Available online at www.sciencedirect.com
SCIENCE DIRECT
Bioorganic & Medicinal Chemistry Letters
Bioorganic & Medicinal Chemistry Letters 16 (2006) 705–709

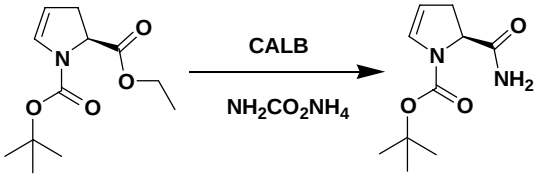
Biocatalytic ammonolysis of (5*S*)-4,5-dihydro-1*H*-pyrrole-1,5-dicarboxylic acid, 1-(1,1-dimethylethyl)-5-ethyl ester: Preparation of an intermediate to the dipeptidyl peptidase IV inhibitor Saxagliptin

Iqbal Gill* and Ramesh Patel

Process Research and Development, Bristol-Myers Squibb Pharmaceutical Research Institute, One Squibb Drive, New Brunswick, NJ 08903, USA

Received 16 September 2005; revised 5 October 2005; accepted 6 October 2005
Available online 27 October 2005

Abstract—An efficient biocatalytic method has been developed for the conversion of (5*S*)-4,5-dihydro-1*H*-pyrrole-1,5-dicarboxylic acid, 1-(1,1-dimethylethyl)-5-ethyl ester (**1**) into the corresponding amide (5*S*)-5-aminocarbonyl-4,5-dihydro-1*H*-pyrrole-1-carboxylic acid, 1-(1,1-dimethylethyl)ester (**2**), which is a critical intermediate in the synthesis of the dipeptidyl peptidase IV (DPP4) inhibitor Saxagliptin (**3**).



Candida antartica lipase B mediates ammonolysis of the ester with ammonium carbamate as ammonia donor to yield up to 71% of the amide. The inclusion of Ascarite and calcium chloride as adsorbents for carbon dioxide and ethanol byproducts, respectively, increases the yield to 98%, thereby offering an efficient and practical alternative to chemical routes which yield 57–64%.

The enzyme dipeptidyl peptidase IV (DPP4), which regulates plasma levels of the insulinotropic proGlucagon Like Peptide-1(7-36) (GLP-1(7-36)) hormone, has emerged as a novel therapeutic target for the treatment of type 2 diabetes.[1–4] Inhibitors of DPP4 have been shown to elevate levels of GLP-1(7-36) and promote insulin secretion and thereby regulate blood glucose levels.[5–7] Recently, methanoproline nitrile-based small molecule inhibitors of DPP4, such as Saxagliptin (**3**), have emerged as potent, long-lasting, selective, and orally active therapeutics for type 2 diabetes.[8,9] During the course of a program to develop Saxagliptin-type DPP4 inhibitors, it was required to devise an efficient process for the conversion of (5*S*)-4,5-dihydro-1*H*-pyrrole-1,5-dicarboxylic acid, 1-(1,1-dimethylethyl)-5-ethyl ester (**1**) (synthesized from the readily available (*S*)-pyroglutamic acid via a sequence of esterification, Boc protection, carbonyl reduction, and dehydration reactions) into the corresponding amide (5*S*)-5-aminocarbonyl-4,5-dihydro-1*H*-pyrrole-1-carboxylic acid, 1-(1,1-dimethylethyl)ester (**2**) (Fig. 1). Direct chemical ammonolyses were hindered by the requirement for aggressive reaction conditions which resulted in unacceptable levels of amide racemization and side-product formation, while milder two-step hydrolysis–condensation protocols using coupling agents, such as 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT–MM),[10] were compromised by reduced overall yields of 57–64%. To address this issue, a biocatalytic procedure

Keywords: *Candida antartica* lipase; CALB; Enzymatic; Biocatalytic; Ammonolysis; DPP4.
* Corresponding author. e-mail: iqba.gill@bms.com

0960-894X/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2005.10.021

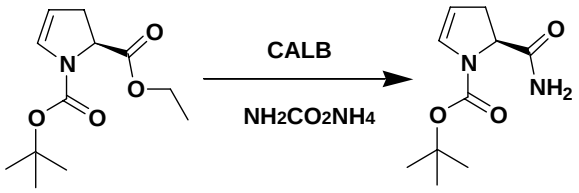
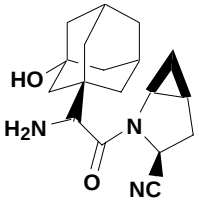


Figure 1. Biocatalytic ammonolysis of BMS-404764 to BMS-562419.

was developed, based upon the *Candida antartica* lipase B (CALB)-mediated ammonolysis of **1** with ammonium carbamate to furnish **2** without racemization and with low levels of side-product formation (Fig. 2).

Commercial hydrolases were initially screened for the ammonolysis of **1**. The biocatalysts comprised lipases (Amano lipases A, AK, AP12, AY30, D, F, FAP15, G, GC20, M, MAP10, N, PS, PS30, and R, Biocatalyst lipases ANL, CCL, and RJL, Enzymatix lipases B1 and F5, Europa lipases 4, 13, 14, and 21, Julich lipases RN and RO, Meito Sangyo lipases AL, MY, OF, PL, QLM, SL, TL, and UL, Sigma lipases CRL and PPL, Sepracor lipase OF, Biocatalysts Lipomod 200, Boehringer Chirazyme L2, Novo Lipolase 30T, Novozym lipase CALB, Lipozyme IM60, and Novozym-435), proteases (Sigma papain, bromelain, subtilisin, α-chymotrypsin, pronase E, and proteinase K) and esterases (Sigma Pig Liver esterase, Fluka *B. thermoglucisdasus*, *B. stearothermophilus*, *C. lipolytica*, *M. miehei*, *R. oryzae*, *S. cerevisiae*, *S. diastatochromogenes*, and *T. lanuginosus* esterases, and Julich esterases BS1, BS2, BS3, SD, and PF). Screening experiments utilized process stream ester feed, which consisted of ca. 22% w/v (0.91 M) of the ester in toluene. In the interest of preserving high productivity and process telescoping, it was decided to use this feed without dilution or cosolvents. Since the latter precluded the use of free ammonia due to its low solubility in toluene, solid ammonium carbamate, which has been shown to be an effective ammonia source in enzymatic ammonolyses, was employed instead.[11] Reactions were performed using a mixture of neat process feed, ammonium carbamate (71 g/L, 2 mol equiv of ammonia), and biocatalyst (25 g/L), shaken at 400 rpm, 50 °C. Under these conditions, CALB and its immobilized forms Novozym 435 and Chirazyme L2 provided racemization-free amide with yields of 69%, 43%, and 40%, together with 21%, 18%, and 22% of side-products (by HPLC), respectively, while all other biocatalysts furnished less than 5% of the desired product. This result was in accordance with literature reports demonstrating the unique effectiveness of CALB in the ammonolysis and aminolysis of esters.[12–27]



(3)

Figure 2. Saxagliptin.

The ammonolysis reaction with free CALB was then optimized with regard to the ammonia source, temperature, and the CALB and ammonium carbamate loads (Tables 1 and 2). A screening of ammonia sources showed that ammonium carbamate and ammonium carbonate were the best cosubstrates (Table 1). Other ammonium salts, amides, ammonia generated in situ, and gaseous ammonia provided low ammoniolysis rates and product yields. The poor results obtained with free ammonia as opposed to ammonia slowly generated from ammonium carbamate/carbonate may reflect inhibition of CALB by high concentrations of ammonia. With regard to temperature, the initial rate of amide formation increased from 20 to 70 °C, initially being fairly linear over 20–40 °C, then leveling off at higher temperatures

Table 1. Effect of ammonia source on the CALB-mediated ammonolysis of **1**[a]

Ammonia source[a]	Initial rate[b] (mmol h^{-1} g^{-1})	Amide[c] (%)	Side products[c] (%)
$NH_2CO_2NH_4$	2.88	70	22
$(NH_4)_2CO_3$	2.61	62	18
HCO_2NH_4	0.06	2	18
$MeCO_2NH_4$	0.08	5	12
$(NH_4)_2HPO_4$	0.05	3	8
NH_3 gas (batch addition)[d]	0.17	16	9
NH_3 gas (bubbled at 5 rvm)[e]	0.23	13	14
NH_4Cl + *t*-BuONa[f]	0.08	4	41
$HCONH_2$	0.04	4	22
$MeCONH_2$	0.06	3	12

[a] 0.5 mL of process stream ester (22% w/v, 0.91 M in toluene), amount of ammonia donor corresponding to 2 mol equiv ammonia, and 25 mg enzyme (23% w/w of ester load), in a 2 mL vial, stirred at 400 rpm, 20–70 °C, 72 h. Reactions were analyzed by HPLC.[29]

[b] The initial rates were measured at 2 h and are quoted as millimole of amide formed per hour per gram of CALB.

[c] Yields as measured by HPLC at 72 h. The side products appeared to be oligomeric compounds formed via addition/condensation reactions of **1** and were not further characterized.

[d] A 25 mL Teflon flask was charged with substrate and biocatalyst, then purged thoroughly with ammonia gas and sealed.

[e] Dry ammonia gas was bubbled at ca. 5 reaction volumes per min from a lecture bottle.

[f] 2.1 and 2.0 mol equiv of ammonium chloride and sodium *t*-butoxide, respectively, were added to a mixture of substrate and biocatalyst.

Table 2. Effect of temperature, catalyst, and ammonium carbamate loads on the CALB-mediated ammonolysis of **1**[a]

[CALB] (g/L)	[$NH_2CO_2NH_4$] (g/L (mol equiv NH_3)	Temp (°C)	Initial rate[b] (mmol h^{-1} g^{-1})	Amide[c] (%)	Side products[c] (%)
25	71 (2.0)	20	0.47	17	6
25	71 (2.0)	30	0.95	24	9
25	71 (2.0)	40	1.79	37	11
25	71 (2.0)	50	2.81	58	13
25	71 (2.0)	60	3.45	53	17
25	71 (2.0)	70	3.88	35	39
20	71 (2.0)	50	2.89	56	10
25	71 (2.0)	50	2.65	65	13
30	71 (2.0)	50	2.38	63	7
35	71 (2.0)	50	2.31	68	16
40	71 (2.0)	50	2.26	66	14
45	71 (2.0)	50	2.20	67	16
50	71 (2.0)	50	2.07	70	19
60	71 (2.0)	50	2.05	71	18
25	35 (1.0)	50	3.26	49	18
25	53 (1.5)	50	2.80	55	16
25	89 (2.5)	50	2.23	68	15
25	107 (3.0)	50	1.73	60	12
25	124 (3.5)	50	0.71	57	10
25	142 (4.0)	50	0.71	53	11

[a] 0.5 mL of process stream ester (22% w/v, 0.91 M in toluene), 18–70 mg ammonium carbamate (1–4 mol equiv ammonia), and 10–30 mg enzyme (9–27% w/w of ester load), in a 2 mL vial, stirred at 400 rpm, 20–70 °C, 72 h. Reactions were analyzed by HPLC.[29]

[b] The initial rates were measured at 2 h and are quoted as millimole of amide formed per hour per gram of CALB.

[c] Yields as measured by HPLC at 72 h. The side products appeared to be oligomeric compounds formed via addition/condensation reactions of **1** and were not further characterized.

(Table 2). The amide yield was maximal at 50–60 °C and dropped off sharply on either side, while side-product formation increased with temperature. Where the CALB load was concerned, the initial rate gradually decreased from 2.89 mmol h^{-1} g^{-1} at 20 g CALB/L (9% w/w of ester input) to 2.05 mmol h^{-1} g^{-1} at 60 g CALB/L (27% w/w of ester input). In contrast, the amide yield showed the reverse trend, increasing from 56% to 71%, with side products varying from 7% to 19%. For ammonium carbamate load, the initial rate decreased from 3.26 mmol h^{-1} g^{-1} at 1.0 mol equiv of ammonia donor to 0.71 mmol h^{-1} g^{-1} at 4 mol equiv, while the amide yield was maximal at 2–3 mol equiv of ammonia. The exact origins of these trends were unclear, although one could reasonably invoke the effects of ammonia, the formed amide, and the released ethanol and carbon dioxide on CALB activity.

In view of the above results, the inclusion of various additives was investigated with the aim of ameliorating potential inhibitory phenomena, shifting the equilibrium toward amide synthesis and reducing side-product formation (Table 3). Drying agents such as molecular sieves and Drierite did not have an appreciable effect on the reaction, providing amide yields of 58–71% with 28–37% of side products, but a significant improvement (79% amide and 13% side products) was observed with calcium chloride added at 100 g/L. The latter is known to complex alcohols as well as act as a desiocant, and its presumed binding of ethanol released during the course of amide formation may have served to mitigate any deleterious effects of this alcohol on CALB catalysis. In this context, it should be noted that the inclusion of as little as 5% of ethanol reduced the amide yield by 16–24% in the absence of additives. More interestingly, a dramatic increase in amide yield to 84% and 95% was achieved by including sodalime and Ascarite, respectively, at 200 g/L in the reaction headspace, this presumably by way of adsorption of carbon dioxide liberated from the decomposition of ammonium carbamate. A further slight increase in yield to 98% was attained via the combined use of 100 g/L calcium chloride and 200 g/L Ascarite. It should be noted that the strong basicity of sodalime and Ascarite precluded their direct use in the reaction mixture so as to avoid decomposition of **1** and **2**. It should also be mentioned that attempts to shift the equilibrium via the application of vacuum or a stream of nitrogen meant to remove carbon dioxide and ethanol byproducts failed, presumably due to the rapid loss of ammonia from the reaction medium (Table 4).

A prep-scale reaction was conducted with the process ester feed to test the utility of the approach.[28] Ester (220 g/L) was reacted with 90 g/L (1.25 mol equiv) of ammonium carbamate, 33 g/L (15% w/w of ester input) CALB, 110 g/L calcium chloride, and 216 g/L Ascarite (in the headspace), run at 50 °C, 3 d. Complete conversion of ester was achieved, with the formation of 96% (182 g/L) of amide **2** and 4% of side products, and after workup 98% potency amide of >99.9% ee was isolated in 81% yield.

In conclusion, an efficient biocatalytic process has been developed for the conversion of ethyl ester **1** into the amide **2**. By employing CALB-mediated ammonolysis with ammonium carbamate as ammonia donor in the presence of calcium chloride and Ascarite, a 220 g/L ester feed was converted up to 98% yield into the desired amide. This furnished high-purity amide

Table 3. Effect of additives on the CALB-catalyzed ammoniolysis of **1** with ammonium carbamate[a]

Additive	[Additive] (g/L)	Initial rate[b] (mmol h^{-1} g^{-1})	Amide[c] (%)	Side products[c] (%)
None	—	2.76	67	28
3 Å Molecular sieves	100	2.71	61	28
4 Å Molecular sieves	100	2.64	63	27
5 Å Molecular sieves	100	2.73	58	34
13X Molecular sieves	100	2.82	70	29
$CaSO_4$ (Drierite)	100	2.54	62	35
$CaCl_2$	50	2.80	75	11
$CaCl_2$	100	2.91	79	13
$CaCl_2$	150	2.93	77	16
Sodalime (headspace)	100	2.75	78	22
Sodalime (headspace)	200	2.78	83	17
Ascarite (headspace)	100	2.97	84	14
Ascarite (headspace)	150	3.02	89	11
Ascarite (headspace)	200	3.13	95	5
Ascarite (headspace)	250	3.15	94	6
Ascarite (headspace)	300	3.20	91	9
$CaCl_2$ + Ascarite (headspace)	50 + 100	2.95	87	13
$CaCl_2$ + Ascarite (headspace)	50 + 200	3.07	94	6
$CaCl_2$ + Ascarite (headspace)	100 + 200	3.11	98	2

[a] 0.2 mL of process ester (22% w/v, 0.91 M in toluene), 14 mg ammonium carbamate (2 mol equiv ammonia), additive (indicated amount), and 5 mg enzyme (12.5% w/w of ester load) in a 4 mL vial were stirred at 400 rpm, 50 °C, 72 h. For Ascarite, a perforated Teflon thimble was used to hold the additive ca. 1 cm above the reaction mixture surface. Reactions were analyzed by HPLC.[29]

[b] The initial rates were measured at 2 h and are quoted as millimole of amide formed per hour per gram of CALB.

[c] Yields as measured by HPLC at 72 h. The side products appeared to be oligomeric compounds formed via addition/condensation reactions of **1** and were not further characterized.

Table 4. Effect of vacuum and nitrogen purging on the CALB-catalyzed ammoniolysis of **1** with ammonium carbamate[a]

Conditions	[Additive] (g/L)	Initial rate[b] (mmol h^{-1} g^{-1})	Amide[c] (%)	Side products[c] (%)
Closed vessel	—	2.60	65	23
Closed vessel + $CaCl_2$ + Ascarite	100 + 200	3.18	96	4
Vessel open to air	—	0.79	19	42
Vessel under vacuum (200 mbar)	—	0.23	12	17
Vessel under vacuum (100 mbar)	—	0.11	5	16
Vessel under vacuum (50 mbar)	—	0.08	4	19
Headspace purged with N_2 (5 rvm)[d]	—	0.29	12	13
Headspace purged with N_2 (20 rvm)[d]	—	0.21	9	16
Reaction mix purged with N_2 (5 rvm)[d]	—	0.19	7	13
Reaction mix purged with N_2 (20 rvm)[d]	—	0.05	3	15

[a] 0.2 mL of process ester (22% w/v, 0.91 M in toluene), 14 mg ammonium carbamate (2 mol equiv ammonia), additive (indicated amount), and 5 mg enzyme (12.5% w/w of ester load) in a 4 mL vial were stirred at 400 rpm, 50 °C, 72 h. For Ascarite, a perforated Teflon thimble was used to hold the additive ca. 1 cm above the reaction mixture surface. Reactions were analyzed by HPLC.[29]

[b] The initial rates were measured at 2 h and are quoted as millimole of amide formed per hour per gram of CALB.

[c] Yields as measured by HPLC at 72 h. The side products appeared to be oligomeric compounds formed via addition/condensation reactions of **1** and were not further characterized.

[d] rvm, reaction volumes per minute.

with no accompanying racemization, with minimal side-product formation, and provided an economic and scalable process suitable for industrial use. Also, the application of Ascarite and calcium chloride as carbon dioxide and alcohol adsorbents, respectively, may provide a general method for increasing yields in the enzymatic ammoniolysis of esters with ammonium carbamate/carbonate.

References and notes

1. Lambeir, A.-M.; Durinx, C.; Scharpé, S.; De Meester, I. *Crit. Rev. Clin. Lab. Sci.* **2003**, *40*, 209.
2. Takei, I.; Kasatani, T. *Biomed. Pharmacother.* **2004**, *58*, 578.
3. Knudsen, L. B. *J. Med. Chem.* **2004**, *47*, 4128.
4. Weber, A. E. *J. Med. Chem.* **2004**, *47*, 4135.
5. Demuth, H.-U.; McIntosh, C. H. S.; Pederson, R. A. *Biochim. Biophys. Acta Proteins & Proteomics* **2005**, *1751*, 33.
6. Cheon, H. G.; Kim, S. S.; Kim, K.-R.; Rhee, S.-D.; Yang, S.-D.; Ahn, J. H.; Park, S. D.; Lee, J. M.; Jung, W. H.; Lee, H. S.; Kim, H. Y. *Biochem. Pharmacol.* **2005**, *70*, 22.
7. Brandt, I.; Joossens, J.; Chen, X.; Maes, M.-B.; Scharpé, S.; De Meester, I.; Lambeir, A.-M. *Biochem. Pharmacol.* **2005**, *70*, 134.
8. Magnin, D. R.; Robl, J. A.; Sulsky, R. B.; Augeri, D. J.; Huang, Y.; Simpkins, L. M.; Taunk, P.; Betebenner, D. A.; Robertson, J. G.; Abboa-Offei, B.; Wang, A.; Cap, M.;

Xing, L.; Tao, L.; Sitkoff, D. F.; Malley, M. F.; Gougoutas, J. Z.; Khanna, A.; Huang, Q.; Han, S.-P.; Parker, R. A.; Hamann, L. G. *J. Med. Chem.* **2004**, *47*, 2587.
9. Augeri, D. J.; Robl, J. A.; Betebenner, D. A.; Magnin, D. R.; Khanna, A.; Robertson, J. G.; Simpkins, L. M.; Taunk, P. C.; Huang, Q.; Han, S.-P.; Abboa-Offei, B.; Wang, A.; Cap, M.; Xin, L.; Tao, L.; Tozzo, E.; Welzel, G. E.; Egan, D. M.; Marcinkeviciene, J.; Chang, S. Y.; Biller, S. A.; Kirby, M. S.; Parker, R. A.; Hamann, L. G. *J. Med. Chem.* **2005**, *48*, 5025.
10. Kunishima, M.; Kawachi, C.; Hioki, K.; Terao, K.; Tani, S. *Tetrahedron* **2001**, *57*, 1551.
11. Litjens, M. J. J.; Straathof, A. J. J.; Jongejan, J. A.; Heijnen, J. J. *Chem. Commun.* **1999**, 1255.
12. Prasad, A. K.; Husain, M.; Singh, B. K.; Gupta, R. K.; Manchanda, V. K.; Olsen, C. E.; Parmar, V. S. *Tetrahedron Lett.* **2005**, *46*, 4511.
13. Levinson, W. E.; Kuo, T. M.; Kurtzman, C. P. *Enzyme Microb. Technol.* **2005**, *37*, 126.
14. González-Sabín, J.; Gotor, V.; Rebolledo, F. *Tetrahedron: Asymmetry* **2004**, *15*, 481.
15. Jacobsen, E. E.; Hoff, B. H.; Moen, A. R.; Anthonsen, T. *J. Mol. Catal. B.* **2003**, *21*, 55.
16. Slotema, W. F.; Sandoval, G.; Guieysse, D.; Straathof, A. J. J.; Marty, A. *Biotechnol. Bioeng.* **2003**, *82*, 664.
17. Baldessari, A.; Mangone, C. P. *J. Mol. Catal. B.* **2001**, *11*, 335.
18. Gotor, V. *Bioorg. Med. Chem.* **1999**, *7*, 2189.
19. Conde, S.; Lopez-Serrano, P.; Martinez, A. *J. Mol. Catal. B.* **1999**, *7*, 299.
20. Wegman, M. A.; Hacking, M. A. P. J.; Rops, J.; Pereira, P.; van Rantwijk, F.; Sheldon, R. A. *Tetrahedron: Asymmetry* **1999**, *10*, 1739.
21. Garcia-Urdiales, E.; Rebolledo, F.; Gotor, V. *Tetrahedron: Asymmetry* **1999**, *10*, 721.
22. Starmans, W. A. J.; Doppen, R. G.; Thijs, L.; Zwananburg, B. *Tetrahedron: Asymmetry* **1998**, *9*, 429.
23. Hacking, M. A. P. J.; Wegman, M. A.; Rops, J.; van Ranwijk, F.; Sheldon, R. A. *J. Mol. Catal. B.* **1998**, *5*, 155.
24. Vörde, C.; Högberg, H.-E.; Hedenström, E. *Tetrahedron: Asymmetry* **1996**, *7*, 1507.
25. Chamorro, C.; González-Muñiz, R.; Conde, S. *Tetrahedron: Asymmetry* **1995**, *6*, 2343.
26. Garcia, M. J.; Rebolledo, F.; Gotor, V. *Tetrahedron Lett.* **1993**, *34*, 6141.
27. Garcia, M. J.; Rebolledo, F.; Gotor, V. *Tetrahedron* **1994**, *50*, 6935.
28. Preparative scale ammonolysis of (5*S*)-4,5-dihydro-1*H*-pyrrole-1,5-dicarboxylic acid (**1**): A 500 mL PTFE flask was charged with process ester feed (72.4 g, ca. 60 mL, containing 13.2 g, 54.8 mmol **1**), ammonium carbamate (5.4 g, 69 mmol, 2.5 mol equiv of ammonia), anhydrous calcium chloride beads (6.6 g), and CALB powder (2.0 g, 15% w/w of ester input), then a 40 mL glass fiber thimble containing 13 g Ascarite was placed in the neck of the flask, the flask sealed, and the whole setup was shaken at 150 rpm, 50 °C, 3 d. HPLC analysis indicated that 100% conversion of ester had been achieved with the formation of 96% of amide and 4% of side products.[29] The reaction mixture was diluted with ethyl acetate (30 mL) and filtered through a pad of silica gel 60 (10 g), and the filter cake was washed with 1:1 toluene/ethyl acetate (2× 10 mL). Acetic acid (0.5 mL) was added to the combined filtrates and the solution was evaporated at 50 °C to yield a viscous brown liquid. This was diluted with ethyl acetate (30 mL), the suspension was filtered through a pad of silica gel 60 (10 g), and the pad was washed with ethyl acetate (1× 10 mL). The filtrate was evaporated at 30 °C to ca. 25 mL; the residue was diluted with cyclohexane (ca. 75 mL), seeded with crystalline amide, and the mixture was stirred at 100 rpm, rt, 5 h, then at 100 rpm, 5 °C, 22 h. The suspension was filtered, and the cake was washed with 4:1 cyclohexane/ethyl acetate (3× 10 mL), and then dried under vacuum at rt to furnish the amide as off-white crystals, 9.42 g, 81% yield, 98% potency, 99% AP (by HPLC), >99.9% ee. ^{1}H NMR (DMSO-d_6, 25 °C, 400 MHz) δ 1.39 (s, 9H), 2.48 (m, 1H), 2.94 (m, 1H), 4.37 (dd, 1H), 4.96 (m, 1H), 6.50 (br d, 1H), 6.96 (br d, 1H), 7.38 (m, 1H) ppm; ^{13}C NMR (DMSO-d_6, 25 °C, 125 MHz) δ 28.7, 31.1, 58.1, 71.2, 111.7, 23.0, 152.2, 177.4 ppm.
29. Analytical methods: Samples for HPLC analysis were diluted to 2 mM with methanol containing 1.0% acetic acid, filtered (0.5 μm PTFE), and then analyzed on a Shimadzu LC-10 system equipped with a Phenomenex Synergi Max-RP (4 μm, 2× 50 mm) column. The column was eluted with 10% to 100% B over 8 min, where A = 8:2 (v/v) water/methanol with 0.05% TFA and B = 8:2 (v/v) acetonitrile/methanol with 0.05% TFA. The flow rate was 0.6 mL min^{-1}, injection volume 5 μL, column temperature 30 °C, and the detection wavelength 225 nm. The ester and amide eluted at 6.9 and 2.0 min, respectively. Amide enantiopurity was determined by chiral HPLC analysis on a Shimadzu LC-10 system equipped with a Chiralpak AS-H (5 μm, 4.6× 150 mm) column. The column was eluted with 40% B over 30 min, where A = heptane and B = 1:1 (v/v) heptane/isopropanol. The flow rate was 1.0 mL min^{-1}, injection volume 20 μL, the column kept at room temperature, and the detection wavelength 225 nm. The (*R*)- and (*S*)-amide enantiomers eluted at 7.2 and 15.6 min, respectively.