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CD4 mimics targeting the mechanism of HIV entry

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ABSTRACT

A structure–activity relationship study was conducted of several CD4 mimicking small molecules which block the interaction between HIV-1 gp120 and CD4. These CD4 mimics induce a conformational change in gp120, exposing its co-receptor-binding site. This induces a highly synergistic interaction in the use in combination with a co-receptor CXCR4 antagonist and reveals a pronounced effect on the dynamic supramolecular mechanism of HIV-1 entry.

Recently, remarkable success has attended the clinical treatment of HIV-infected and AIDS patients, with 'highly active antiretroviral therapy (HAART)'. This approach involves a combination of two or three agents from two categories: reverse transcriptase inhibitors and protease inhibitors.[1] In addition, the molecular mechanism involved in HIV-entry and -fusion into host cells has been described in detail.[2] The complex interactions of surface proteins on cellular and viral membranes, which are designated as a dynamic supramolecular mechanism of HIV entry, are reported to be crucial to the viral infection. In a first step, an HIV envelope protein, gp120 interacts with a cell surface protein, CD4, leading to a conformational change in gp120 followed by subsequent binding of gp120 to a co-receptor CCR5[3] or CXCR4.[4] CCR5 and CXCR4 are the major co-receptors for the entry of macrophage-tropic (R5-) and T cell line-tropic (X4-) HIV-1, respectively. The interaction of gp120 with CCR5 or CXCR4 triggers entry of another envelope protein, gp41 to the cell membrane and formation of a gp41 trimer-of-hairpins structure, which causes fusion of HIV/cell-membranes and completes the infection.

Informed by this mechanism, a fusion inhibitor, enfuvirtide (fuzeon, Trimers & Roche)[5] and a CCR5 antagonist, maraviroc (Pfizer)[6] in addition to an integrase inhibitor, raltegravir (Merck)[7] have been used clinically. However, serious problems with chemotherapy still persist, including the emergence of viral strains with multi-drug resistance (MDR), considerable adverse effects and high costs. Consequently, development of novel drugs possessing mechanisms of action different from those of the above inhibitors is currently required. We have previously developed selective CXCR4 antagonists[8] and fusion inhibitors.[9] Furthermore, *N*-(4-Bromophenyl)-*N'*-(2,2,6,6-tetramethylpiperidin-4-yl)-oxalamide (**1**) and *N*-(4-chlorophenyl)-*N'*-(2,2,6,6-tetramethylpiperidin-4-yl)-oxalamide (**2**) were previously found using chemical library screening to inhibit syncytium formation by other researchers.[10] **1** and **2** bind to gp120 with binding affinities of K_d = 2.2 μM and 3.7 μM, respectively, blocking the interaction of gp120 with CD4 in the first step of an HIV-1 entry. Thus, in the present study we focus on the development of CD4 mimics that can block the interaction between gp120 and CD4. We have investigated the effect of CD4 mimics on conformational changes of gp120 and on their use in combination use with a CXCR4 antagonist.

Initially, molecular modeling of compound **2** docked into gp120 was carried out using docking simulations performed by the FlexSIS module of SYBYL 7.1 (Tripos, St. Louis) (Fig. 1).[11] The atomic coordinates of the crystal structure of gp120 with soluble CD4 (sCD4) were retrieved from Protein Data Bank (PDB) (entry 1RZJ) (Fig. 1a) and it was observed that Phe^{43} and Arg^{59} of the CD4 have multiple contacts with Asp^{368}, Glu^{370} and Trp^{427} of gp120, which are all conserved residues. An inspection of the environment of compound **2** docked in gp120 revealed the presence of a large cavity around the *p*-position of the phenyl ring of compound **2**, which could interact with the viral surface protein gp120 (Fig. 1b and c). Several analogs of **2** with substituents on the phenyl ring were therefore synthesized.

All compounds except **12** were synthesized by previously published methods (Scheme 1).[10b,12,13] Aniline derivatives (**3**) were coupled with ethyl oxalyl chloride to yield the corresponding ethyl oxalamates **4**. Saponification of the above oxalamates to the corresponding free acids and the subsequent coupling with 4-ami-

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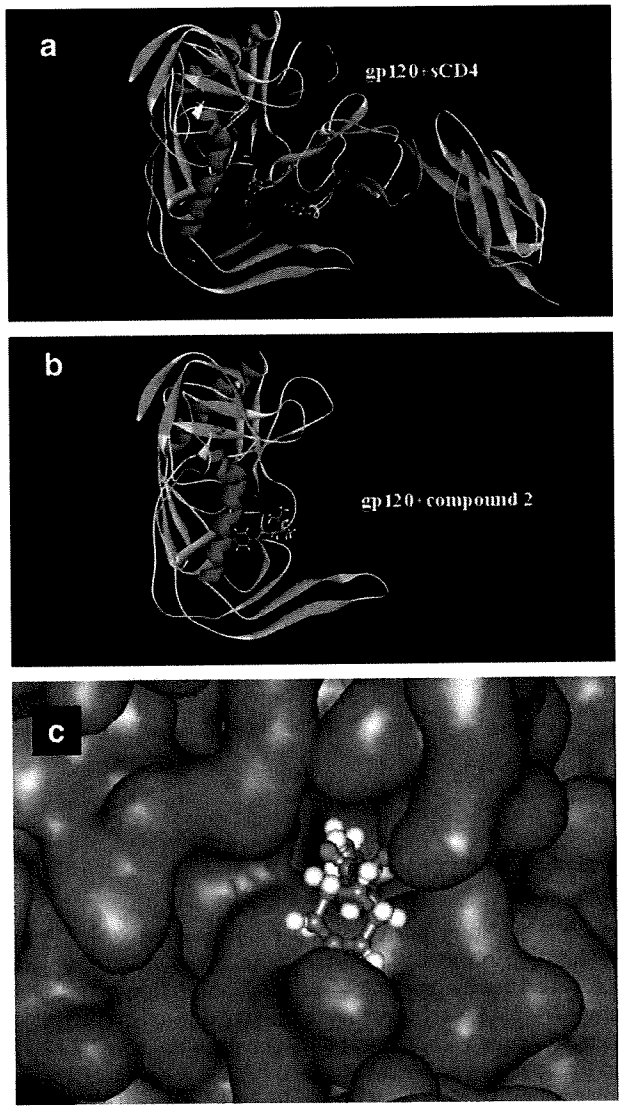
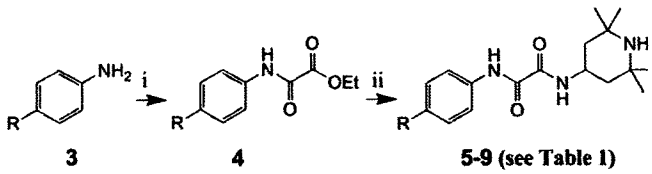
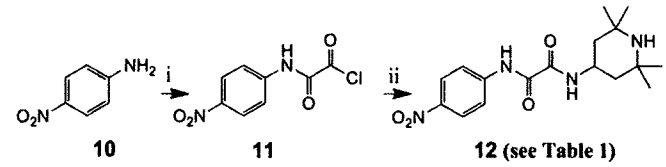


Figure 1. (a) The crystal structure of gp120 with soluble CD4 (sCD4) retrieved from the PDB (entry 1RZJ); (b) docking structure of compound **2** and gp120; (c) a focused figure of (b) shown by space-filling model.



Scheme 1. Reagents and conditions: (i) ethyl oxalyl chloride, Et_3N; (ii) 1 M NaOH; 4-amino-2,2,6,6-tetramethylpiperidine, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride, 1-hydroxybenzotriazole, Et_3N.

no-2,2,6,6-tetramethylpiperidine using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and 1-hydroxybenzotriazole (HOBt) yielded compounds **5–9**. In the case of compound **12**, whose amide bond is not stable during the reaction of the saponification of the corresponding oxalamates, an alternative synthetic scheme was used (Scheme 2).[14] The reaction of *p*-nitroaniline (**10**) with oxalyl chloride gave the corresponding oxoacetamide **11**, which was subsequently coupled with 4-amino-2,2,6,6-tetramethylpiperidine to yield the desired compound **12**.



Scheme 2. Reagents and conditions: (i) oxalyl chloride, Et_3N; (ii) 4-amino-2,2,6,6-tetramethylpiperidine, Et_3N.

The anti-HIV activity of the synthetic compounds was evaluated against various viral strains including both laboratory and primary isolates (Table 1). IC_{50} values were determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method[15] as the concentrations of the compounds which conferred 50% protection against HIV-1-induced cytopathogenicity in PM1/CCR5 cells. Cytotoxicity of the compounds based on the viability of mock-infected PM1/CCR5 cells was also evaluated using the MTT method. CC_{50} values were determined as the concentrations achieving 50% reduction of the viability of mock-infected cells. Compounds **1** and **2** showed potent anti-HIV activity against laboratory isolates, IIIB (X4, Sub B) and 89.6 (dual, Sub B) strains, and compound **2** also possessed potent activity against a primary isolate, an fTOI strain (R5, Sub B). All of the IC_{50} values were between 4 μM and 10 μM. Compound **1** was not tested against primary isolates. The potencies of compounds **1** and **2** are comparable to the reported binding affinities for gp120 (K_d = 2.2 and 3.7 μM, respectively).[10] Several of the new analogs of compounds **1** and **2** showed significant anti-HIV activity. Compound **5**, which has a phenyl group in place of the *p*-chlorophenyl group of compound **2**, did not show significant anti-HIV activity at concentrations below 100 μM against all strains tested except for an fTOI strain (R5, Sub B). This result suggests that a substituent at the *p*-position of the phenyl ring is critical for potent activity. Compound **6**, which has a fluorine atom at the *p*-position of the phenyl ring, showed moderate anti-HIV activity against laboratory isolates, IIIB (X4, Sub B) and 89.6 (dual, Sub B) strains (IC_{50} = 61 and 81 μM, respectively), but, at concentrations below 100 μM, failed to show significant anti-HIV activity against a primary isolate, a KYAG strain (R5, Sub B). Among halogen atoms, fluorine is less suitable than bromine or chlorine as a substituent at the *p*-position of the phenyl ring, as evidenced by compound **6**, which is 8–15-fold less potent than compounds **1** and **2** against IIIB (X4, Sub B) and 89.6 (dual, Sub B) strains. Compound **7**, which has a methyl group at the *p*-position of the phenyl ring, showed relatively more potent activity against IIIB (X4, Sub B) and 89.6 (dual, Sub B) strains (IC_{50} = 23 and 41 μM, respectively) than compound **6**. Compound **7** also showed significant anti-HIV activity against primary isolates, fTOI (R5, Sub B) and KYAG (R5, Sub B) strains (IC_{50} = 16 and 51 μM, respectively). Compound **8**, with a methoxy group at the *p*-position of the phenyl ring, did not show significant anti-HIV activity against all strains tested until a concentration of 100 μM was reached. In the biological assays, derivatives having electron-withdrawing substituents such as bromine, chlorine and fluorine at the *p*-position of the phenyl ring are relatively potent, whereas derivatives having electron-donating groups such as methoxy at this position are not potent. Furthermore, the steric effect of a substituent at the *p*-position of the phenyl ring appears to be critical to anti-HIV activity. The sum of Hammett constants (σ) of benzoic acid substituents[16] shown in Table 1 can be used to evaluate the electron-withdrawing or -donating effect of the substituents on the aromatic ring. The Taft E_s values[16a,17] were used as steric parameters for substituents at the *p*-position of the phenyl ring. The order of potency found for the halogen-containing derivatives in anti-HIV activity against laboratory isolates, IIIB (X4, Sub B) and 89.6 (dual, Sub B), is: compound **1** (R = Br) (σ = 0.23, E_s = −1.16), **2**

Table 1
Hammett constants (σ) and steric effects (E_s) of substituted aromatic rings and anti-HIV activity and cytotoxicity of synthetic compounds

Compd	R[a]	σ[b]	E_s[c]	IC_{50}[e] (μM)				CC_{50}[e] (μM)
				Lab. isolates		Primary isolates		
				IIIB (X4)	89.6 (dual)	fTOI (R5)	KYAG (R5)	
1	Br	0.23	−1.16	4	9	ND	ND	150
2	Cl	0.23	−0.97	8	10	5	>30	170
5	H	0	0	>100	>100	81	>100	350
6	F	0.06	−0.46	61	81	ND	>100	320
7	CH_3	−0.17	−1.24	23	41	16	51	210
8	OCH_3	−0.27	−0.55	>100	>100	ND	>100	340
9	CF_3	0.54	−2.40	ND	27	ND	ND	72
12	NO_2	0.78	−1.77[d]	ND	42	ND	ND	230
sCD4				0.010	0.021	0.0044	ND	ND

[a] See Schemes 1 and 2.
[b] σ = Hammett constant of a substitutent on a benzoic acid derivative.[16]
[c] E_s = steric effect of a substituent at the *para* position on the aromatic ring.[16a,17]
[d] The average value of −1.01 and −2.52, which are E_s values of the NO_2 group, −1.77, was used.
[e] Values are means of at least three experiments (ND = not determined).

(R = Cl) (σ = 0.23, E_s = −0.97), **6** (R = F) (σ = 0.06, E_s = −0.46) and **5** (R = H) (σ = 0, E_s = 0). This is the order of substituents' electron-withdrawing ability and also of their size. Methyl (σ = −0.17, E_s = −1.24) is an electron-donating group, but is almost as bulky as a bromine atom. Thus, the *p*-methyl derivative **7** has relatively potent anti-HIV activity against laboratory isolates, IIIB (X4, Sub B) and 89.6 (dual, Sub B), higher than that of compound **6** (R = F) but lower than that of compound **1** (R = Br) or **2** (R = Cl). The electron-donating ability of a methoxy group is stronger (σ = −0.27), but the bulk size is smaller (E_s = −0.55), than that of a methyl group. Thus, the *p*-methoxy derivative **8** has no significant anti-HIV activity against all strains tested at concentrations below 100 μM. Two derivatives containing bulkier and more potent electron-withdrawing substituents such as trifluoromethyl (R = CF_3) (σ = 0.54, E_s = −2.40) and nitro (R = NO_2) (σ = 0.78, E_s = −1.77) at the *p*-position of the phenyl ring were evaluated. Compounds **9** (R = CF_3) and **12** (R = NO_2) showed significant anti-HIV activity against an 89.6 (dual, Sub B) strain. These are less potent than compounds **1** and **2** and this is perhaps due to the excessive size of the substituents at the *p*-position. This suggests that a certain level of the bulk size and a potent electron-withdrawing ability of the substituents are preferable for anti-HIV activity. It is estimated that a cavity around the *p*-position of the phenyl ring of CD4 mimicking compounds would be optimally filled by bromine (E_s = −1.16) or a methyl group (E_s = −1.24) at *p*-position, and that an electron-deficient aromatic ring might interact tightly with a negatively charged group such as carboxy of Glu^{370}. In isothermal titration calorimetry (ITC) experiments reported elsewhere,[10c] compound **5** (R = H) does not have significant affinity for gp120, and compound **6** (R = F) has less potent affinity for gp120 than compound **2**, consistent with the present data. In all but one of the compounds, no significant cytotoxicity was detected (CC_{50} >150 μM, Table 1), the exception being compound **9** (R = CF_3) (CC_{50} = 72 μM). Compounds **7** and **12** have relatively low cytotoxicities, compared to compounds **1** and **2**.

Fluorescence activated cell sorting (FACS) analysis was performed[15] to investigate whether these synthetic compounds interact with gp120 inducing the conformational change necessary for the approach of an anti-envelope antibody or a co-receptor to the gp120. The profile of binding of an anti-envelope CD4-induced monoclonal antibody, 4C11, to the Env-expressing cell surface (an R5-HIV-1 strain, JR-FL,-infected PM1 cells) pretreated with the above CD4 mimic analogs was examined. Comparison of the binding of 4C11 to the cell surface was measured in terms of the mean fluorescence intensity (MFI), and is shown in Figure 2. Pretreatment of the Env-expressing cells with compound **2** (MFI = 38.42) produced a remarkable increase in binding affinity for 4C11, similar to that observed in pretreatment with sCD4 (MFI = 37.90). This is consistent with the results in the previous paper[10] where it was reported that compound **2** enhances the binding of gp120 to the 17b monoclonal antibody which recognizes the co-receptor binding site of gp120. Env-expressing cells, which were not pretreated with sCD4 or a CD4 mimic compound, did not show significant binding affinity for 4C11 (Fig. 2, blank). The increase in binding affinity for monoclonal antibodies may be due to conformational changes in gp120, which were caused by the interaction of sCD4 or a CD4 mimic with gp120. It is hypothesized that such conformational changes involve the exposure of the co-receptor binding site of gp120 (the V3 loop), which is hidden internally, since the binding of gp120 to 17b is enhanced. Compound **5**, which failed to show significant anti-HIV activity, and compounds **7**, **9** and **12**, which had significant anti-HIV activity, were assessed in the FACS analysis. The profile of the binding of 4C11 to the Env-expressing cell surface pretreated with compound **5** (MFI = 14.34) was similar to that of the blank (MFI = 11.24), suggesting that compound **5** offers no significant enhancement of binding affinity for 4C11. This result is compatible with the anti-HIV activity of compound **5**. The profile of the binding of 4C11 to the Env-expressing cell surface pretreated with compound **7** (MFI = 38.33) was entirely similar to that of compound **2** used as a pretreatment. Pretreatment of the cell surface with compounds **9** and **12** (MFI = 29.09 and 30.01, respectively) produced a slightly lower enhancement of binding affinity for 4C11, compared to those of compounds **2** and **7** as pretreatments. However, in the ITC experiments reported elsewhere,[10c] compound **9** (R = CF_3) has a high affinity for gp120, comparable to that of compound **2**, but compound **12** (R = NO_2) does not have significant affinity for gp120, indicating that these are not consistent with the current FACS studies, possibly due to the difference in the assay systems. Although the anti-HIV activity of **7** is weaker than that of compound **2**, the level of compound **7** inducing an enhancement of binding affinity of gp120 for 4C11 is comparable to that of compound **2**. The concentration of compounds used in the FACS analysis was 100 μM, much beyond the IC_{50} values of compounds **2** and **7**. A concentration of 100 μM would be also sufficient for the expression of anti-HIV activity caused by compounds **2** and **7**.

An effect on the use of compound **2** combined with another entry inhibitor was investigated. Analysis of the synergistic effects of anti-HIV agents was performed according to the median effect principle using the CalcuSyn version 2 computer program[18] to estimate IC_{50} values of compounds in different combinations. Combination indices (CI) were estimated from the data evaluated using the MTT assay

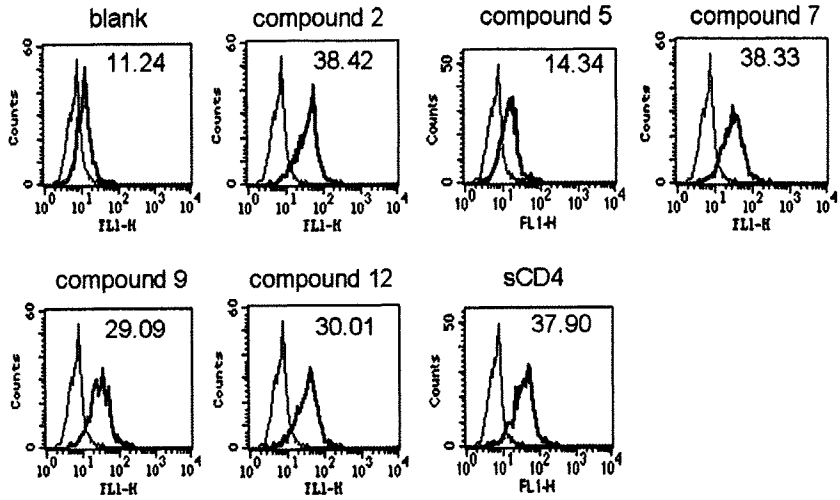


Figure 2. JR-FL (R5, Sub B) chronically infected PM1 cells were preincubated with 100 μM of a CD4 mimic or sCD4 (11 nM) for 15 min, and then incubated with an anti-HIV-1 mAb, 4C11, at 4 °C for 15 min. The cells were washed with PBS, and fluorescein isothiocyanate (FITC)-conjugated goat anti-human IgG antibody was used for antibody-staining. Flow cytometry data for the binding of 4C11 (green lines) to the Env-expressing cell surface in the presence of sCD4 or a CD4 mimic are shown among gated PM1 cells along with a control antibody (anti-human CD19: black lines). Data are representative of the results from a minimum of two independent experiments. The number at the top of each graph shows the mean fluorescence intensity (MFI) of the antibody 4C11.

Table 2
Combination indices (CI) for compound **2** or sCD4 and a CXCR4 antagonist, T140, against an HIV IIIB strain

Combination	HIV strain	CI values at different IC[a]		
		IC_{50}	IC_{75}	IC_{90}
2 + T140	IIIB	0.786	0.713	0.655
sCD4 + T140	IIIB	0.705	0.528	0.400

[a] The multiple-drug effect analysis reported by Chou et al. was used to analyze the effects of combinational uses of compounds.[18] CI <0.9: synergy, 0.9 < CI < 1.1: additivity, CI >1.1: antagonism.

(Table 2).[15] Compound **2** showed a highly remarkable synergistic anti-HIV activity with a co-receptor CXCR4 antagonist, T140,[8a] against an X4-HIV-1 strain, IIIB at various IC values (IC_{50}, IC_{75} and IC_{90}). However, sCD4 exhibited a higher synergistic effect (lower CI values) with T140 (Table 2). The interaction of sCD4 or a CD4 mimic with gp120 would expose the co-receptor-binding site of gp120, and the co-receptor CXCR4 could then easily approach gp120. Thus, an inhibitory effect of a CXCR4 antagonist would be meaningful, and a significant synergistic effect might also be brought about by a combination of sCD4 or a CD4 mimic and T140.

In summary, a series of CD4 mimic compounds were synthesized and evaluated for their anti-HIV activity. Several compounds showed significant anti-HIV activity with relatively low cytotoxicity. SAR studies showed that a certain level of size and electron-withdrawing ability of the substituents at the *p*-position of the phenyl ring are suitable for potent anti-HIV activity. In addition, the treatment of Env-expressing cells with several CD4 mimicking compounds causes a conformational change, exposing the co-receptor-binding site of gp120 externally. Thus, a CD4 mimic exhibited a remarkable synergistic effect with a co-receptor antagonist. These compounds are essential probes directed to the dynamic supramolecular mechanism of HIV entry, and important leads for the cocktail therapy of AIDS.

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11. The structure of compound **2** was built in Sybyl and minimized with the MMFF94 force field and partial charges. (see: Halgren, T. A. *J. Comput. Chem.* **1996**, *17*, 490.) Docking was then performed using FlexSIS through its SYBYL

module, into the crystal structure of gp120 (PDB, entry 1RZJ). The binding site was defined as residues Val^{255}, Asp^{368}, Glu^{370}, Ser^{375}, Ile^{424}, Trp^{427}, Val^{430} and Val^{475}, and included residues located within a radius 4.4 Å. The ligand was considered to be flexible, and all other options were set to their default values. Figures were generated with ViewerLite version 5.0 (Accelrys Inc., San Diego, CA).

12. *For example, the synthesis of compound* **7**: To a solution of ethyl oxalyl chloride (0.400 mL, 3.48 mmol) in THF (20 mL) were added triethylamine (Et_3N) (0.480 mL, 3.48 mmol) and *p*-toluidine (373 mg, 3.48 mmol) with stirring at 0 °C. The reaction mixture was allowed to warm to room temperature, and then stirred for 6 h. After removal by filtration of the resulting salts, the filtrate was concentrated under reduced pressure. The residue was extracted with EtOAc (50 mL), and the extract was washed successively with brine (20 mL), 1 M HCl (20 mL × 2), brine (20 mL), saturated $NaHCO_3$ (20 mL × 2) and brine (20 mL × 3), then dried over $MgSO_4$. Concentration under reduced pressure gave the crude ethyl oxalamate, which was used without further purification. To a solution of the crude ethyl oxalamate (640 mg, 3.09 mmol) in THF (30 mL) were added aqueous 1 M NaOH (3.40 mL, 3.40 mmol), water (50 mL) and MeOH (20 mL) with stirring at 0 °C. The reaction mixture was allowed to warm to room temperature, and then stirred for 20 h. After the addition of aqueous 1 M HCl (5 mL), MeOH and THF were evaporated under reduced pressure. The residue was acidified to pH 2 with 1 M HCl, and extracted with EtOAc (50 mL × 2). The combined organic layer was washed with brine (20 mL × 3), and dried over $MgSO_4$. Concentration under reduced pressure gave the crude acid, which was used for the next reaction without further purification. To a solution of the above crude acid (514 mg, 2.87 mmol) in THF (10 mL) were added 1-hydoxybenzotriazole (484 mg, 3.16 mmol), 4-amino-2,2,6,6-tetramethylpiperidine (446 μL, 2.58 mmol), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (606 mg, 3.16 mmol) and Et_3N (0.439 mL, 3.16 mmol) with stirring at 0 °C. The reaction mixture was allowed to warm to room temperature, and then stirred for 20 h. After evaporation of THF, the residue was dissolved in $CHCl_3$ (50 mL). The mixture was washed with saturated $NaHCO_3$ (20 mL × 2) and brine (20 mL × 3), and dried over $MgSO_4$. Concentration under reduced pressure gave the crude crystalline mass. The usual work-up followed by recrystallization from EtOAc–*n*-hexane gave the title compound **7** (363 mg, 1.14 mmol, 39.8%) as colorless crystals, mp = 176 °C; δ_H (400 MHz; $CDCl_3$) 1.07 (1H, m, NH), 1.16 (6H, s, CH_3), 1.29 (6H, s, CH_3), 1.44 (2H, m, CH_2), 1.91 (1H, d, *J* 3.7, C*H*H), 1.94 (1H, d, *J* 3.7, CH*H*), 2.34 (3H, s, CH_3), 4.25 (1H, m, CH), 7.17 (2H, d, *J* 8.3, ArH), 7.33 (1H, m, NH), 7.50 (2H, d, *J* 8.4, ArH), 9.18 (1H, s, NH); HRMS (FAB), *m/z* calcd for $C_{18}H_{28}N_3O_2$ $(MH)^+$ 318.2182, found 318.2173.
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14. *The synthesis of compound* **12**: To a solution of Et_3N (417 μL, 3.00 mmol) and 4-nitroaniline (138 mg, 1.00 mmol) in THF (1.3 mL) was added oxalyl dichloride (85.8 μL, 1.00 mmol) with stirring at 0 °C. After being stirred for 30 min at 0 °C, Et_3N (167 μL, 1.20 mmol) and 4-amino-2,2,6,6-tetramethylpiperidine (156 μL, 0.90 mmol) were added. The reaction mixture was stirred for 6 h at 0 °C. After removal by filtration of the resulting salts, the filtrate was concentrated under reduced pressure. The residue was dissolved in $CHCl_3$ (20 mL), and the mixture was washed successively with brine (10 mL), saturated $NaHCO_3$ (10 mL × 2) and brine (10 mL × 3), and dried over $MgSO_4$. Concentration under reduced pressure followed by flash chromatography over silica gel with $CHCl_3$–MeOH (9:1) gave 42.4 mg (0.122 mmol, 13.5%) of the title compound **12** as colorless crystals, mp = 190 °C; δ_H (400 MHz; $CDCl_3$) 1.09 (1H, m, NH), 1.17 (6H, s, CH_3), 1.29 (6H, s, CH_3), 1.43 (2H, m, CH_2), 1.92 (1H, d, *J* 3.8, C*H*H), 1.95 (1H, d, *J* 3.8, CH*H*), 4.28 (1H, m, CH), 7.29 (1H, m, NH), 7.82 (2H, d, *J* 9.1, ArH), 8.28 (2H, d, *J* 9.1, ArH), 9.55 (1H, s, NH); HRMS (FAB), *m/z* calcd for $C_{17}H_{25}N_4O_4$ $(MH)^+$ 349.1876, found 349.1871.
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