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Design, synthesis, and preliminary studies of the activity of novel derivatives of *N*-cinnamoyl-L-aspartic acid as inhibitors of aminopeptidase N/CD13

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ABSTRACT

A series of novel derivatives of *N*-cinnamoyl-L-aspartic acid were designed, synthesized, and assayed for their inhibitory activities against aminopeptidase N. The preliminary biological assay showed that compound **8c** has the most potent inhibitory activity against APN with an IC_{50} of 11.1 ± 0.9 μM, this could be used as the lead compound in future research on anticancer agents.

1. Introduction

Aminopeptidase N (APN), which is identical to the human lymphocyte surface cluster differentiation antigen, CD13, is a zinc-dependent membrane metalloprotease in the M1 family of ectoenzymes.[1] It is responsible for the cleavage of the *N*-amino group from a polypeptide chain, and APN has been shown to participate in the degradation of polypeptides and proteins. APN is over-expressed on tumor cells, and it plays a critical role in tumorigenesis and the regulation of immunological function.[2,3]

Over more than three decades, studies have demonstrated numerous natural and synthetic inhibitors of APN, including AHPA-Val,[4–6] Amastatin,[7] Bestatin,[8] Probestin,[9] Curcumin,[10] and lapstatin.[11] Among these inhibitors, Bestatin is the best known for its application in the clinical treatment of adult acute nonlymphocytic leukemia.

In recent years, our group has reported several new inhibitors of APN, such as L-lysine derivatives,[12] AHPA (3-2-hydroxyl-phenyl butanoic acid) derivatives,[13] 3-phenylpropane-1,2-diamine derivatives,[14] cinnamic acid and caffeic acid with hydroxyproline derivatives,[15] and L-iso-glutamine derivatives.[16] In a previous study, the interaction of an APN inhibitor and the active site of APN was reported. Kiyoshi[17] showed the co-crystal complex of APN and Bestatin. According to the literature, the active site of APN can be divided into three parts: part A (the S_1 pocket) is to the left of part B, which contains a zinc ion, and part C (the S_1' pocket) is on the right. The phenyl ring of Bestatin interacts with the S_1 pocket, the hydroxyl group and the carbonyl group chelate with the zinc ion, and the leucine residue is inserted into the S_1' pocket.

In this work, we aimed to develop a novel framework for an inhibitor of APN. Wang et al.[16] had reported that L-glutamine derivatives could serve as potential antitumor agents that are directed toward APN. We chose L-aspartic acid as the initial material, and a cinnamic acid moiety was introduced on to the scaffold. Cinnamic acid is a natural differential inductor that can inhibit the proliferation of insensitive prostatic carcinomas and can reverse the transformation and induce differentiation of human lung cancer cells, liver cancer, and human promyelocytic leukemia cells.[18–21] In addition, different building blocks (such as amines) were introduced to interact with the S_1' pocket of APN. The phenyl ring of cinnamic acid was inserted into the pocket S_1, and the carboxy group or the hydroxymate served as the zinc-binding group. In this report, we describe the synthesis and evaluation of the enzymatic activity of the *N*-cinnamoyl-L-aspartic acid derivatives, and docking studies of the interaction will also be discussed.

2. Chemistry

The target compounds were synthesized efficiently, following the procedures shown in Scheme 1.

The starting material, compound **2**, was prepared from L-aspartic acid according to methods described in the literature.[22] The key intermediate **6** was obtained by the anhydration of compound **5**.

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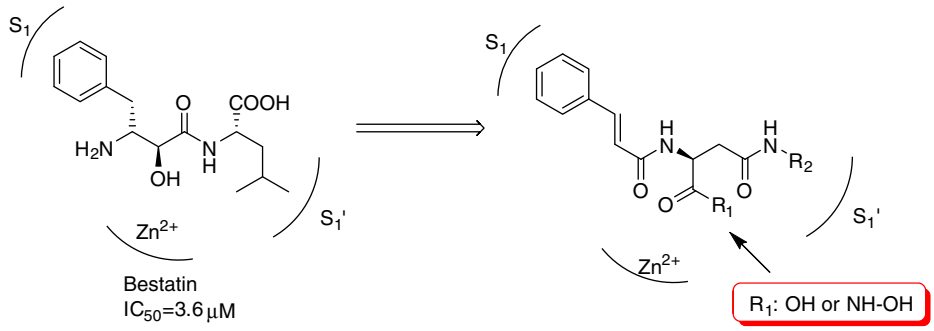


The compound **6** was reactivated with amineõ to yield **7a–7o**. Compounds **7a–7o** can be activated with hydroxylamine hydrochloride to obtain **8a**–**8o**.

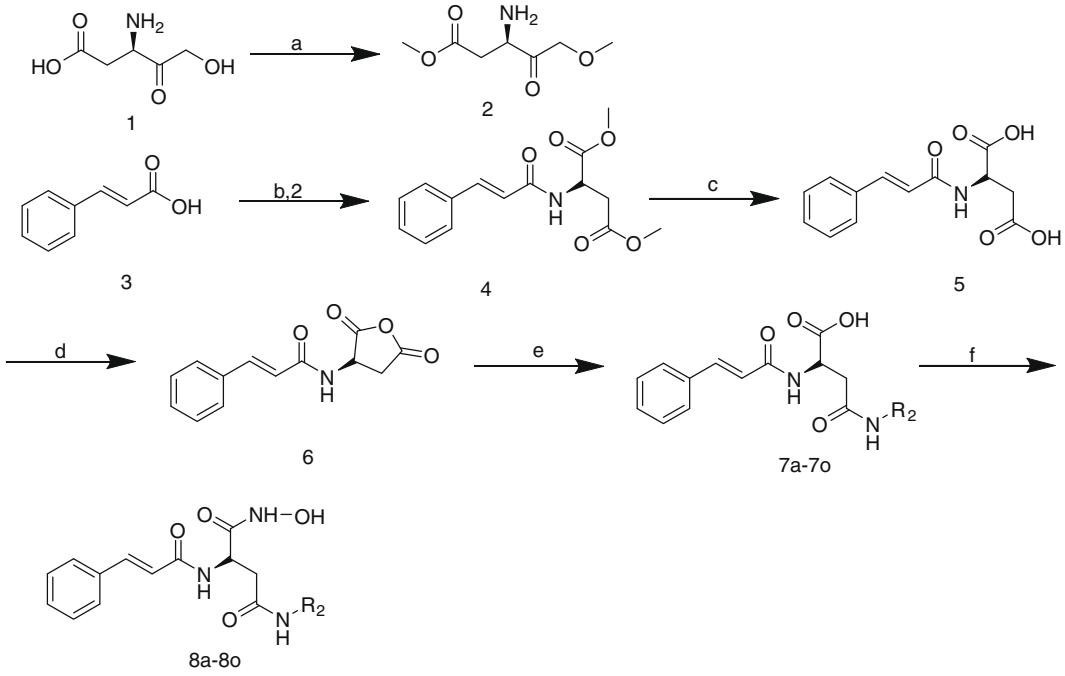
3. Results and discussion

In order to identify the activities of our compounds, preliminary pharmacological studies were performed against APN and MMP-2 in vitro. Similar to APN, MMP-2 is a zinc-dependent metalloproteinase that is associated with malignant tumors. According to the results of the enzyme assay, most compounds exhibited better inhibitory activity against APN than MMP-2.

Comparison of compounds **7a–7o** and **8a–8o** showed that the introduction of a stronger zinc-binding group, hydroxymate, could significantly increase the inhibitory activities against APN. The compounds **7a–7o**, which had a carboxy zinc-binding group, possessed inhibitory activities but most of them were of low potency for the inhibition of APN. As shown in Table 1, compounds **8c**–**8f** exhibited well activity towards APN among these target compounds. The IC_{50} values for these four inhibitors of APN ranged between 11.11 and 18.55 μM. In addition, the effects of **8c**–**8f** on the proliferation of HL-60 cells, compared with Bestatin, are shown in Figure 1. Cell viability was assessed by the MTT method. Compound **8c** exhibited an inhibitory effect on cell proliferation with an IC_{50} value of 0.78 ± 0.11 mM, and showed greater potency than Bestatin, which had an IC_{50} value of 0.92 ± 0.12 mM.

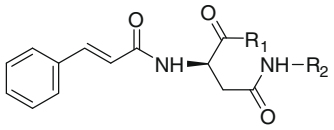
The results of the assay of enzyme inhibition showed that when the R_1 group was fixed, the R_2 was altered from aliphatic to aromatic. The compounds bearing aliphatic amines were found to be more potent than those bearing aromatic amines. This result may be owing to the fact that aliphatic amines are flexible and can be inserted into the S_1' pocket more easily than the aromatic amines. Moreover, the length of the side chain will partly influence the activities of the compounds. The R_2 group of the most potent compound, **8c,** is a butyl group, which is similar to the isobutyl side chain of Bestatin. When the R_2 group was converted to undecane, the compound **8g**, possessed a fortieth of the activity of compound **8c**.

In order to investigate the interaction of the target compounds with APN, the representative compound **8c** was constructed with a Sybyl/Sketch module and optimized using Powell's method with a Tripos force field with the convergence criterion set at 0.05 kcal/(Å mol), and assigned with the Gasteiger–Hückel method. The docking study was performed using a Sybyl/FlexX module: the residues in a radius of 7.0 Å around Bestatin in the co-crystal structure (PDB code: 2DQM) were selected as the active site. Other docking



Scheme 1. Reagents and conditions: (a) MeOH, HCl, ice bath; (b) isobutyl chloroformate, *N*-methylmorpholine, THF, −15 °C; (c) MeOH, NaOH, HCl; (d) Ac_2O, 55–60 °C; (e) AcOH, Et_3N; (f) isobutyl chloroformate, Et_3N, THF, MeOH, NH_2OH, HCl.

Table 1
The structures and IC_{50} values of the target compounds



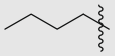
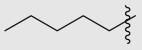
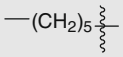
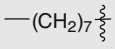
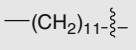
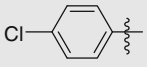
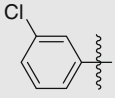
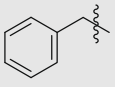
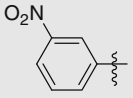
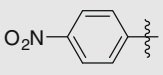
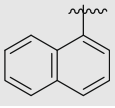
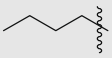
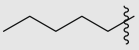
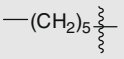
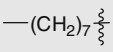
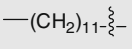
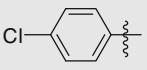
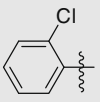
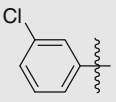
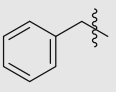
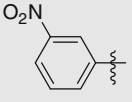
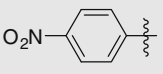
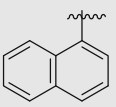
Compound	R_1	R_2	$IC_{50}{}^{\alpha}$ (μM)	
			APN	MMP-2
7a	–OH		9108.6 ± 8.2	>1000
7b	–OH		2199.7 ± 8.1	>1000
7c	–OH		189.7 ± 2.3	594.3 ± 3.7
7d	–OH		204.3 ± 1.5	>1000
7e	–OH		387.8 ± 2.4	>1000
7f	–OH		232.2 ± 2.1	>1000
7g	–OH		962.0 ± 4.0	176.5 ± 2.1
7h	–OH		348.5 ± 4.9	>1000
7i	–OH		263.4 ± 3.6	>1000
7j	–OH		718.6 ± 3.2	>1000
7k	–OH		206.8 ± 1.4	999 ± 3.2
7l	–OH		232.4 ± 1.2	>1000
7m	–OH		1781 ± 4.7	>1000
7n	–OH		273.1 ± 2.0	>1000
7o	–OH		739.2 ± 6.8	>1000

Table 1 (*continued*)

Compound	R_1	R_2	$IC_{50}{}^{\alpha}$ (μM)	
			APN	MMP-2
8a	NH–OH		36.0 ± 1.1	131.0 ± 2.3
8b	NH–OH		65.9 ± 1.5	166.6 ± 2.7
8c	NH–OH		11.1 ± 0.9	232.5 ± 1.8
8d	NH–OH		17.4 ± 1.4	196.5 ± 2.5
8e	NH–OH		18.1 ± 2.2	178.6 ± 1.5
8f	NH–OH		18.5 ± 2.0	285.9 ± 3.0
8g	NH–OH		438.2 ± 3.4	>1000
8h	NH–OH		191.5 ± 2.7	196.6 ± 1.8
8i	NH–OH		86.6 ± 1.3	>1000
8j	NH–OH		1206 ± 2.7	>1000
8k	NH–OH		65.1 ± 1.6	447.2 ± 2.8
8l	NH–OH		115.0 ± 1.2	324.4 ± 2.0
8m	NH–OH		1440 ± 5.2	>1000
8n	NH–OH		189.1 ± 2.1	>1000
8o	NH–OH		2050 ± 4.6	>1000
Bestatin			3.60 ± 0.6	162.0 ± 4.8

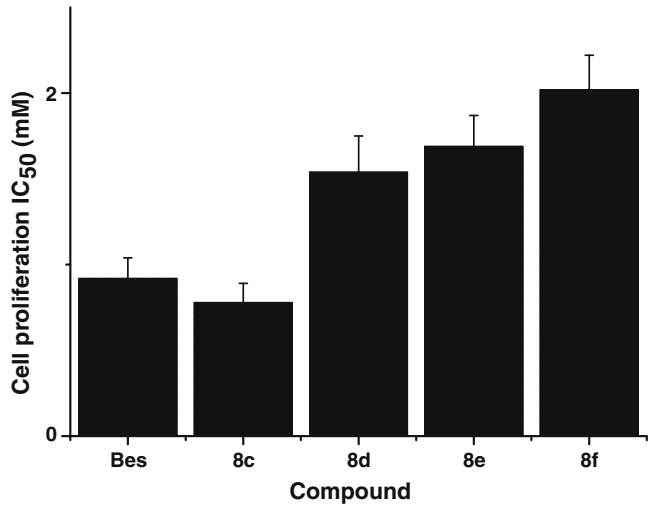


Figure 1. Effects of Bestatin and compounds **8c**–**8f** on proliferation of the HL-60 cell line. Each column represents the mean and SE of five independent experiments.

parameters available in the program were maintained as the defaults. The docking studies showed that the phenyl group of the cinnamic acid moiety was inserted into the S_1 pocket, and the butyl group plunged into the pocket S_1' (Fig. 2a). The carbonyl group and the oxygen atoms in hydroxymate (OH) can interact with the zinc ion with a distance of 1.97 and 1.74 Å, respectively. In addition, the amino group of compound **8c** formed a hydrogen bond with the oxygen atom of Tyr^{381} with a distance of 1.84 Å (Fig. 2b). Tyr^{381} has been reported to be beneficial to the stabilization of the reaction intermediate with the zinc ion.[17] The hydroxymate group can form a hydrogen bond with the carboxyl group of Glu^{298} with a spaced length of 1.89 Å. Glu^{298} is a strong base that is capable of mediating the attack of a water molecule upon the carbonyl carbon of the substrate in the catalytic mechanism of APN.

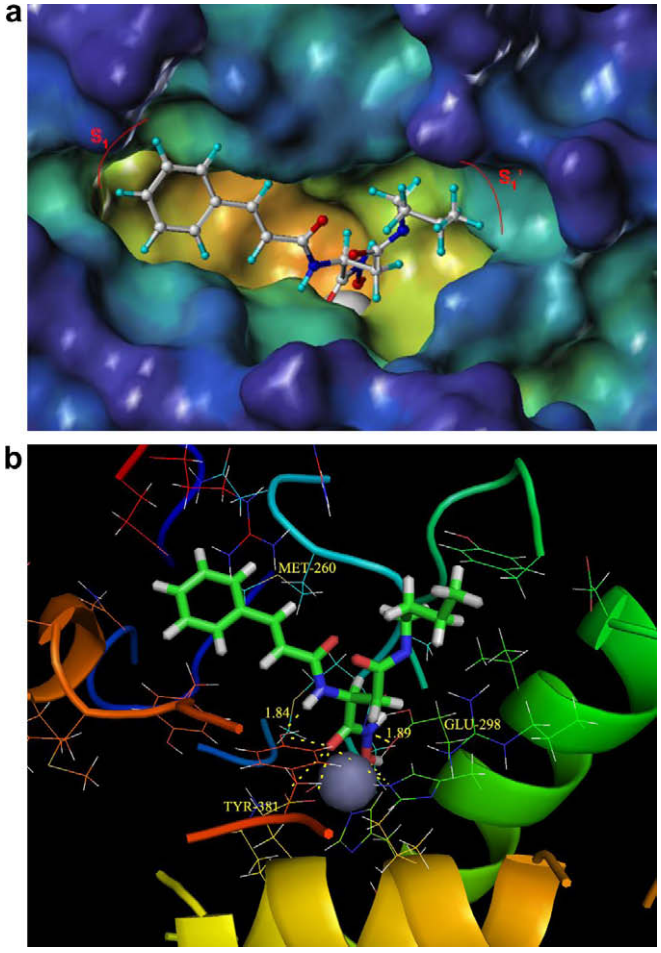


Figure 2. (a) The FlexX docking result of **8c** with APN; (b) The FlexX docking result of **8c** shown by PyMOL.

4. Conclusion

In conclusion, we have described the synthesis of a series of derivatives of *N*-cinnamoyl-L-aspartic acid and their properties as inhibitors of APN. Most of the target compounds have potent inhibitory activities against APN and the most effective compound, **8c**, exhibited excellent enzymatic inhibition activity and selectivity for APN. Compound **8c** could be used as a lead compound for the development of small molecular peptidomimetic inhibitors of APN for investigation as potential anticancer agents in the future.

5. Experimental

5.1. APN inhibition assay

IC_{50} values against APN were determined as previously described, with the use of L-Leu-*p*-nitroanilide as a substrate and microsomal aminopeptidase from Porcine Kidney microsomes (Sigma) as the enzyme, in 50 μM PBS, pH 7.2, at 37 °C. The hydrolysis of the substrate was monitored by following the change in the absorbance measured at 405 nm with a UV–vis spectrophotometer, Pharmacia LKB, Biochrom 4060. All the solutions of the inhibitors were prepared in the assay buffer, and the pH was adjusted to 7.5 by the addition of 0.1 M HCl or 0.1 M NaOH. All the inhibitors were preincubated with APN for 30 min at room temperature. The assay mixture, which contained the inhibitor solution (the concentration of which was dependent on the inhibitor), the enzyme solution (4 mg/ml final concentration), and the assay buffer, was adjusted to 200 μl.

5.2. MMP inhibition assay

Gelatinase A (MMP-2) and TNBS were purchased from Sigma, and the substance was synthesized as described by Vijaykumar et al. The gelatinase, substance, and inhibitor were dissolved in sodium borate (pH 8.5, 50 mmol/l) and incubated for 30 min at 37 °C. Subsequently, 0.03% TNBS was added and incubated for another 20 min: the resulting solution was investigated using a wavelength of 450 nm to measure absorption.

5.3. MTT assay

HL-60 cells were grown in RPMI1640 medium containing 10% FBS at 37 °C in a humidified incubator with 5% CO_2. Cell proliferation was determined by the MTT (3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide) assay. Briefly, cells were plated in a 96-well plate at 10,000 cells per well, cultured for 4 h in complete growth medium, then treated with 1600, 800, 400, 200, or 100 μg/ml of the compounds for 48 h. Following this, 0.5% MTT solution was added to each well. After further incubation for 4 h, the formazan formed from MTT was extracted by adding DMSO and mixing for 15 min. The optical density was read with an ELISA reader at 570 nm.

5.4. Chemistry: general procedures

All the materials we used were commercially available. All the solvents were distilled before use. All reactions were monitored by thin-layer chromatography on 0.25-mm silica gel plates (60GF-254) and visualized with UV light or ferric chloride. Silica gel of 200–300 mesh size, was used in column chromatography to depurate the end products. The proton NMR spectra were determined on a Brucker DRX spectrometer (300 MHz), δ in parts per million and J in Hertz, and TMS was used as an internal standard. ESI-MS were determined on an API 4000 spectrometer. Measurements were made in CD_3OD solutions. Melting points were determined using an electrothermal melting point apparatus, and were uncorrected.

5.4.1. Dimethyl L-aspartate hydrochloride (2)

L-Aspartic acid (**1**) (5 g, 37.6 mmol) was suspended in methanol (300 ml) and the suspension was stirred in an ambient ice bath under a chloride hydrogen atmosphere until the suspension became clear. The product solution was rotary evaporated under reduced pressure. Methanol was then added and rotary evaporated to remove the hydrogen chloride. A mass of white solid was obtained and was crystallized in diethyl ether to produce 5.73 g (yield: 81.4%) of white crystal, mp: 114–117 °C.

5.4.2. Dimethyl 2-cinnamamidosuccinate (4)

N-Methylmorpholine (1.23 ml, 11.2 mmol) in THF (30 ml) was added isobutyl chloroformate (1.44 ml, 11.2 mmol) at −15 °C. The mixture was stirred for 30 min at the same temperature. A solution of the compound dimethylster 2-aspartic acid hydrochlorate (3.02 g, 15.3 mmol) in THF (20 ml) was added dropwise to the reaction mixture. The stirring was continued for 1 h at −15 °C and then the mixture was removed from the cooling bath. The reaction was continued for 4 h and the mixture was filtrated. After filtration, the filtrate was concentrated with a rotary evaporator. The residue was dissolved in EtOAc and washed with 5% $NaHCO_3$, 10% citric acid, and saturated brine in turn. The EtOAc solution was dried over Na_2SO_4 and concentrated with a rotary evaporator to afford the crude product. The crude product was recrystallized by EtOAc to afford pure title compound **4**, 2.00 g, yield: 67.54%, mp: 123–125 °C. ESI-MS: *m/z*: 292.3 $[M+H]^+$; ^1H NMR (CD_3OD) δ 2.89 (s, 2H), 3.75 (s, 6H), 4.97–5.10 (m, 1H), 6.71 (d, J = 16,1H), 7.28–7.44 (m, 4H), 7.61–7.66 (m, 2H). Anal. Calcd for $C_{15}H_{17}NO_5$: C, 61.85; H, 5.88; N, 4.81. Found: C, 61.67; H, 5.85; N, 4.85.

5.4.3. 2-Cinnamamidosuccinic acid (5)

To a stirred solution of compound **4** (2 g, 6.9 mmol) in MeOH (20 ml) was added 1 M NaOH (10 ml). The reaction mixture was stirred for 3 h. The pH of the reaction mixture was adjusted to 5–6 with 1 M HCl. After removal of solvents, the residue was partitioned between EtOAc and 10% citric acid. The aqueous phase was extracted twice with EtOAc. The combined organic phase was washed with water and brine, dried over Na_2SO_4, filtered, and concentrated with a rotary evaporator to give 1.74 g of crude oil. The crude oil was purified by flash column chromatography on silica gel (CH_2Cl_2/MeOH. 50/1) to afford compound **5**, 1.47 g, yield: 81.2%, mp: 102–105 °C; $[\alpha]_D^{25}$ +23.8 (*c* l, MeOH); ESI-MS: *m/z*: 264.2 $[M+H]^+$; ^1H NMR (CD_3OD) δ 2.90–2.93 (m, 2H), 4.89 (d, J = 16,1H), 7.39–7.41 (m, 3H), 7.55–7.61 (m, 3H). Anal. Calcd for $C_{13}H_{13}NO_5$: C, 59.31; H, 4.98; N, 5.32. Found: C, 59.49; H, 4.94; N, 5.35.

5.4.4. *N*-(2,5-Dioxotetrahydrofuran-3-yl) cinnamamide (6)

Compound **5** (10 g, 38.0 mmol) was added to 80 ml acetic anhydride. The reaction mixture was stirred at 55–60 °C for 5 h. The mixture was filtered while the solution was hot, to the filtrate was added some anhydrous diethyl ether and this was placed in a refrigerator to obtain the white crystal: 4.75 g (Compound **6**), yield: 51%, mp: 102–105 °C; $[\alpha]_D^{25}$ +36.3 (*c* l, MeOH); ESI-MS: *m/z*: 245.3 $[M+H]^+$; ^1H NMR (CD_3OD) δ 3.10–3.45 (m, 2H), 5.09–5.21 (m, 1H), 6.70 (d, J = 16, 1H), 7.35–7.44 (m, 3H), 7.59–7.63 (m, 3H). Anal. Calcd for $C_{13}H_{11}NO_4$: C, 63.67; H, 4.52; N, 5.71. Found: C, 63.86; H, 4.49; N, 5.74.

5.4.5. 2-Cinnamamido-4-oxo-4-(propylamino) butanoic acid (7a)

Compound **6** (2.45 g, 10 mmol) and *n*-propylamine (0.65 g, 11 mmol) were suspended in glacial acetic acid (30 ml). The suspension was stirred at room temperature. The reaction was monitored by thin-layer chromatography in order to ensure complete reaction. The product solution was rotary evaporated under reduced pressure. To the residue was added some water, and the product was left in a refrigerator to obtain the white crystal, which was filtered, dried and weighed: 2.45 g (**7a**), yield: 80.59%, mp: 116–118 °C; $[\alpha]_D^{25}$ 19.8 (*c* l, MeOH); ESI-MS: *m/z*: 320.4 $[M+H]^+$; ^1H NMR (CD_3OD) δ 0.89–0.95 (m, 3H), 1.48–1.58 (m, 2H), 2.73–2.93 (m, 2H), 3.13–3.21 (m, 2H), 4.84–4.92 (m, 1H), 6.70 (d, J = 16, 1H), 7.38–7.44 (m, 3H), 7.54–7.61 (m, 3H). Anal. Calcd for $C_{16}H_{20}N_2O_4$: C, 63.14; H, 6.62; N, 9.20. Found: C, 62.95; H, 6.59; N, 9.27.

Compounds **7b–7o** were synthesized following the procedure described above.

5.4.6. 2-Cinnamamido-4-(isopropylamino)-4-oxobutanoic acid (7b)

2.51 g, yield: 82.57%, mp: 130–133 °C, $[\alpha]_D^{25}$ +31.2 (*c* l, MeOH); ESI-MS: *m/z*: 320.4 $[M+H]^+$; ^1H NMR (CD_3OD) δ 0.99 (s, 6H), 2.67–2.74 (m, 2H), 3.85–3.89 (m, 1H), 4.78–4.82 (m, 1H), 6.72 (d, J = 16, 1H), 7.41–7.43 (m, 3H), 7.52–7.59 (m, 3H). Anal. Calcd for $C_{16}H_{20}N_2O_4$: C, 63.14; H, 6.62; N, 9.20. Found: C, 62.96; H, 6.57; N, 9.26.

5.4.7. 4-(Butylamino)-2-cinnamamido-4-oxobutanoic acid (7c)

2.66 g, yield: 83.65%, mp: 124–126 °C, $[\alpha]_D^{25}$ +41.3 (*c* l, MeOH); ESI-MS: *m/z*: 319.3 $[M+H]^+$; ^1H NMR (CD_3OD) δ 0.91 (t, J = 9,3H), 1.26–1.47 (m, 4H), 2.57–2.79 (m, 2H), 3.13–3.29 (m, 2H), 4.83–4.89 (m, 1H), 6.69 (d, J = 16, 1H), 7.38–7.41 (m, 3H), 7.56–7.62 (m, 3H). Anal. Calcd for $C_{17}H_{22}N_2O_4$: C, 64.13; H, 6.97; N, 8.80. Found: C, 64.32; H, 7.01; N, 8.75.

5.4.8. 2-Cinnamamido-4-oxo-4-(pentylamino) butanoic acid (7d)

2.75 g, yield: 82.83%, mp: 124–125 °C, $[\alpha]_D^{25}$ +32.4 (*c* l, MeOH); ESI-MS: *m/z*: 333.4 $[M+H]^+$; ^1H NMR (CD_3OD) δ 0.93 (t, J = 7.5, 3H), 1.27–1.35 (m, 4H), 1.49–1.55 (m, 2H), 2.56–2.74 (m, 2H), 3.06–3.12 (m, 2H), 4.80–4.87 (m, 1H), 6.469 (d, J = 16, 1H), 7.40–7.43 (m, 3H), 7.56–7.60(m, 3H). Anal. Calcd for $C_{18}H_{24}N_2O_4$: C, 65.04; H, 7.28; N, 8.43. Found: C, 64.85; H, 7.22; N, 8.36.

5.4.9. 2-Cinnamamido-4-(hexylamino)-4-oxobutanoic acid (7e)

2.80 g, yield: 80.92%, mp: 112–114 °C, $[\alpha]_D^{25}$ +20.8 (*c* l, MeOH); ESI-MS: *m/z*: 347.3 $[M+H]^+$; ^1H NMR (CD_3OD) δ 0.94 (t, J = 9, 3H), 1.25–1.37 (m, 6H), 1.50–1.54 (m, 2H), 2.59–2.76 (m, 2H), 3.12–3.17 (m, 2H), 4.80–4.86 (m, 1H), 6.71 (d, J = 16, 1H), 7.35–7.43 (m, 3H), 7.57–7.64 (m, 3H). Anal. Calcd for $C_{19}H_{26}N_2O_4$: C, 65.87; H, 7.56; N, 8.09. Found: C, 65.66; H, 7.60; N, 8.13.

5.4.10. 2-Cinnamamido-4-(octylamino)-4-oxobutanoic acid (7f)

3.21 g, yield: 85.83%, mp: 108–110 °C, $[\alpha]_D^{25}$ +29.7 (*c* l, MeOH); ESI-MS: *m/z*: 375.5 $[M+H]^+$; ^1H NMR (CD_3OD) δ 0.91 (t, J = 6, 3H), 1.28–1.62 (m, 12H), 2.34–2.56 (m, 2H), 3.15–3.28 (m, 2H),

4.58–4.67 (m, 1H), 6.69 (d, J = 16, 1H), 7.34–7.38 (m, 3H), 7.40–7.54 (m, 3H). Anal. Calcd for $C_{21}H_{30}N_2O_4$: C, 67.35; H, 8.07; N, 7.48. Found: C, 67.56; H, 8.13; N, 7.52.

5.4.11. 2-Cinnamamido-4-(dodecylamino)-4-oxobutanoic acid (7g)

3.57 g, yield: 83.02%, mp: 82–86 °C, $[\alpha]_D^{25}$ +17.8 (*c* l, MeOH); ESI-MS: *m/z*: 431.3 $[M+H]^+$; ^1H NMR (CD_3OD) δ 0.94 (t, J = 9.6, 3H), 1.23–1.45 (m, 20H), 2.64–2.72 (m, 2H), 3.09–3.16 (m, 2H), 4.60–4.69 (m, 1H), 6.70 (d, J = 16, 1H), 7.34–7.44 (m, 3H), 7.54–7.57 (m, 3H). Anal. Calcd for $C_{25}H_{38}N_2O_4$: C, 69.74; H, 8.90; N, 6.51. Found: C, 69.51; H, 8.97; N, 6.46.

5.4.12. 4-(4-Chlorophenylamino)-2-cinnamamido-4-oxobutanoic acid (7h)

3.27 g, yield: 87.90%, mp: 152–155 °C, $[\alpha]_D^{25}$ +32.6 (*c* l, MeOH); ESI-MS: *m/z*: 373.2 $[M+H]^+$; ^1H NMR (CD_3OD) δ 2.79–3.02 (m, 2H), 4.89–5.02 (m, 1H), 6.71 (d, J = 16, 1H), 7.30–7.33 (m, 3H), 7.39–7.41 (m, 3H), 7.58–7.63 (m, 4H). Anal. Calcd for $C_{19}H_{17}ClN_2O_4$: C, 61.03; H, 4.60; N, 7.51. Found: C, 59.49; H, 4.63; N, 7.46.

5.4.13. 4-(2-Chlorophenylamino)-2-cinnamamido-4-oxobutanoic acid (7i)

3.13 g, yield: 84.14%, mp: 148–151 °C, $[\alpha]_D^{25}$ +20.0 (*c* l, MeOH); ESI-MS: *m/z*: 373.2 $[M+H]^+$; ^1H NMR (CD_3OD) δ 2.76–2.89 (m, 2H), 4.76–4.92 (m, 1H), 6.69 (d, J = 16, 1H), 7.21–7.32 (m, 2H), 7.30–7.34 (m, 3H), 7.39–7.42 (m, 3H), 7.98 (s, 1H). Anal. Calcd for $C_{19}H_{17}ClN_2O_4$: C, 61.03; H, 4.60; N, 7.51. Found: C, 59.53; H, 4.68; N, 7.43.

5.4.14. 4-(3-Chlorophenylamino)-2-cinnamamido-4-oxobutanoic acid (7j)

3.29 g, yield: 88.44%, mp: 140–144 °C, $[\alpha]_D^{25}$ +24.6 (*c* l, MeOH); ESI-MS: *m/z*: 373.2 $[M+H]^+$; ^1H NMR (CD_3OD) δ 2.67–2.78 (m, 2H), 4.70–4.75 (m, 1H), 6.68 (d, J = 16, 1H), 7.23–7.47 (m, 3H), 7.40–7.45 (m, 3H), 7.56–7.64 (m, 3H), 7.99 (s, 1H). Anal. Calcd for $C_{19}H_{17}ClN_2O_4$: C, 61.03; H, 4.60; N, 7.51. Found: C, 59.47; H, 4.66; N, 7.56.

5.4.15. 4-(Benzylamino)-2-cinnamamido-4-oxobutanoic acid (7k)

2.87 g, yield: 81.53%, mp: 148–151 °C, $[\alpha]_D^{25}$ +30.4 (*c* l, MeOH); ESI-MS: *m/z*: 353.4 $[M+H]^+$; ^1H NMR (CD_3OD) δ 2.58–2.71 (m, 2H), 4.33 (s, 2H), 4.85–4.91 (m, 1H), 6.70 (d, J = 16, 1H), 7.21–7.29 (m, 5H), 7.37–7.42 (m, 3H), 7.55–7.62 (m, 3H). Anal. Calcd for $C_{20}H_{20}N_2O_4$: C, 68.17; H, 5.72; N, 7.95. Found: C, 67.97; H, 5.69; N, 7.90.

5.4.16. 2-Cinnamamido-4-oxo-4-(phenylamino) butanoic acid (7l)

2.71 g, yield: 80.18%, mp: 166–168 °C, $[\alpha]_D^{25}$ +19.0 (*c* l, MeOH); ESI-MS: *m/z*: 339.4 $[M+H]^+$; ^1H NMR (CD_3OD) δ 2.67–2.80 (m, 2H), 4.87–4.96 (m, 1H), 6.69 (d, J = 16, 1H), 7.30–7.34 (m, 3H), 7.37–7.62 (m, 5H), 7.62–7.65 (m, 3H). Anal. Calcd for $C_{19}H_{18}N_2O_4$: C, 67.44; H, 5.36; N, 8.28. Found: C, 67.64; H, 5.41; N, 8.34.

5.4.17. 2-Cinnamamido-4-(3-nitrophenylamino)-4-oxobutanoic acid (7m)

3.11 g, yield: 81.20%, mp: 140–142 °C, $[\alpha]_D^{25}$ +26.4 (*c* l, MeOH); ESI-MS: *m/z*: 384.3 $[M+H]^+$; ^1H NMR (CD_3OD) δ 2.67–2.74 (m, 2H), 4.78–4.86 (m, 1H), 6.71 (d, J = 16, 1H), 7.40–7.41 (m, 3H), 7.56–7.61 (m, 3H), 7.76–7.99 (m, 3H), 8.69 (s, 1H). Anal. Calcd for $C_{19}H_{17}N_3O_6$: C, 59.53; H, 4.47; N, 10.96. Found: C, 59.34; H, 4.54; N, 11.04.

5.4.18. 2-Cinnamamido-4-(4-nitrophenylamino)-4-oxobutanoic acid (7n)

3.04 g, yield: 79.37%, mp: 156–159 °C, $[\alpha]_D^{25}$ +28.9 (*c* l, MeOH); ESI-MS: *m/z*: 384.4 $[M+H]^+$; ^1H NMR (CD_3OD) δ 2.61–2.84 (m, 2H), 4.62–4.90 (m, 1H), 6.70 (d, J = 16, 1H), 7.38–7.42 (m, 3H), 7.56–7.60 (m, 3H), 7.85–7.92 (m, 2H), 8.22–8.25 (m, 2H). Anal. Calcd for $C_{19}H_{17}N_3O_6$: C, 59.53; H, 4.47; N, 10.96. Found: C, 59.41; H, 4.56; N, 11.00.

5.4.19. 2-Cinnamamido-4-(naphthalene-1-ylamino)-4-oxobutanoic acid (7o)

3.23 g, yield: 83.25%, mp: 188–190 °C, $[\alpha]_D^{25}$ +34.5 (*c* l, MeOH); ESI-MS: *m/z*: 389.3 $[M+H]^+$; ^1H NMR (CD_3OD) δ 2.66–2.86 (m, 2H), 4.86–4.92 (m, 1H), 6.67 (d, J = 16, 1H), 6.91 (d, J = J = 12, 1H), 7.29–7.37 (m, 3H), 7.49–7.54 (m, 3H), 7.56–7.59 (m, 4H), 8.54–8.61 (m, 2H). Anal. Calcd for $C_{23}H_{20}N_2O_4$: C, 71.12; H, 5.19; N, 7.21. Found: C, 71.35; H, 5.24; N, 7.13.

5.4.20. 2-Cinnamamido-N^1-hydroxy-N^4-propylsuccinamide (8a)

Compound **7a** (1.0 g, 2.4 mmol) was suspended in THF (20 ml) and isobutyl chloroformate (1.44 ml, 11.2 mmol) and Et_3N (2 ml) were added; the suspension was stirred at room temperature and the reaction was monitored by thin-layer chromatography in order to ensure complete reaction. Hydroxylamine hydrochloride (0.41 g, 5.9 mmol) was dissolved in absolute methanol (20 ml) and Et_3N (1.56 ml); the mixture was stirred for 20 min, then added the mixture to the above-mentioned reaction dropwise. The reaction was monitored by thin-layer chromatography. The product solution was rotary evaporated under reduced pressure. The residue was dissolved in EtOAc (50 ml) and washed with 1 N HCl and distilled water in turn, dried over Na_2SO_4, filtered, and concentrated with a rotary evaporator to give 0.74 g of crude oil. The crude oil was purified by column chromatography on silica gel (CH_2Cl_2/MeOH, 50/1) to afford compound **8a**: 0.69 g, yield: 65.78%. mp: 145–147 °C; $[\alpha]_D^{25}$ +22.3 (*c* l, MeOH); ESI-MS: *m/z*: 320.4 $[M+H]^+$; ^1H NMR (CD_3OD) δ 0.93 (t, J = 7.5, 3H), 1.51–1.58 (m, 2H), 2.52–2.76 (m, 2H), 3.15–3.17 (m, 2H), 4.85–4.93 (m, 1H), 6.72 (d, J = 16, 1H), 7.34–7.42 (m, 3H), 7.56–7.61 (m, 3H). Anal. Calcd for $C_{16}H_{21}N_3O_4$: C, 60.17; H, 6.63; N, 13.16. Found: C, 60.35; H, 6.67; N, 13.08.

5.4.21. 2-Cinnamamido-N^1-hydroxy-N^4-isopropylsuccinamide (8b)

0.65 g, yield: 61.96%, mp: 163–164 °C; $[\alpha]_D^{25}$ +34.8 (*c* l, MeOH); ESI-MS: *m/z*: 320.4 $[M+H]^+$; ^1H NMR (CD_3OD) δ 1.24 (d, J = 9.6, 6H), 2.50–2.78 (m, 2H), 3.85–4.01 (m, 1H), 4.76–4.85 (m, 1H), 6.67 (d, J = 16, 1H), 7.30–7.42 (m, 3H), 7.58–7.66 (m, 3H). Anal. Calcd for $C_{16}H_{21}N_3O_4$: C, 60.17; H, 6.63; N, 13.16. Found: C, 60.37; H, 6.59; N, 13.27.

5.4.22. N^4-Butyl-2-cinnamamido-N^1-hydroxysuccinamide (8c)

0.71 g, yield: 67.81%, mp: 153–154 °C; $[\alpha]_D^{25}$ +38.6 (*c* l, MeOH); ESI-MS: *m/z*: 334.7 $[M+H]^+$; ^1H NMR (CD_3OD) δ 0.95 (t, J = 6.3, 3H), 1.15–1.51 (m, 4H), 2.57–2.79 (m, 2H), 3.16–3.34 (m, 2H), 4.85–4.93(m, 1H), 6.68 (d, J = 16, 1H), 7.40–7.41 (m, 3H), 7.54–7.59 (m, 3H). Anal. Calcd for $C_{17}H_{23}N_3O_4$: C, 61.25; H, 6.95; N, 12.60. Found: C, 61.08; H, 6.89; N, 12.46.

5.4.23. 2-Cinnamamido-N^1-hydroxy-N^4-pentylsuccinamide (8d)

0.86 g, yield: 82.30%, mp: 169–170 °C; $[\alpha]_D^{25}$ +30.9 (*c* l, MeOH); ESI-MS: *m/z*: 348.5 $[M+H]^+$; ^1H NMR (CD_3OD) δ 0.94 (t, J = 7.5, 3H), 1.23–1.38 (m, 4H), 1.50–1.56 (m, 2H), 2.56–2.81 (m, 2H), 3.15–3.18 (m, 2H), 4.82–4.89 (m, 1H), 6.72 (d, J = 16, 1H), 7.34–7.43 (m, 3H), 7.58–7.63 (m, 3H). Anal. Calcd for $C_{18}H_{25}N_3O_4$: C, 62.23; H, 7.25; N, 12.10. Found: C, 62.42; H, 7.31; N, 12.01.

5.4.24. 2-Cinnamamido-N^4-hexyl-N^1-hydroxysuccinamide (8e)

0.82 g, yield: 78.62%, mp: 178–180 °C; $[\alpha]_D^{25}$ +22.3 (*c* l, MeOH); ESI-MS: *m/z*: 362.6 $[M+H]^+$; ^1H NMR (CD_3OD) δ 0.94 (t, J = 7.5, 3H), 1.25–1.34 (m, 6H), 1.50–1.56 (m, 2H), 2.55–2.82 (m, 2H), 3.18–3.22 (m, 2H), 4.81–4.88 (m, 1H), 6.71 (d, J = 16, 1H), 7.34–7.43 (m, 3H), 7.56–7.62 (m, 3H). Anal. Calcd for $C_{19}H_{27}N_3O_4$: C, 63.14; H, 7.53; N, 11.63. Found: C, 62.95; H, 7.46; N, 11.51.

5.4.25. 2-Cinnamamido-N^1-hydroxy-N^4-octylsuccinamide (8f)

0.85 g, yield: 81.73%, mp: 156–158 °C, $[\alpha]_D^{25}$ +32.0 (*c* l, MeOH); ESI-MS: *m/z*: 390.4 $[M+H]^+$; ^1H NMR (CD_3OD) δ 0.91 (t, J = 6, 3H), 1.25–1.77 (m, 12H), 2.27–2.50 (m, 2H), 3.27–3.45 (m, 2H), 4.59–4.69 (m, 1H), 6.70 (d, J = 16, 1H), 7.28–7.35 (m, 3H), 7.40–7.56 (m, 3H). Anal. Calcd for $C_{21}H_{31}N_3O_4$: C, 64.76; H, 8.02; N, 10.79. Found: C, 64.96; H, 8.06; N, 10.72.

5.4.26. 2-Cinnamamido-N^4-dodecyl-N^1-hydroxysuccinamide (8g)

0.70 g, yield: 68.43%, mp: 155–157 °C, $[\alpha]_D^{25}$ +19.2 (*c* l, MeOH); ESI-MS: *m/z*: 446.6 $[M+H]^+$; ^1H NMR (CD_3OD) δ 0.86 (t, J = 9, 3H), 1.20–1.35 (m, 20H), 2.60–2.80 (m, 2H), 3.15–3.25 (m, 2H),4.70–4.80 (m, 1H), 6.72 (d, J = 16, 1H), 7.30–7.42 (m, 3H), 7.52–7.58 (m, 3H). Anal. Calcd for $C_{25}H_{39}N_3O_4$: C, 67.39; H, 8.82; N, 9.43. Found: C, 67.18; H, 8.75; N, 9.51.

5.4.27. N^4-(4-Chlorophenyl)-2-cinnamamido-N^1-hydroxysuccinamide (8h)

0.79 g, yield: 75.96%, mp: 154–156 °C, $[\alpha]_D^{25}$ +29.5 (*c* l, MeOH); ESI-MS: *m/z*: 388.5 $[M+H]^+$; ^1H NMR (CD_3OD) δ 2.67–2.79 (m, 2H), 4.93–5.01 (m, 1H), 6.71 (d, J = 16, 1H), 7.30–7.33 (m, 3H), 7.40–7.59 (m, 4H), 7.60–7.68 (m, 3H). Anal. Calcd for $C_{19}H_{18}ClN_3O_4$: C, 58.84; H, 4.68; N, 10.84. Found: C, 58.66; H, 4.73; N, 10.76.

5.4.28. N^4-(2-Chlorophenyl)-2-cinnamamido-N^1-hydroxysuccinamide (8i)

0.82 g, yield: 78.85%; mp: 157–159 °C, $[\alpha]_D^{25}$ +23.2 (*c* l, MeOH); ESI-MS: *m/z*: 388.5 $[M+H]^+$; ^1H NMR (CD_3OD) δ 2.67–2.76 (m, 2H), 4.92–5.02 (m, 1H), 6.68 (d, J = 16, 1H), 7.30–7.33 (m, 3H), 7.40–7.59 (m, 4H), 7.60–7.68 (m, 3H). Anal. Calcd for $C_{19}H_{18}ClN_3O_4$: C, 58.84; H, 4.68; N, 10.84. Found: C, 58.68; H, 4.75; N, 10.74.

5.4.29. N^4-(3-Chlorophenyl)-2-cinnamamido-N^1-hydroxysuccinamide (8j)

0.78 g, yield: 75.0%, mp: 149–152 °C, $[\alpha]_D^{25}$ +26.1 (*c* l, MeOH); ESI-MS: *m/z*: 388.5 $[M+H]^+$; ^1H NMR (CD_3OD) δ 2.66–2.77 (m, 2H), 4.73–4.78 (m, 1H), 6.70 (d, J = 16, 1H), 7.18–7.39 (m, 3H), 7.40–7.44 (m, 4H), 7.55–7.63 (m, 3H). Anal. Calcd for $C_{19}H_{18}ClN_3O_4$: C, 58.84; H, 4.68; N, 10.84. Found: C, 58.67; H, 4.74; N, 10.78.

5.4.30. N^4-Benzyl-2-cinnamamido-N^1-hydroxysuccinamide (8k)

0.80 g, yield: 76.70%, mp: 136–138 °C, $[\alpha]_D^{25}$ +33.2 (*c* l, MeOH); ESI-MS: *m/z*: 368.5 $[M+H]^+$; ^1H NMR (CD_3OD) δ 2.61–2.70 (m, 2H), 4.45 (s, 2H), 4.88–4.93 (m, 1H), 6.71 (d, J = 16, 1H), 7.23–7.32 (m, 5H), 7.40–7.41 (m, 3H), 7.55–7.59 (m, 3H). Anal. Calcd for $C_{20}H_{21}N_3O_4$: C, 65.38; H, 5.76; N, 11.44. Found: C, 65.59; H, 5.80; N, 11.36.

5.4.31. 2-Cinnamamido-N^1-hydroxy-N^4-phenylsuccinamide (8l)

0.77 g, yield: 73.75%, mp: 166–168 °C; $[\alpha]_D^{25}$ +21.8 (*c* l, MeOH); ESI-MS: *m/z*: 354.5 $[M+H]^+$; ^1H NMR (CD_3OD) δ 2.66–2.78 (m, 2H), 4.90–4.99 (m, 1H), 6.64 (d, J = 16, 1H), 7.14–7.34 (m, 3H), 7.36–7.60 (m, 5H), 7.60–7.68 (m, 3H). Anal. Calcd for $C_{19}H_{19}N_3O_4$: C, 64.58; H, 5.42; N, 11.89. Found: C, 64.37; H, 5.46; N, 11.81.

5.4.32. 2-Cinnamamido-N^1-hydroxy-N^4-(3-nitrophenyl) succinamide (8m)

0.70 g, yield: 67.37%, mp: 153–155 °C, $[\alpha]_D^{25}$ +23.9 (*c* l, MeOH); ESI-MS: *m/z*: 399.4 $[M+H]^+$; ^1H NMR (CD_3OD) δ 2.71–2.79 (m, 2H), 4.85–4.94 (m, 1H), 6.67 (d, J = 16, 1H), 7.40–7.41 (m, 3H), 7.56–7.61 (m, 3H), 7.74–7.78 (m, 3H), 8.68 (s, 1H). Anal. Calcd for $C_{19}H_{118}N_4O_6$: C, 57.28; H, 4.55; N, 14.06. Found: C, 57.10; H, 4.52; N, 14.14.

5.4.33. 2-Cinnamamido-N^1-hydroxy-N^4-(4-nitrophenyl) succinamide (8n)

0.74 g, yield: 71.22%, mp: 206–207 °C, $[\alpha]_D^{25}$ +31.2 (*c* l, MeOH); ESI-MS: *m/z*: 399.4 $[M+H]^+$; ^1H NMR (CD_3OD) δ 2.61–2.84 (m, 2H), 4.61–4.93 (m, 1H), 6.73 (d, J = 16, 1H), 7.40–7.42 (m, 3H), 7.59–7.62 (m, 3H), 7.87–7.90 (m, 2H), 8.22–8.25 (m, 2H). Anal. Calcd for $C_{19}H_{118}N_4O_6$: C, 57.28; H, 4.55; N, 14.06. Found: C, 57.09; H, 4.50; N, 14.12.

5.4.34. 2-Cinnamamido-N^1-hydroxy-N^4-(naphthalene-1-yl) succinamide (8o)

0.66 g, yield: 63.52%, mp: 170–172 °C; $[\alpha]_D^{25}$ +36.8 (*c* l, MeOH); ESI-MS: *m/z*: 404.6 $[M+H]^+$; ^1H NMR (CD_3OD) δ 2.66–2.86 (m, 2H), 4.93–4.96 (m, 1H), 6.65 (d, J = 16, 1H), 6.82 (d, J = 12, 1H), 7.21–7.22 (m, 3H), 7.39–7.41 (m, 3H), 7.44–7.56 (m, 4H), 8.64–8.71 (m, 2H). Anal. Calcd for $C_{23}H_{21}N_3O_4$: C, 68.47; H, 5.25; N, 10.42. Found: C, 68.26; H, 5.28; N, 10.49.

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