauma, where they showed substantial beneficial effects. Continuous infusion of TRH or bolus injections of analogues with intact C-termini and longer half-lives improved recovery across species in models of traumatic injury to the brain or spinal cord.[6] Such benefits may be related to reversal of pathophysiological secondary events (i.e., release of glutamate, generation of free radicals) that are initiated by the insult and evolve from minutes to days later. For example, TRH or TRH analogues stabilize levels of catecholamines, restore cationic imbalances, and antagonize the pathophysiological effects of endogenous opioids, leukotrienes and platelet-activating factor that occur after injury.[7]

Whether synthesized endogenously or injected, TRH is rapidly metabolized in blood and CNS tissue. The cyclic dipeptide cyclo[His-Pro] (CHP) is generated by primary cleavage of the pyroglutamyl residue from TRH by the abundant and relatively non-specific enzyme pyroglutamyl aminopeptidase.[8] CHP is also synthesized de novo endogenously, where it is both detectable in CNS tissue by radioimmunoassay and has demonstrable bioactivity. Because CHP and TRH share similarities in biological activity, and exogenous administration of TRH can increase CHP, it has been hypothesized that at least some of the effects of TRH administration may occur through CHP.[9] Therefore, we designed a novel

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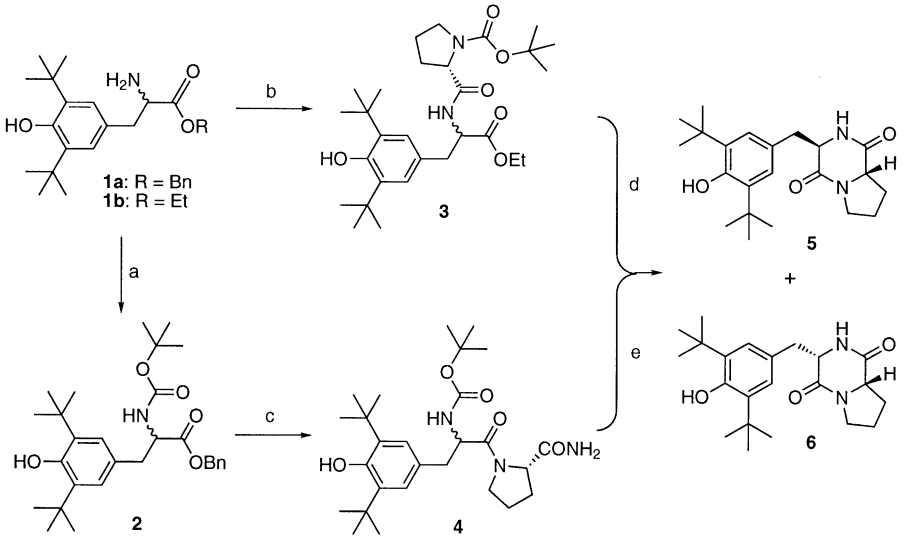

Scheme 1. Reagents and conditions: (a) $(BOC)_2O$, $^iPr_2NEt$, rt; (b) DCC, HOBt, *N*-(*tert*-butoxycarbonyl)-L-proline; (c) (i) H_2/Pd/C; (ii) L-prolinamide, DCC, HOBt; (d) (i) CF_3COOH, CH_2Cl_2 (ii) NaCN, MeOH; (e) (i) CF_3COOH, CH_2Cl_2 (ii) Et_3N, CH_2Cl_2.

cyclic dipeptide related to CHP in which the imidazole moiety is replaced by a di-*tert*-butylphenol (i.e., the histidine residue is replaced by 3,5-di-*tert*-butyltyrosine). Our rationale was that the known structure of CHP, combined with the ability of certain phenols to act as scavengers for reactive oxygen species (ROS) might yield compounds with novel neuroprotective potential. We report the route of synthesis of the two diastereomeric cyclic dipeptides, cyclo[(di-*tert*-Bu)Tyr-Pro], together with details of their action in several different in vitro models of neuronal injury. These molecules appear to provide an interesting profile of action that warrants further study.

Chemistry

The diketopiperazines **5** and **6** were prepared in optically pure form starting from the benzyl or ethyl ester of 3′,5′-di-*tert*-butyltyrosine (**1a**/**1b**)[10] in two different ways (Scheme 1). Compound **1a** was prepared in racemic form by reaction of the anion of benzyl *N*-(diphenylmethylene)glycinate with 4-(bromomethyl)-2,6-di-*tert*-butylphenol followed by HCl workup. Next, the free amine **1a** was treated with $(BOC)_2O$ in the presence of diisopropylethylamine to afford the amino-protected tyrosine derivative **2**. The benzyl ester group of **2** was cleaved by hydrogenolysis over Pd/C to obtain the free acid, which was then coupled with L-prolinamide in presence of HOBt and DCC to give **4** as a diastereomeric mixture. Compound **4** was further converted to **5** and **6** by treatment with trifluoroacetic acid followed by triethylamine. The two diastereomers (**5** and **6**) were separated by column chromatography.

As an alternative and shorter route to the same cyclic peptides, the ethyl ester **1b** was coupled with the *N*-(*tert*-butoxycarbonyl)-L-proline in the presence of HOBt and DCC in CH_2Cl_2 to yield the dipeptide **3** in 80% yield. This intermediate was then treated with TFA in methylene chloride followed by NaCN in MeOH to provide the diketopiperazines **5** and **6** in good yield. Chromatographic purification of the crude diastereomeric mixture gave equal amounts of **5** and **6**. The structures of compounds **5** and **6** were established by NMR analysis, and additionally, a single-crystal X-ray analysis was carried out on **6** (Fig. 1). The second route is the more efficient one of the two, as only three synthetic steps are required from the starting amino acid **1b**.

Biological studies — activity in a model of CNS injury

To evaluate whether **5** and **6** showed neuroprotective activity we tested them in several models of cell death. 3,5-Di-*tert*-butyltyrosine (DBT) and cyclo(Gly-Pro) (CGP),[11] the components of the new drugs **5** and **6**, were used as controls. The in vitro mechanical punch model has been used by our group and others to mimic both acute cell destruction and secondary loss associated with traumatic injury in vivo.[12] In this model, neuronal-glial co-cultures are subjected to physical

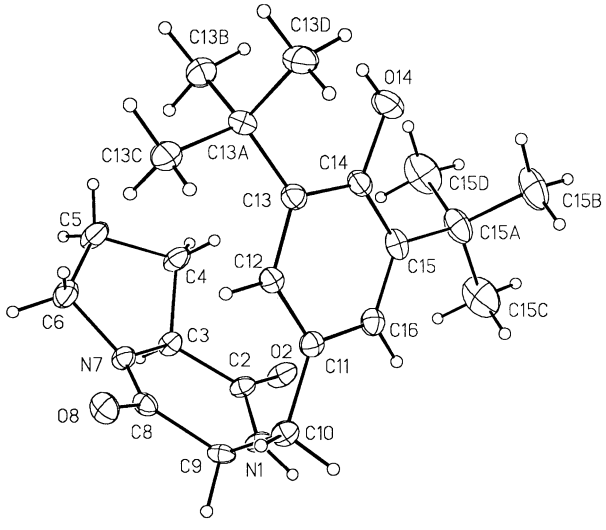

Figure 1. Single-crystal X-ray structure of the diketopiperazine **6**.

disruption with a device that rapidly deploys a series of 28 parallel blades to the culture surface. Cells under the blades are destroyed on contact, and the cultures are then washed to remove resultant debris. However, over the course of the ensuing 24 h after impact, neurons throughout the culture slowly begin to die; calcein staining reveals that the highest proportion of cell death occurs at and near the site of impact, with progressively lower and more delayed cell death/injury taking place as the distance from impact increases. Because this injury is both delayed and indirect, it serves as a useful model of secondary injury mechanisms that contribute to impairment in vivo.[12] In our co-culture system, activity of the enzyme lactate dehydrogenase in the culture media correlates with total neuronal death. Treatment with either **5** or **6** reduced cell death in this model (Fig. 2); this activity followed an inverted U-shaped dose-response curve. Neither DBT nor CGP prevented cell death in this model, suggesting that neither the 3,5-di-*tert*-butyltyrosine nor the cyclo(Gly-Pro) components of **5** and **6** were singularly involved in their neuroprotective activity.

Multiple factors have been implicated in the secondary injury response. They include free radicals, glutamate, influx and cellular dysregulation of sodium and calcium, breakdown of phospholipids with the generation of toxic by-products, activation of inflammatory cascades and alterations in signal transduction (i.e., protein kinase C and others), and induction of programmed (apoptotic)[13,14] cell death cascades. Therefore, to characterize the neuroprotective activity of **5** and **6** further, we evaluated whether they would prevent cell death induced by several of these injury-associated factors/mechanisms.

Calcium-induced necrosis

Maitotoxin is a marine, algal, polyether type neurotoxin that induces cell death via external calcium influx and mobilization of intracellular calcium stores.[15] It produces rapid deterioration and necrotic morphology, as well as activation of enzymes associated with necrotic cell death.[15] Compounds **5** and **6** were very effective at preventing death induced by a maitotoxin pulse (Fig. 3). High concentrations of DBT and CGP also showed some neuroprotection in this model, but it was 4-fold less than that provided by equal concentrations of **5** or **6**. These data suggest that both the DBT and CGP moieties of **5** and **6** may each contribute, albeit minimally, to the observed neuroprotective properties of **5** and **6** against cell death induced by maitotoxin. MK-801 showed no activity.

Excitotoxic cell death

The next objective was to determine whether **5** and **6** prevented excitotoxic cell death induced by a glutamate pulse. Neither compound prevented this type of cell death. Higher concentrations of these drugs also had no effect (data not shown). MK-801, a selective inhibitor of the NMDA subtype of glutamate receptors, served as a positive control.

Apoptotic death

Staurosporine induces apoptotic death and activation of enzymes (caspases) specifically associated with this type of cell self-destruction in cultured neurons.[14] Therefore we examined whether **5** and **6** could prevent this type of cell death in our culture system. The drugs had no effect on death induced by 0.1 μM staurosporine at concentrations of 0.1–100 μM (data not shown). MK-801 was also ineffective in this model.

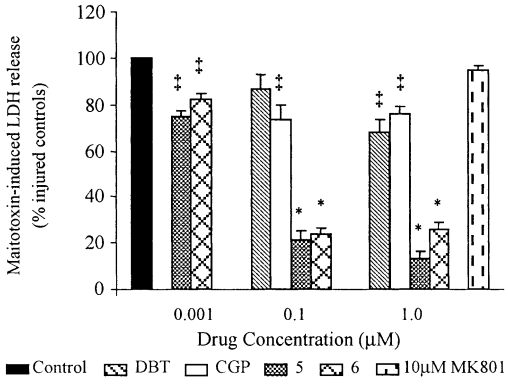

Figure 3. Bars represent the mean ± SEM for $n = 40$–46 wells per condition. $^{\ddagger}p < 0.01$, $p < 0.0001$ for *t*-test with Bonferroni correction.

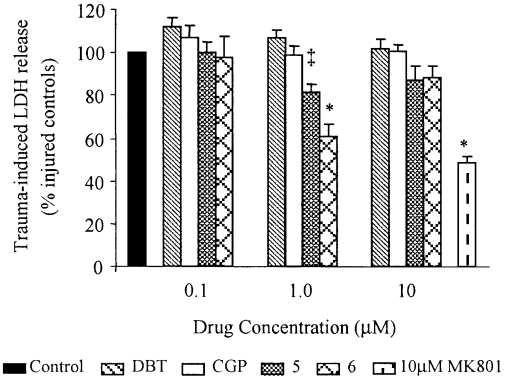

Figure 2. Bars represent the mean ± SEM for $n = 40$–46 wells per condition. $^{\ddagger}p < 0.01$, $^{\star}p < 0.0001$ for *t*-test with Bonferroni correction.

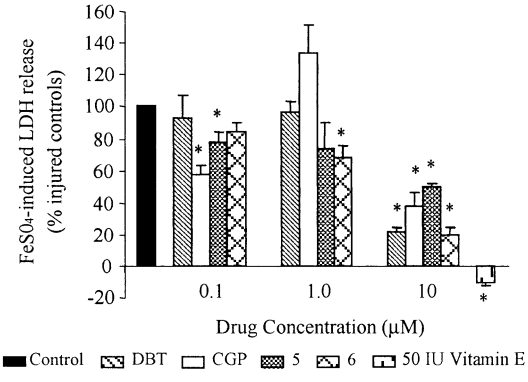

Figure 4. Bars represent the mean ± SEM for $n = 40$–46 wells per condition. $^{+}p < 0.05$, $^{\ddagger}p < 0.01$, $^{\star}p < 0.0001$ for *t*-test with Bonferroni correction.

Free-radical-induced death

As anticipated based upon the presence of the di-*tert*-butylphenol group in these cyclic dipeptides, both compounds **5** and **6** increased survival in a model of free radical induced cell death[16] initiated by $FeSO_4$ (Fig. 4). However, in contrast to the results seen with the mechanical punch, the neuroprotective efficacy in this model followed a dose-dependent activity curve. DBT and CGP showed neuroprotective activity as well, with DBT and **6** providing the best protection at the highest concentrations tested. As glycine itself is known in the literature[17] to serve as an antioxidant, it was not surprising to find that CGP, a cyclic derivative of glycine, shows significant neuroprotection even at a concentration of 0.1 μM in this model. These data indicate that not only does the DBT moiety contribute to the free-radical scavenging properties of **5** and **6**, but that the CGP moiety may do so as well. Vitamin E, a well-described anti-oxidant, was utilized as a positive control.

Conclusion

In summary, the novel cyclic dipeptides reported herein show neuroprotective properties in a neuronal-glial model of trauma, which involves activation of multiple secondary injury factors. They are also able to block calcium-induced necrotic cell death initiated by maitotoxin as well as death induced by $FeSO_4$, a free radical generator. Secondary tissue damage and associated cell death after acute CNS insults thought to reflect a multifactorial biochemical process that together cause cellular necrosis or apoptosis.[18] Development of compounds **5** and **6**, like other diketopiperazines or related tripeptides,[19] was based upon the concept that effective treatment of CNS injury requires either use of multiple neuroprotective agents with complementary mechanisms or use of single compounds (like TRH) with multifactorial actions. In the present studies, compounds **5** and **6** attenuated both trauma and maitotoxin induced cell death in vitro; each of these models causes almost exclusively necrotic cell death. Data from the DBT and CGP controls suggest that each of these components contributes to the neuroprotective effects of **5** and **6**. In contrast, the compounds did not inhibit glutamate induced cell death, which can cause either necrotic or apoptotic cell death, depending upon concentration, duration of treatment or age of cultures. They also did not inhibit a classical apoptotic cell death model. Free radicals may cause either necrotic or apoptotic cell death through different mechanisms.[20] Taken together, the ability of compounds **5** or **6** to modify three different in vitro models, all of which may be associated with cellular necrosis, suggests that the protective actions reflect antinecrotic properties. Yet their inability to modify glutamate induced changes indicates that other pro-necrotic factors may be involved, such as free radical induced lipid peroxidation[20] or effects of calpains.[15] Thus, the unique combination of these moieties yields compounds with a pharmacological profile that indicates an action through several different secondary injury mechanisms. When tested at concentrations as high as 100 μM, these compounds failed to show any cytotoxicity. Therefore, these agents may have potential utility as treatments for acute CNS injuries such as stroke and trauma.

Experimental

Chemical methods

General. Starting materials were obtained from the Aldrich Chemical Co. or from other commercial suppliers. Solvents were purified as follows: diethyl ether was distilled from phosphorus pentoxide; THF was freshly distilled under nitrogen from sodium/benzophenone. ^1H and ^{13}C NMR spectra were obtained with a Varian Unity Inova instrument at 300 and 75.46 MHz, respectively. ^1H chemical shifts (δ) are reported in ppm downfield from internal TMS. ^{13}C chemical shifts are referenced to $CDCl_3$ (central peak, δ = 77.0 ppm). NMR assignments were made with the help of COSY, NOESY, DEPT, and HETCOR experiments. Melting points were determined in Pyrex capillaries with a Thomas-Hoover Unimelt apparatus and are uncorrected. Mass spectra were measured in the EI mode at an ionization potential of 70 eV. TLC was performed on Merck silica gel $60F_{254}$ glass plates; column chromatography was performed using Merck silica gel (60–200 mesh). Abbreviations: THF, tetrahydrofuran; LDA, lithium diisopropylamide; DCC, dicyclohexylcarbodiimide; DMF, dimethylformamide; HOBt, 1-hydroxybenzotriazole.

3′,5′-Di-*tert*-butyltyrosine benzyl ester (1a).[10] To a solution of benzyl *N*-(diphenylmethylene)glycinate[18] (1.3 g, 4.0 mmol) in THF (20 mL) at −78 °C was added under a N_2 atmosphere LiHMDS (5.0 mL, 1.0 M solution in THF, 5.0 mmol). After stirring for 15 min at −78 °C, 4-(bromomethyl)-2,6-di-*tert*-butylphenol (1.2 g, 4.0 mmol) was added dropwise, and the resulting mixture was stirred for an additional 8 h. The solvent was removed under reduced pressure, and the residue was stirred with ether/1 N HCl (20 mL, 1:1 mixture) overnight at rt. The reaction mixture was neutralized to pH 7.0 and extracted with ether (3 × 100 mL). The combined ether layers were washed with brine, dried (Na_2SO_4), and concentrated. The residue on purification by flash chromatography (hexane/ethyl acetate 1:1) yielded the ester **1a** (0.84 g) in 54% yield: ^1H NMR ($CDCl_3$) δ 1.43 (s, 18H), 2.88 (dd, J = 7.5, 13.5 Hz, 1H), 3.05 (dd, J = 5.7, 13.5 Hz, 1H), 3.79 (dd, J = 5.7, 7.2 Hz, 1H), 5.13 and 5.19 (ABq, J = 12.3 Hz, 2H), 7.00 (s, 2H), 7.33 (m, 5H). ^{13}C NMR ($CDCl_3$) δ 30.51, 34.47, 41.27, 56.29, 66.83, 125.99, 127.70, 128.28, 128.49, 128.77, 135.75, 136.20, 152.90, 175.40. MS (EI) m/z 383 (M^+, 3%), 219 (100%).

3′,5′-Di-*tert*-butyltyrosine ethyl ester (1b)..[10] To a solution of ethyl *N*-(diphenylmethylene)glycinate[18] (1.3 g, 3.9 mmol) in THF (20 mL) at −78 °C was added LDA (5.0 mL, 1.0 M solution in THF, 5.0 mmol) under a N_2 atmosphere. After stirring for 15 min at −78 °C, a solution of 4-(bromomethyl)-2,6-di-*tert*-butylphenol (1.2 g,

4.0 mmol) in THF (5 mL) was added. The resulting mixture was stirred for an additional 5 h at −78 °C. The solvent was removed. To the resulting residue was added ether (10 mL) followed by HCl (1 N, 10 mL), and the mixture was stirred at room temperature overnight. The reaction mixture was neutralized to pH 7.0 and extracted with ether (3 × 100 mL). The combined ether layers were washed with brine, dried (Na_2SO_4), and concentrated. The residue was purified by column chromatography on silica gel (EtOAc/MeOH 15:1) to give the ester **1b**. Yield: 1.0 g (65%). ^{1}H NMR ($CDCl_3$) δ 1.27 (t, J = 7.2 Hz, 3H), 1.43 (s, 18H), 2.84 (dd, J = 7.8, 13.8 Hz, 1H), 3.02 (dd, J = 5.4, 13.8 Hz, 1H), 3.70 (dd, J = 5.4, 7.5 Hz, 1H), 4.18 (q, J = 7.2 Hz, 2H), 7.00 (s, 2H). ^{13}C NMR ($CDCl_3$) δ 14.37, 30.46, 34.43, 41.16, 56.12, 60.98, 125.95, 127.75, 136.13, 152.80, 175.43. MS (EI) m/z 321 (M^+, 1%), 219 (100%).

***N*-(*tert*-Butoxycarbonyl)- 3′,5′-di-*tert*-butyltyrosine benzyl ester (2).** To a solution of compound **1a** (0.42 g, 1.09 mmol) in CH_3CN (10 mL) was added at room temperature under N_2, $^iPr_2NEt$ (0.18 g, 1.4 mmol), followed by $(BOC)_2O$ (0.305 g, 1.4 mmol). The mixture was stirred overnight. The solvent was removed under reduced pressure, and the residue was purified by flash chromatography (hexane/ethyl acetate 15:1) to give the pure product (**2**, 0.48 g) in 90% yield: ^{1}H NMR ($CDCl_3$) δ 1.44 (s, 18H), 1.47 (s, 9H), 3.06 (m, 2H), 4.65 (dd, J = 6.3, 14.1 Hz, 1H), 5.13 (m, 4H), 6.95 (s, 2H), 7.27 (m, 2H), 7.35 (m, 3H). ^{13}C NMR ($CDCl_3$) δ 28.52, 30.48, 34.43, 54.82, 67.08, 79.94, 126.04, 126.54, 128.10, 128.47, 128.74, 135.39, 136.11, 153.01, 155.17, 172.24. MS (EI) m/z 483 (M^+, 5%), 219 (100%).

***N*-[*N*-(*tert*-Butoxycarbonyl)prolyl]-3′,5′-di-*tert*-butyltyrosine ethyl ester (3).** To a solution of *N*-(*tert*-butoxycarbonyl)proline (53 mg, 0.24 mmol) in DMF (3 mL) was added HOBt (35 mg, 0.26 mmol), followed by DCC (50 mg, 0.24 mmol). The mixture was stirred for 2 h, then a solution of 3,5-di-*tert*-butyltyrosine ethyl ester (**1b**) (80 mg, 0.29 mmol) and triethylamine (0.3 mL) in DMF (5 mL) was added, and the resulting mixture was stirred for 12 h at room temperature. The solvent was pumped off, and the residue was purified by flash chromatography on silica gel (hexane/ethyl acetate 1:1) to give **3** (125 mg, 97%) as a white foam. ^{1}H NMR ($CDCl_3$) δ 1.10–1.14 (dt, J = 7.2 Hz, 3H), 1.39 (s, 27H), 1.90 (m, 4H), 3.00 (m, 2H), 3.36 (m, 3H), 4.18 (m, 2H), 4.76 (m, 1H), 5.16 (s, 1H), 6.36 (br s, 1H), 6.95 (s, 1H), 7.00 (s, 1H).

Cyclo[(*R*)-3′,5′-di-*tert*-butyl-Tyr-L-Pro] (5) and cyclo[(*S*)-3′,5′-di-*tert*-butyl-Tyr-L-Pro] (6). From compound 2. To a solution of compound **2** (480 mg, 1.0 mmol) in *tert*-butyl alcohol (20 mL) was added 10% Pd/C (40 mg). The resulting mixture was stirred at room temperature under an H_2 atmosphere for 2 h, and the catalyst was filtered off. After removal of the solvent, the residue was dissolved in DMF/EtOAc (1:5, 12 mL), and to this solution was added HOBt (162 mg, 1.2 mmol), followed by DCC (254 mg, 1.23 mmol). The resulting solution was stirred for 2 h, and then prolinamide (124 mg, 1.08 mmol) was added. The mixture was stirred overnight at room temperature. The precipitate was filtered off, and the solution was diluted with CH_2Cl_2 (500 mL), washed with water, dried (Na_2SO_4), and concentrated. The residue was passed through a short column of silica gel to give the crude product **4** (470 mg, 97%). This material was dissolved in CH_2Cl_2/CF_3COOH (10:1, 5 mL) at 0 C, and the solution was stirred for 2 h at the same temperature. The solvent was removed under reduced pressure, the residue was dissolved in CH_2Cl_2/triethylamine (5:1, 10 mL), and the resulting mixture was stirred overnight. The reaction mixture was diluted with CH_2Cl_2, washed with saturated $NaHCO_3$, dried (Na_2SO_4), and concentrated to give an oil. Purification by column chromatography on silica gel [$CHCl_3$/MeOH (9:1)] gave the pure compounds **5** (80 mg, 21% yield) and **6** (121 mg, 32% yield).

From compound 3. A solution of compound **3** (16 mg, 0.03 mmol) in CH_2Cl_2/CF_3COOH (10:1, 5 mL) was stirred for 2 h at 0 °C. The reaction mixture was diluted with CH_2Cl_2, washed with saturated $NaHCO_3$, dried (Na_2SO_4), and concentrated to give an oil. The residue was dissolved in MeOH (5 mL), NaCN (2 mg) was added, and the resulting mixture was stirred at 45 °C overnight. The solvent was removed under reduced pressure, and the residue was purified by column chromatography on silica gel [$CHCl_3$/MeOH (9:1)] to give the compounds **5** and **6** (5 mg, 43%, and 5.2 mg, 45%, respectively).

5: $[\alpha]_D^{25}$ −137° (c 0.93, $CHCl_3$). Mp 194–197 °C. ^{1}H NMR ($CDCl_3$) δ 1.45 (s, 18H), 1.98 (m, 3H), 2.37 (m, 1H), 2.69 (dd, J = 10.8, 14.4 Hz, 1H), 3.59 (m, 3H), 4.12 (t, J = 7.2 Hz, 1H), 4.27 (d, J = 8.7 Hz, 1H), 5.21 (s, 1H), 5.68 (s, 1H), 7.00 (s, 2H). ^{13}C NMR ($CDCl_3$) δ 22.70, 28.62, 30.41, 34.52, 37.12, 45.53, 56.58, 59.34, 125.73, 126.37, 136.94, 153.33, 165.46, 169.48. Anal. calcd for $C_{22}H_{32}N_2O_3$: C, 70.92; H, 8.66; N, 7.52. Found: C, 71.13; H, 8.50; N, 7.63.

6: $[\alpha]_D^{25}$ −23° (c 1.07, $CHCl_3$). Mp 216–218 °C. ^{1}H NMR ($CDCl_3$) δ 1.44 (s, 18H), 1.76 (m, 2H), 1.95 (m, 1H), 2.16 (m, 1H), 2.64 (dd, J = 6.6, 10.5 Hz, 1H), 2.93 (dd, J = 3.9, 13.8 Hz, 1H), 3.13 (dd, J = 5.7, 13.5 Hz, 1H), 3.42 (m, 1H), 3.63 (m, 1H), 4.23 (q, J = 5.1 Hz, 1H), 5.23 (s, 1H), 5.80 (s, 1H), 6.99 (s, 2H). ^{13}C NMR ($CDCl_3$) δ 21.95, 29.11, 30.49, 34.43, 40.54, 45.27, 57.90, 59.43, 125.55, 126.83, 136.65, 153.50, 165.21, 169.39. Anal. calcd for $C_{22}H_{32}N_2O_3$: C, 70.92; H, 8.66; N, 7.52. Found: C, 70.66; H, 8.45; N, 7.65.

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