Bis-Substituted Malonic Acid Hydroxamate Derivatives as Inhibitors of Human Neutrophil Collagenase (MMP8)

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Malonic acid hydroxamate derivatives bis-substituted at the methylene group were synthesized as potential nonpeptidic inhibitors of human neutrophil collagenase (MMP8). The presence of an aromatic residue both at the C2 malonic acid position and in the C-terminal tail for hydrophobic interactions with the surface-exposed S1 binding site and the S1′ pocket of the enzyme, respectively, was found to be sufficient for submicromolar inhibition potencies. For optimal insertion of the aryl amide group into the hydrophobic S1′ pocket, spacing of the C-terminal phenyl group by at least a 3C-chain was required. In view of these results the achiral indan-2,2-dicarboxylic acid was used to mimic the 2-benzyl-2-methylmalonic acid residue, and its derivatization to the 3-phenylpropyl amide hydroxamate produced a potent, achiral, low-mass inhibitor of MMP8 (K_i = 0.3 μM), the binding mode of which was unambiguously determined by X-ray crystallographic analysis.

Introduction

The X-ray analyses of complexes of matrix metalloproteinases (MMPs) with synthetic hydroxamate- or thiol-type inhibitors revealed binding of the inhibitors to the active-site cleft of the enzymes in extended conformations and in a manner similar to the binding mode of the substrates,[1−15] except for the thermolysin inhibitor (2*R*,*S*)-HONH-Mal(*i*-Bu)-Ala-Gly-NH_2.[16,17] This malonic acid-based hydroxamate binds to human neutrophil collagenase (MMP8) with the hydroxamate acting as a bidentated chelator of the active-site Zn^{2+} ion.[15] The hydrophobic substituent at the malonic acid moiety is directed toward the edge strand of the active-site cleft in a hydrophobic interaction with the S1 subsite of the enzyme. In contrast to the succinic acid hydroxamates which represent the most potent MMPs inhibitors known so far,[18] the malonic acid-based inhibitor lacks a spacer between the chelating group and the hydrophobic group. Correspondingly, the position of the side chain of the subsequent amino acid residue, i.e., of Ala, becomes similar but not identical to that of P2′ residues in substrates, while the residual peptidic tail is inserted into the S1′ pocket, thus leading to an overall β-turn-like conformation of the inhibitor in the enzyme-bound state.

In a previous study we have analyzed the possibility of improving the binding affinity of malonic acid-based compounds for MMP8 by iterative changes in the peptidic tail at the pseudo-P1′ residue and in the P2′ residue, as well as varying the methylene substituent of the malonic acid residue as a pseudo-P1 residue.[19] Thereby X-ray structural analyses of selected MMP8/inhibitor complexes fully confirmed the non-substrate-like binding mode of these malonic acid-based inhibitors.[20] By conversion of the peptidic to nonpeptidic malonic acid derivatives, submicromolar inhibitory potencies were obtained if the following two conditions were met: (i) sufficiently large hydrophobic pseudo-P1′ residues to ensure strong interactions with the hydrophobic S1′ pocket of MMP8 and (ii) monosubstitution at the methylene group of the malonic acid moiety with alkyl or aryl groups for additional binding to the surface-exposed S1 subsite.[19]

In the present study a bis-substitution at the methylene group of the malonic acid hydroxamate was analyzed as a possible approach to improve inhibition of MMP8 by low-molecular-weight nonpeptidic hydroxamates.

Results and Discussion

Chemistry. Monoalkylation of diethyl malonate at the C2 position was performed by standard methods with alkyl halides.[19] In a subsequent alkylation step the 2,2-bis-substituted diethyl malonates were obtained following essentially known procedures as outlined in Scheme 1.[21−23] Conversely, the cyclic bis-substituted malonic acid diethyl esters were prepared in one step employing suitable dibromo derivatives.[24,25] The resulting 2,2-bis-substituted malonic acid diethyl esters were then hydrolyzed with 1 equiv of KOH to produce the monoethyl esters which in turn were condensed with (benzyloxy)amine using EDCI/HOBT as coupling reagent to avoid the guanidine adduct formation.[19] After an additional saponification step with excesses of KOH, the resulting benzyl-protected malonic acid hydroxamates were amidated with arylamines using again the

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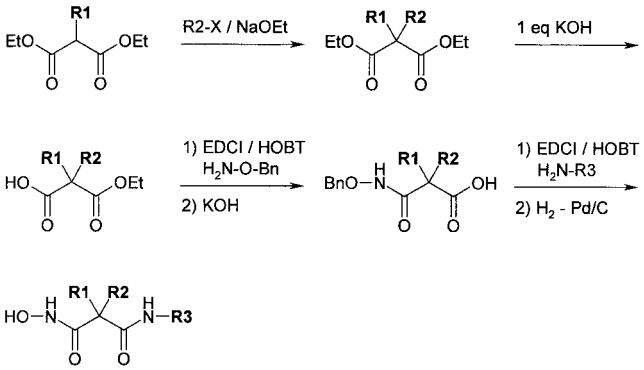
Scheme 1. General Synthetic Route Used for the Synthesis of the Chiral and Achiral Malonic Acid-Based MMP8 Inhibitors

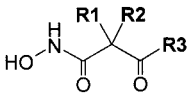


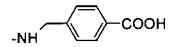
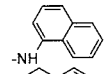
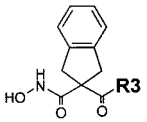
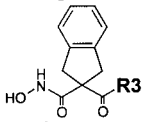
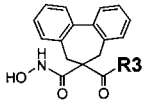
EDCI/HOBt procedure. Finally, hydrogenolytic cleavage of the benzyl group from the hydroxamate over Pd/C catalyst led to the target hydroxamic acids.

MMP8 Inhibition by Bis-Substituted Malonic Acid Hydroxamates. In our previous study on monosubstituted malonic acid-based MMP inhibitors, both branched alkyl and aryl derivatives such as 2-isobutyl, 2-benzyl, or 2-(2-phenyl)ethyl compounds were found to increase the inhibitory potency[19] by hydrophobic interactions with the S1 subsite as clearly revealed by X-ray analysis.[15,20] In an attempt to further exploit the S1 binding site, we have synthesized bis-substituted malonic acid derivatives, and a comparison of the inhibitory potency of 2-isobutyl-2-methylmalonic acid hydroxamate ethyl ester (**2**) (K_i = 41 μM) with that of the related 2-benzyl-2-methyl derivative **5** (K_i = 11 μM) indicated a clear advantage of an aromatic substituent (Table 1). To answer the question of whether the bis-substituted derivatives retain the binding mode of the monosubstituted malonic acid-based inhibitors, X-ray crystallographic analysis of the MMP8/**5** complex was performed. Apparent disorder in the crystals of MMP8/**5** prevented the unambiguous determination of a single structure, possibly because both stereoisomers may be bound in the crystals. An initial difference electron density map supported the binding mode shown in Figure 1 where the hydroxamate group acts as a bidentated chelator of the active-site Zn^{2+} ion, the benzyl group covers a hydrophobic patch on the surface of the enzyme, and the following carbonyl group is hydrogen-bonded to Leu160NH whereas the aliphatic tail extends to the S1′ pocket. However, the resolution of the electron density map does not rule out binding of the alternate stereoisomer with the aromatic group inserted into the S1′ pocket and the ethyl ester group interacting at the exposed site. Indeed, the electron density map of a second crystal seems to favor this interpretation although again not unambiguously. Simultaneous binding of both stereoisomers would support the hypothesis of similar binding free energies for both. Since from volume considerations the aliphatic binding in S1′ is likely weaker than that of the aromatic group, the hydrophobic interactions of the exposed benzyl group would have to compensate for this loss.

In view of these results, in a first series of compounds the methyl/benzyl substitution pattern at the malonic acid was retained and the effect of different C-terminal derivatizations for interaction with the S1′ subsite of

Table 1. Inhibition of MMP8 with Chiral and Achiral Bis-Substituted Nonpeptidic Malonic Acid Hydroxamates



R1	R2	R3	Compound	K_i [μM]*
Me	*i*-Butyl	O-Ethyl	**2**	41
Me	CH_2-Ph	O-Ethyl	**5**	11
Me	CH_2-Ph	NH-Ph	**7**	44
Me	CH_2-Ph	NH-CH_2-Ph	**8**	1.1
Me	CH_2-Ph	NH-$(CH_2)_2$-Ph	**9**	9.6
Me	CH_2-Ph	NH-$(CH_2)_3$-Ph	**10**	0.24
Me	CH_2-Ph	NH-$(CH_2)_4$-Ph	**11**	0.38
Me	CH_2-Ph		**12**	41
Me	CH_2-Ph		**13**	45
Me	CH_2-Ph		**14**	8.1
NH-$COCH_3$	CH_2-Ph	NH-CH_2-Ph	**16**	2.3
OH	CH_2-Ph	NH-CH_2-Ph	**19**	1.9
		NH-CH_2-Ph	**21**	1.5
		NH-$(CH_2)_3$-Ph	**22**	0.30
		NH-$(CH_2)_3$-Ph	**24**	1.9

*For ±standard errors of the K_i values, see the Experimental Section.

MMP8 was analyzed. Taking into account the beneficial effect of C-terminal aromatic amides observed for of monosubstituted malonic acid hydroxamates,[19] the amides **7**−**11** were synthesized which differ in the number of methylene groups as spacers of the phenyl group from the amide. While the anilide **7** showed a 4-fold weaker inhibitory potency (K_i = 44 μM) than the ester **5**, with the benzyl amide **8** (K_i = 1.1 μM) a remarkably more potent inhibitor with a 10-fold enhanced binding affinity was obtained. Interestingly, the affinity for the enzyme was found to decrease again remarkably with the homobenzyl amide **9** (K_i = 9.6 μM), while further extension of the C-terminus with three (**10**) or four (**11**) methylene groups resulted in significantly improved inhibitory potencies with K_i values in the submicromolar range, i.e., 0.24 and 0.38 μM, respectively. Modeling experiments performed with compounds **10** and **11** using the binding mode of **5** as template indicate better occupancy of the S1′ pocket and thus improved hydrophobic interactions with this enzyme subsite than with **8**. Attempts to similarly improve inhibition of MMP8 with *para*-, *meta*-, or *ortho*-chloro substitutions in the benzyl amide **8** failed (K_i

Figure 1. One of the two possible binding modes of (2*R*,*S*)-HONH-Mal(Me/Bn)-OEt (**5**) to MMP8 as derived from X-ray crystallographic analysis. In the binding mode represented in this figure, the benzyl group interacts with the S1 subunit and the ethyl ester is inserted into the S1′ pocket.

values in the range of 1.8−1.7 μM). Similarly, with *para*-, *meta*-, or *ortho*-methyl derivatives of **8** the K_i values remained in the range of 1.4−9 μM. Introduction of a *para*-carboxyl group into the benzyl amide **8**, as a potential interaction partner for the Arg222 residue on the bottom of the S1′ pocket of MMP8, led to a K_i value of 41 μM. This result would suggest that in contrast to that expected from the modeling experiments described in the Experimental Section, a buried salt bridge between the *para*-carboxyl group and the Arg side chain does not occur and that interactions with the S1′ subsite are significantly reduced. By constraining conformationally the C-terminal benzyl amide residue as an α-naphthyl amide (**13**) weak inhibition was again obtained. Similarly unsuccessful was the derivatization of the bis-substituted malonic acid hydroxamate to the 1,2,9,10-tetrahydroisochinolide (**14**) as a rigid mimetic of the benzyl or 2-phenylethyl amide; the inhibition potency was found to be identical to that of the 2-phenylethyl amide **9**.

Attempts to improve the solubility of the inhibitors as well as the hydrogen-bonding network with the edge strand of the active-site cleft of MMP8 led us to replace the 2-methyl group of compound **8** with an acetamido (**16**) or hydroxy (**19**) group. Both hydroxamates showed slightly reduced inhibitory potencies compared with the parent compound **8** (Table 1).

MMP8 Inhibition by Achiral Cyclic 2,2-Bis-Substituted Malonic Acid Hydroxamates. The bis-substituted malonic acid derivatives described above were all obtained as racemates. To bypass this drawback achiral 2,2-cyclic derivatives of the malonic acid hydroxamates were examined for their affinity for MMP8. For this purpose the indan-2,2-dicarboxylic acid was prepared as a mimetic of the 2-benzyl-2-methyl-malonic acid and converted to the hydroxamate benzyl amide **21**. As expected from modeling experiments, it showed an inhibitory potency that compared well to that of compound **8** (Table 1).

In crystals of the MMP8/**21** complex the structure of the achiral inhibitor could be unambiguously determined, although the electron density around the indan C2 carbon is somewhat weaker (Figure 2). Minor adjustments of the malonic acid moiety and of the substituent, correlated with the indan pucker, might be a reasonable explanation. A comparison of the position of inhibitor **21** in the active-site cleft of the enzyme with that of compound **5** (Figure 3) shows an overall translation of the common groups away from the S1′ pocket; however, hydrogen bonding of the malonic acid carbonyl to Leu160NH is retained and the indan group interacts with the hydrophobic surface formed mainly by the side chain of Ile159, whereas the C-terminal benzyl group inserts into the S1′ pocket.

Similarly to what was observed for the 2-benzyl-2-methyl-substituted derivatives, replacement of the C-terminal benzyl residue with the 3-phenylpropyl group (**22**) leads to improved interactions in the hydrophobic S1′ binding site with a K_i value of 0.3 μM that is almost identical with that of compound **10**. An enhanced hydrophobic interaction at the S1 site was attempted with the 6,7-dihydro-5*H*-dibenzo[*a*,*f*]cycloheptene-6,6-dicarboxylic acid as an achiral mimetic of a 2,2-dibenzylmalonic acid moiety. Its conversion to the hydroxamate 3-phenylpropyl amide **24**, however, led to inhibition of MMP8 with a K_i of 1.9 μM which is 15-fold higher than that of the related indan derivative **22**. Modeling experiments were predicting for compound **24** the binding of one aromatic group in a manner very similar to that of the indan moiety and an additional hydrophobic interaction by the second benzene moiety with the edge of the hydrophobic S1′ pocket. Therefore, the experimentally observed lower inhibition potency of **24** might be attributed more to energetic than entropic factors since the side chain of compound **24** should be almost as rigid as in **22**.

Experimental Section

Materials and Methods. Solvents and reagents used in the synthesis were of the highest quality commercially available. Dnp-Pro-Leu-Gly-Leu-Trp-Ala-D-Arg-NH_2 was purchased from Bachem (Heidelberg, Germany). TLC silica gel 60 plates were from Merck AG (Darmstadt, Germany), and compounds were visualized with the chlorine/tolidine or permanganate reagent. FAB-MS spectra were recorded on a Finnigan MAT 900 spectrometer and the NMR spectra on AMX 400 and AMX 500 (Bruker) spectrometers.

Enzyme Assay. The catalytic domain of MMP8 (Phe^{79},-Gly^{242}-MMP8) was used for the inhibition experiments. Enzyme assays were performed at 25 °C in 10 mM $CaCl_2$, 100 mM NaCl, 50 mM Tris/HCl (pH 7.6) using 8 nM enzyme concentration and the fluorogenic substrate Dnp-Pro-Leu-Gly-Leu-Trp-Ala-D-Arg-NH_2 (1×10^{-5} M). Enzyme kinetics was performed essentially as described by Stack and Gray,[26] and the increase in fluorescence at 350 nm was monitored over a period of 100 s with a luminescence spectrometer LS 50B (Perkin-Elmer) to determine initial rates of hydrolysis. Evaluation of the kinetic data was performed as reported by Copeland et al.,[27] and the IC_{50} values were determined with the program Enzfitter (Version 1.05, 1987, Elsevier-Biosoft); average standard errors for the IC_{50} values were ±5−10%. Assuming Michaelis−Menten kinetics, the K_i values were then calculated from the IC_{50} values according to the equation: $K_i = IC_{50} \cdot K_m/(K_m + [S])$.

X-ray Crystallography. Crystals of the MMP8 (Phe^{79},-Gly^{242}-MMP8) complexed with inhibitor **21** were grown with sitting drop vapor diffusion using conditions similar to those

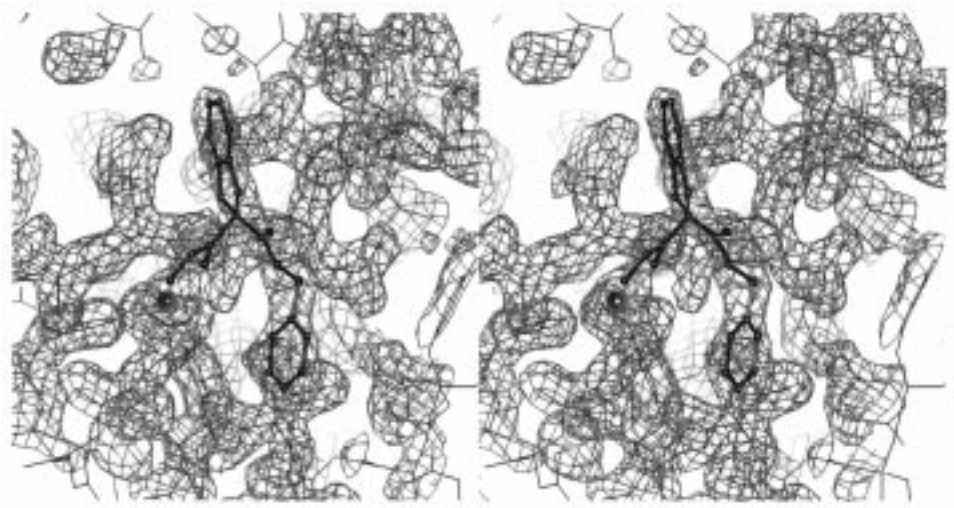


Figure 2. X-ray structure of HONH-Ind-NHBn (**21**) in the MMP8/inhibitor complex superimposed with the 2.0 Å $2F_o - F_c$ electron density map.

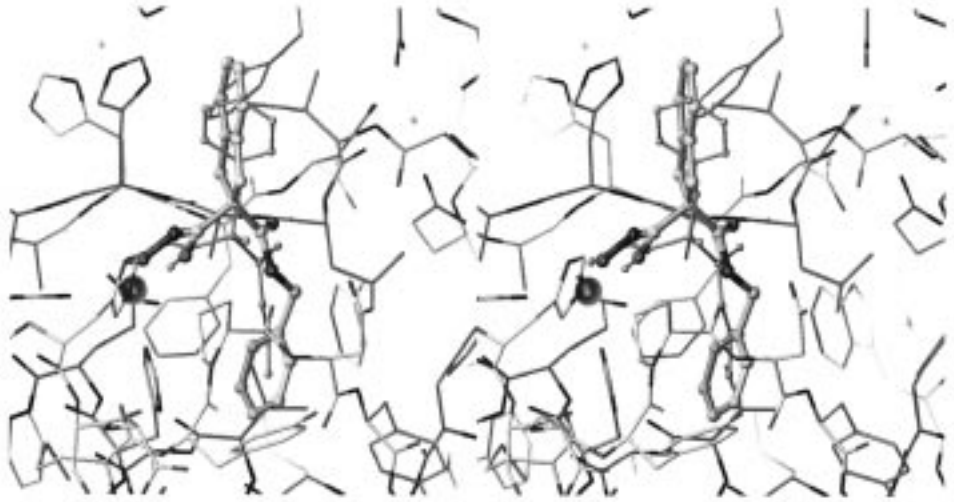


Figure 3. Comparison of the binding mode of the inhibitors **5** and **21** to the active site of MMP8.

previously reported.[15,20] Drops consisting of 2 μL of inhibitor solution (50 mM in aqueous MeOH), 2 μL of enzyme solution (4.5 mg/mL in MES/NaOH, pH 6), and 3 mL of precipitant (0.1 M MES/NaOH, pH 6.0, 10% PEG 6000, 3% MPD) were equilibrated at room temperature against a reservoir solution of 0.8 M phosphate buffer at pH 6. Crystals of *P*212121 symmetry and cell dimensions $a, b, c = 33.0, 69.5, 72.7$ Å grew out of precipitated protein after several weeks. A total of 8075 nonredundant reflections were processed for overall completeness of 71% in the resolution range 6−2 Å after application of a 2σ threshold; the *R*-merge was 6% overall and 27% in the highest resolution shell.

Two crystals of the MMP8/**5** complex were obtained by soaking MMP8 crystals in inhibitor solution for several hours. Data were measured to a theoretical completeness of at least 90%, but poorer crystal quality compared to the MMP/**21** crystals resulted in an overall completeness of 50% to 2.3 Å after 2σ cutoff for crystal no. 1 (cell dimensions 33.1, 69.0, 72.7) and 74% to 2.3 Å for crystal no 2 (cell dimensions 33.0, 69.1, 72.7). All data were collected using a Siemens ×1000 multiwire area detector using Siemens data collection and processing software (FRAMBO/SADIE/ASTRO/SAINT).

The refinement was performed with XPLOR, and the structures have R-factors of 23.5% for MMP/**21** to 2.0 Å (without solvent) and 21.2% for crystal no. 1 and 23.5% for crystal no. 2 of MMP/**5** to 2.3 Å (with 42 solvent molecules).

Computational Methods. Modeling experiments were performed on the crystal structure of MMP8[15] using the program package INSIGHT/DISCOVER (version 2.96, MSI Technologies, San Diego, CA) with the implemented force field CVFF and a dielectric constant of 1. By keeping the protein structure fixed and the hydroxamate function as a ligand of the active-site Zn^{2+} as determined in the MMP8/**5** or MMP8/**21** complexes as a constraint, alternative orientations and conformations of potential new inhibitors were generated and energy-minimized to convergence. For inspection of the possible binding mode of newly designed ligands and to gain information about hydrogen-bonding and hydrophobic interactions, interaction sites have been generated with the program LUDI.[28]

Synthesis. (2*R*,*S*)-BnONH-Mal(Me/*i*-Bu)-OEt (1). Diethyl 2-isobutyl-2-methylmalonate[21] (8.98 g, 39.0 mmol) was saponified in 100 mL of EtOH/water (4:1) with KOH (2.18 g, 39.0 mmol) to the monoethyl ester by refluxing the mixture for 2 days. The solvent was evaporated, and (2*R*,*S*)-KO-Mal-(Me/*i*-Bu)-OEt was precipitated from EtOH with ether: yield 5.41 g (58%). To (2*R*,*S*)-KO-Mal(Me/*i*-Bu)-OEt (3.60 g, 15.0 mmol) and (benzyloxy)amine hydrochloride (2.40 g, 15 mmol) in 15 mL of THF were added HOBT (1.5 g, 10.0 mmol) and EDCI (3.30 g, 17 mmol). The reaction mixture was stirred overnight at room temperature, diluted with AcOEt, and washed with 5% $KHSO_4$, 5% $NaHCO_3$, and water. The organic layer was dried over $MgSO_4$ and evaporated to dryness. The crude product was purified by flash chromatography on silica gel (100 g, AcOEt/*n*-hexane, 1:4): yield 2.20 g (48%) as a colorless oil; TLC (AcOEt/*n*-hexane, 1:2) R_f 0.5; FAB-MS *m*/*z*

308.2 [M + H$^+$]; M_r = 307.17 calcd for $C_{17}H_{25}NO_4$; ^{1}H NMR (DMSO-d_6) δ 11.05 (s, 1H, N*H*OBn), 7.30−7.45 (m, 5H, C_6H_5), 4.86 (s, 2H, C*H*$_2$−C_6H_5), 4.05 (q, 2H, C*H*$_2$CH$_3$), 1.73 (dd, 2H, CH$_2$ (*i*-Bu)), 1.58 (m, 1H, CH (*i*-Bu)), 1.30 (s, 3H, CH$_3$ (Mal)), 1.18 (m, 3H, CH$_2$C*H*$_3$), 0.8 (2d, 6H, 2 CH$_3$ (*i*-Bu)).

(2*R*,*S*)-HONH-Mal(Me/*i*-Bu)-OEt (2). Compound **1** (0.15 g, 0.50 mmol) was hydrogenated in MeOH over Pd/C catalyst under a low stream of H_2 for 1 h. The catalyst was filtered off, and the filtrate was evaporated to dryness: yield 0.105 g (93%); TLC (AcOEt/*n*-hexane, 1:1) R_f 0.5; FAB-MS *m*/*z* 218.1 [M + H$^+$]; M_r = 217.1 calcd for $C_{10}H_{19}NO_4$; ^{1}H NMR (DMSO-d_6) δ 10.58 (s, 1H, N*H*OH), 8.69 (s, 1H, NHO*H*), 4.15 (q, 2H, C*H*$_2$CH$_3$), 1.73 (dd, 2H, CH$_2$ (*i*-Bu)), 1.58 (m, 1H, CH (*i*-Bu)), 1.30 (s, 3H, CH$_3$ (Mal)), 1.18 (m, 3H, CH$_2$C*H*$_3$), 0.90 (d, 6H, 2 CH$_3$ (*i*-Bu)).

(2*R*,*S*)-HO-Mal(Me/Bn)-OEt (3). Diethyl 2-benzyl-2-methylmalonate[22] (4.0 g, 15.0 mmol) was saponified in EtOH/water (4:1) with KOH (0.84 g, 15.0 mmol) at room temperature for 2 days to the monoethyl ester. The bulk of the solvent was evaporated, and the solution was diluted with water and extracted with ether. The aqueous layer was acidified with 2 N HCl to pH 1.5 and extracted with ether; the combined extracts were washed with water, dried over $MgSO_4$, and evaporated to dryness: yield 2.3 g (64%) of colorless crystals; FAB-MS *m*/*z* 237.1 [M + H$^+$]; M_r = 236.1 calcd for $C_{13}H_{16}O_4$; ^{1}H NMR (DMSO-d_6) δ 12.95 (s br, 1H, COOH), 7.1−7.3 (m, 5H, C_6H_5), 4.12 (m, 2H, C*H*$_2$CH$_3$), 3.1 (2d, 2H, C*H*$_2$C$_6$H$_5$), 1.18 (m, 6H, CH$_3$ (Mal), CH$_3$ (Et)).

(2*R*,*S*)-BnONH-Mal(Me/Bn)-OEt (4). To compound **3** (1.20 g, 5.0 mmol) and (benzyloxy)amine hydrochloride (0.96 g, 6.0 mmol) in THF was added NMM (1.01 g, 10 mmol) followed by HOBT (0.65 g, 5.0 mmol) and EDCI (0.97 g, 5.1 mmol). The reaction mixture was stirred overnight at room temperature, diluted with AcOEt, and washed with 5% $KHSO_4$, 5% $NaHCO_3$, and water. The organic layer was dried over $MgSO_4$ and evaporated to dryness: yield 1.05 g (62%) as a colorless oil; TLC (AcOEt/*n*-hexane, 1:2) R_f 0.3; FAB-MS *m*/*z* 342.2 [M + H$^+$]; M_r = 341.2 calcd for $C_{20}H_{23}NO_4$; ^{1}H NMR (DMSO-d_6) δ 11.30 (s, 1H, NH), 7.10−7.30 (m, 10H, 2 C_6H_5), 4.11 (m, 2H, C*H*$_2$CH$_3$), 3.1 (2d, 2H, C*H*$_2$C$_6$H$_5$), 1.15−1.20 (m, 6H, CH$_3$ (Mal), CH$_3$ (Et)).

(2*R*,*S*)-HONH-Mal(Me/Bn)-OEt (5). Compound **4** (0.16 g, 0.47 mmol) was hydrogenated as reported for compound **2**: yield 0.11 g (93%); TLC (AcOEt/*n*-hexane, 1:1) R_f 0.3; mp 89−90 °C; FAB-MS *m*/*z* 252.2 [M + H$^+$]; M_r = 251.1 calcd for $C_{13}H_{17}NO_4$; ^{1}H NMR (DMSO-d_6) δ 10.52 (s, 1H, N*H*OH), 8.84 (s, 1H, NHO*H*), 7.10−7.30 (m, 5H, C_6H_5), 4.11 (m, 2H, C*H*$_2$-CH$_3$), 3.1 (2d, 2H, C_6H_5), 1.18 (m, 6H, CH$_3$ (Mal), CH$_3$ (Et)).

(2*R*,*S*)-BnONH-Mal(Me/Bn)-OH (6). The monoethyl ester **4** (1.02 g, 3.0 mmol) was saponified in THF/water (3:1) with KOH (0.68 g, 12.0 mmol) for 2 h at 80 °C and worked up as described for **3**. The product was isolated by precipitation from ether with *n*-pentane: yield 0.78 g (84.5%); FAB-MS *m*/*z* 314.2 [M + H$^+$]; M_r = 313.1 calcd for $C_{18}H_{19}NO_4$; ^{1}H NMR (DMSO-d_6) δ 13.1 (br, 1H, COOH), 11.30 (s, 1H, NH), 7.10−7.30 (m, 10H, 2 C_6H_5), 4.82 (s, 2H, CH$_2$ (OBn)), 3.1 (2d, 2H, CH$_2$ (Bn)), 1.23 (s, 3H, CH$_3$ (Mal)).

(2*R*,*S*)-HONH-Mal(Me/Bn)-NHPh (7). To **6** (155 mg, 0.5 mmol) and aniline (95 mg, 1.0 mmol) in 2 mL of THF were added HOBT (75 mg, 0.5 mmol) and EDCI (100 mg, 0.6 mmol). After 12 h the reaction mixture was diluted with AcOEt and washed with 5% $KHSO_4$, 5% $NaHCO_3$, and water. The organic layer was dried over $MgSO_4$ and evaporated to dryness. The residue was hydrogenated in MeOH as described for **2**: yield 80 mg (54%) over the two steps; TLC (AcOEt/*n*-hexane, 1:1) R_f 0.2; FAB-MS *m*/*z* 299.1 [M + H$^+$]; M_r = 298.1 calcd for $C_{17}H_{18}N_2O_3$;^{1}H NMR (DMSO-d_6) δ 10.63 (s, 1H, N*H*OH), 9.61 (s, 1H, NHPh), 8.90 (s, 1H, NHO*H*), 7.6 + 7.05−7.35 (m, 10H, 2 C_6H_5), 3.23 (s, 2H, C*H*$_2$C$_6$H$_5$), 1.26 (s, 3H, CH$_3$).

(2*R*,*S*)-HONH-Mal(Me/Bn)-NHBn (8). Coupling of **6** (155 mg, 0.5 mmol) with benzylamine (107 mg, 1.0 mmol) followed by hydrogenation of the *O*-benzylhydroxamate was performed as described for **7**: yield 71 mg (40%) over the two steps; TLC (CHCl$_3$/MeOH, 9:1) R_f 0.5; mp 112−114 °C; FAB-MS *m*/*z* 313.1 [M + H$^+$]; M_r = 312.1 calcd for $C_{18}H_{20}N_2O_3$; ^{1}H NMR (DMSO-d_6) δ 10.50 (s, 1H, N*H*OH), 8.85 (s, 1H, NHO*H*), 8.22 (dd, 1H, NHBn), 7.05−7.35 (m, 10H, 2 C_6H_5), 4.30 (m, 2H, CH$_2$ (NHBn)), 3.09 + 3.17 (2d, 2H, CH$_2$ (Mal(Bn)), 1.17 (s, 3H, CH$_3$ (Mal)).

(2*R*,*S*)-HONH-Mal(Me/Bn)-NH-CH$_2$CH$_2$-Ph (9). The compound **6** (155 mg, 0.5 mmol) was coupled with 2-phenylethylamine (121 mg, 1.0 mmol), and the resulting *O*-benzylhydroxamate was hydrogenated as reported for **7**: yield 120 mg (75%) over the two steps; TLC (CHCl$_3$/MeOH, 9:1) R_f 0.55; FAB-MS *m*/*z* 327.2 [M + H$^+$]; M_r = 326.2 calcd for $C_{19}H_{22}N_2O_3$; ^{1}H NMR (DMSO-d_6) δ 10.42 (s, 1H, N*H*OH), 8.78 (s, 1H, NHO*H*), 7.75 (dd, 1H, N*H*−(CH$_2$)$_2$−Ph), 7.05−7.35 (m, 10H, 2 C_6H_5), 3.28 + 2.70 (m, 4H, (CH$_2$)$_2$−Ph), 3.05 + 3.17 (2d, 2H, CH$_2$ (Mal-(Bn)), 1.13 (s, 3H, CH$_3$ (Mal)).

(2*R*,*S*)-HONH-Mal(Me/Bn)-NH-CH$_2$CH$_2$CH$_2$-Ph (10). The title compound was obtained by amidation of **6** (155 mg, 0.5 mmol) with 3-phenylpropylamine (135 mg, 1.0 mmol) followed by hydrogenation as described for **7**: yield 132 mg (75%) over the two steps; TLC (CHCl$_3$/MeOH, 9:1) R_f 0.6; FAB-MS *m*/*z* 341.2 [M + H$^+$]; M_r = 340.1 calcd for $C_{20}H_{24}N_2O_3$; ^{1}H NMR (DMSO-d_6) δ 10.41 (s, 1H, N*H*OH), 8.87 (s, 1H, NHO*H*), 7.73 (dd, 1H, N*H*−CH$_2$CH$_2$CH$_2$−Ph), 7.10−7.30 (m, 10H, 2 C_6H_5), 3.04 (m, 4H, NH−C*H*$_2$CH$_2$CH$_2$−Ph, CH$_2$ (Bn)), 2.50 (dd, 2H, NH−CH$_2$CH$_2$C*H*$_2$−Ph), 1.71 (q, 2H, NH−CH$_2$C*H*$_2$CH$_2$−Ph), 1.17 (s, 3H, CH$_3$).

(2*R*,*S*)-HONH-Mal(Me/Bn)-NH-CH$_2$CH$_2$CH$_2$CH$_2$-Ph (11). Compound **6** (155 mg, 0.5 mmol) was coupled with 4-phenylbutylamine (147 mg, 1.0 mmol), and the resulting *O*-benzylhydroxamate was hydrogenated as described for **7**: yield 123 mg (66%) over the two steps; TLC (AcOEt/*n*-hexane, 1:1) R_f 0.45; FAB-MS *m*/*z* 355.1 [M + H$^+$]; M_r = 354.2 calcd for $C_{21}H_{26}N_2O_3$; ^{1}H NMR (DMSO-d_6) δ 10.38 (s, 1H, N*H*OH), 8.85 (s, 1H, NHO*H*), 7.73 (dd, 1H, N*H*−CH$_2$CH$_2$CH$_2$CH$_2$−Ph), 7.10−7.35 (m, 10H, 2 C_6H_5), 3.04 (m, 4H, NH−C*H*$_2$CH$_2$CH$_2$-CH$_2$−Ph, CH$_2$ (Bn)), 2.50 (dd, 2H, NH−CH$_2$CH$_2$CH$_2$C*H*$_2$−Ph), 1.58−1.76 (m, 4H, NH−CH$_2$C*H*$_2$C*H*$_2$CH$_2$−Ph), 1.17 (s, 3H, CH$_3$).

(2*R*,*S*)-HONH-Mal(Me/Bn)-NHBn-(*p*-COOH) (12). To **6** (155 mg, 0.5 mmol) in 2 mL of THF was added 4-aminomethylbenzoic acid methyl ester hydrochloride (202 mg, 1.0 mmol) followed by NMM (101 mg, 1.0 mmol), HOBT (75 mg, 0.5 mmol), and EDCI (100 mg, 0.6 mmol). The reaction mixture was stirred overnight, diluted with AcOEt, and washed with 5% $KHSO_4$, 5% $NaHCO_3$, and water. The solution was dried over $MgSO_4$ and evaporated to dryness. The methyl benzoate moiety was saponified in EtOH/water (4:1) with KOH (160 mg, 3.0 mmol) at room temperature for 4 h. The EtOH was evaporated and the solution diluted with water and extracted with ether. The aqueous layer was acidified with 2 N HCl to pH 1.5 and extracted with ether; the combined extracts were washed with water, dried over $MgSO_4$, and evaporated to dryness. The residue was hydrogenated in MeOH as described for **2**: yield 73 mg (41%) over the three steps; TLC (CHCl$_3$/MeOH, 4:1) R_f 0.3; FAB-MS *m*/*z* 357.2 [M + H$^+$]; M_r = 356.1 calcd for $C_{19}H_{20}N_2O_5$; ^{1}H NMR (DMSO-d_6) δ 13.3 (br, 1H, COOH), 10.45 (s, 1H, N*H*OH), 8.73 (br, 1H, NHO*H*), 8.17 (dd, 1H, NHBn), 7.91 + 7.65 (2d, 4H, C_6H_4), 7.05−7.35 (m, 5H, C_6H_5), 4.28 (m, 2H, CH$_2$ (NHBn)), 3.09 + 3.17 (2d, 2H, CH$_2$ (Mal(Bn)), 1.13 (s, 3H, CH$_3$).

(2*R*,*S*)-HONH-Mal(Me/Bn)-α-naphthylamide (13). Coupling of **6** (155 mg, 0.5 mmol) with α-naphthylamine (143 mg, 2.0 mmol) followed by hydrogenation of the *O*-benzylhydroxamate was performed as described for **7**: yield 95 mg (55%) over the two steps; TLC (CHCl$_3$/MeOH, 9:1) R_f 0.5; mp 112−114 °C; FAB-MS *m*/*z* 349.2 [M + H$^+$]; M_r = 348.1 calcd for $C_{21}H_{20}N_2O_3$; ^{1}H NMR (DMSO-d_6) δ 10.85 (s, 1H, N*H*OH), 10.38 (s, 1H, NH (naphthyl)), 9.10 (s, 1H, NHO*H*), 7.17−7.95 (m, 12H, α-naphthyl + C_6H_5), 3.20 + 3.30 (2d, 2H, C*H*$_2$C$_6$H$_5$), 1.17 (s, 3H, CH$_3$).

(2*R*,*S*)-HONH-Mal(Me/Bn)-1,2,9,10-tetrahydroisochinolide (14). The title compound was prepared by coupling **6** (155 mg, 0.5 mmol) with 1,2,9,10-tetrahydroisochinoline (143 mg, 2.0 mmol) and subsequent hydrogenation of the *O*-benzylhy-

droxamate as reported for **7**: yield 105 mg (62%) over the two steps; TLC ($CHCl_3$/MeOH, 9:1) R_f 0.5; mp 112−114 °C; FAB-MS *m/z* 339.2 [M + H^+]; M_r = 338.1 calcd for $C_{20}H_{22}N_2O_3$; ^{1}H NMR (DMSO-d_6) δ 10.65 (s, 1H, N*H*OH), 8.83 (s, 1H, NHO*H*), 7.05−7.25 (m, 9H, arom (tetrahydroisochinolide) + C_6H_5), 4.60 + 3.60 + 2.80 (3m, 6H, 3 CH_2 (tetrahydroisochinolide)), 3.15 (s, 2H, C*H*$_2$C$_6$H$_5$Mal(Bn)), 1.17 (s, 3H, CH_3).

(2*R,S*)-BnONH-Mal(NHAc/Bn)-OEt (15). (2*R,S*)-EtO-Mal(NHAc/Bn)-OH (mp 127−129 °C)[28] (1.40 g, 5.0 mmol) was condensed in THF/DMF (2:1) with (benzyloxy)amine hydrochloride (0.96 g, 6 mmol) by the HOBT/EDCI method in the presence of NMM as auxiliary base and worked up as described for **4**. The product was precipitated from AcOEt with ether/*n*-pentane: yield 0.57 g (30%); TLC (AcOEt/*n*-hexane, 1:1) R_f 0.2; FAB-MS *m/z* 385.2 [M + H^+]; M_r = 384.1 calcd for $C_{21}H_{24}N_2O_5$; ^{1}H NMR (DMSO-d_6) δ 11.44 (s, 1H, NHOBn), 7.67 (s, 1H, NHAc), 7.34−7.38 (m, 5H, C_6H_5 (OBn)), 7.21−7.28 (m, 3H, 2H-3′ + H-4′ (Bn)), 6.99 (d, 2H, 2H-2′ (Bn)), 4.70 + 4.76 (2d, 2H, CH_2 (OBn)), 4.08 (q, 2H, C*H*$_2$CH$_3$), 3.43 + 3.52 (2d, 2H, CH_2 (Bn)), 1.94 (s, 3H, CH_3 (Ac)), 1.16 (t, 3H, CH$_2$C*H*$_3$).

(2*R,S*)-HONH-Mal(NHAc/Bn)-NHBn (16). Compound **15** (1.92 g, 5 mmol) was saponified with KOH (0.80 g, 15.0 mmol) in THF/water (4:1) by refluxing for 10 h. The reaction mixture was diluted with water, extracted with ether, and then acidified to pH 1.5 with 2 N HCl. The product was extracted with AcOEt; the extracts were washed with water, dried with $MgSO_4$, and evaporated to small volume. Upon precipitation with *n*-pentane (2*R,S*)-BnONH-Mal(NHAc/Bn)-OH was obtained as a homogeneous compound (TLC, CH_3CN/H_2O, 4:1; R_f 0.65) in almost quantitative yield.

BnONH-Mal(NHAc/Bn)-OH (178 mg, 0.5 mmol) was condensed with benzylamine (112 mg, 1.0 mmol) by HOBT/EDCI in THF, and the reaction mixture was worked up as described for **7** with additional purification by silica gel chromatography using AcOEt/*n*-hexane (1:2) as eluent. The resulting BnONH-Mal(NHAc/Bn)-NHBn was hydrogenated in MeOH over Pd/C catalyst as described for **2**: yield 70 mg (42%) over the three steps; TLC ($CHCl_3$/MeOH, 9:1) R_f 0.2; dec > 100 °C; FAB-MS *m/z* 356.2 [M + H^+]; M_r = 355.1 calcd for $C_{19}H_{21}N_3O_4$; ^{1}H NMR (DMSO-d_6) δ 10.78 (s, 1H, N*H*OH), 8.92 (s, 1H, NHO*H*), 8.57 (dd, 1H, NHBn), 7.40 (s, 1H, NHAc), 6.90−7.30 (m, 10H, 2 C_6H_5), 4.26 (m, 2H, CH_2 (NHBn)), 3.59 + 3.52 (2d, 2H, CH_2 (Bn)), 1.90 (s, 3H, CH_3).

EtO-Mal(OBn/Bn)-OEt (17). Diethyl benzyloxymalonate[30] (5.33 g, 20 mmol) was added to a solution of sodium (0.51 g, 22 mmol) in EtOH and reacted with benzyl bromide (4.3 g, 25 mmol) under refluxing for 4 h. The solvent was evaporated and the residue dissolved in ether. NaBr was filtered off, the filtrate evaporated, and the product isolated by distillation (180 °C, 0.01 mmHg): yield 4.2 g (59%) of a yellow oil; FAB-MS *m/z* 357.2 [M + H^+]; M_r = 356.2 calcd for $C_{21}H_{24}O_5$; ^{1}H NMR (DMSO-d_6) δ 7.18−7.40 (m, 10H, 2 C_6H_5), 6.31 (s, 2H, CH_2 (OBn)), 4.17 (m, 4H, 2 C*H*$_2$CH$_3$), 3.46 (s, 2H, CH_2 (Bn)), 1.16 (t, 6H, 2 CH$_2$C*H*$_3$).

(2*R,S*)-BnONH-Mal(OBn/Bn)-OEt (18). Compound **17** (3.56 g, 10 mmol) was saponified to the monoethyl ester as described for **3** and then reacted with (benzyloxy)amine hydrochloride following the procedure for **4**: yield 1.17 g of a slightly yellow oil (27% over the two steps); TLC (AcOEt/*n*-hexane, 1:1) R_f 0.7; FAB-MS *m/z* 434.2 [M + H^+]; M_r = 433.2 calcd for $C_{26}H_{27}NO_5$; ^{1}H NMR (DMSO-d_6) δ 11.25 (s, 1H, N*H*OBn), 7.18−7.40 (m, 15H, 3 C_6H_5), 6.31 (s, 2H, CH_2 (NHOBn)), 4.65 (s, 2H, CH_2 (OBn)), 4.23 (m, 2H, C*H*$_2$CH$_3$), 3.42 (s, 2H, CH_2 (Bn)), 1.28 (t, 3H, CH$_2$C*H*$_3$).

(2*R,S*)-HONH-Mal(OH/Bn)-NHBn (19). The ethyl ester **18** (1.17 g, 2.7 mmol) was saponified as described for **6** and then condensed with benzylamine following the procedure reported for **7**. Deprotection was performed as described for **2**: yield 280 mg (33%) over the three steps; TLC ($CHCl_3$/MeOH, 9:1) R_f 0.45; mp 138 °C dec; FAB-MS *m/z* 315.1 [M + H^+]; M_r = 314.1 calcd for $C_{17}H_{18}N_2O_4$; ^{1}H NMR (DMSO-d_6) δ 10.55 (s, 1H, N*H*OH), 8.93 (s, 1H, NHO*H*), 8.30 (dd, 1H, NH−Bn), 7.07−7.30 (m, 10H, 2 C_6H_5), 5.61 (s, 1H, OH), 4.32 + 4.17 (2dd, 2H, CH_2 (NHBn)), 3.13 + 3.25 (2d, 2H, CH_2 (Bn)).

BnONH-Ind-OH (20). Indan-2,2-dicarboxylic acid diethyl ester[24] (4.05 g, 15 mmol) was saponified to the monoethyl ester as described for **3** and then reacted with (benzyloxy)amine hydrochloride following the procedure for **4**. The resulting BnONH-Ind-OEt was again saponified with KOH as described for **6**: yield 1.98 g of soft crystals (43% over the three steps); TLC ($CHCl_3$/MeOH, 4:1) R_f 0.7; FAB-MS *m/z* 312.3 [M + H^+]; M_r = 311.1 calcd for $C_{18}H_{17}NO_4$; ^{1}H NMR (DMSO-d_6) δ 13.0 (br, 1H, COOH), 11.26 (s, 1H, NH), 7.15−7.35 (m, 9H, C_6H_4 + C_6H_5), 4.84 (s, 2H, C*H*$_2$C$_6$H$_5$), 3.56 (m, 4H, CH_2).

HONH-Ind-NHBn (21). Compound **20** (155 mg, 0.5 mmol) was coupled with benzylamine (107 mg, 1.0 mmol) and deprotected by hydrogenation as described for **7**: yield 66 mg (43%) over the two steps; TLC ($CHCl_3$/MeOH, 9:1) R_f 0.4; mp 158 °C dec; FAB-MS *m/z* 311.1 [M + H^+]; M_r = 310.1 calcd for $C_{18}H_{18}N_2O_3$; ^{1}H NMR (DMSO-d_6) δ 10.66 (s, 1H, N*H*OH), 8.83 (s, 1H, NHO*H*), 8.18 (dd, 1H, NHBn), 7.05−7.30 (m, 9H, C_6H_4 + C_6H_5), 4.26 (d, 2H, C*H*$_2$C$_6$H$_5$), 3.45 (s, 4H, 2 CH_2).

HONH-Ind-NH-CH$_2$CH$_2$CH$_2$-Ph (22). BnONH-Ind-OH (**20**) (155 mg, 0.5 mmol) was coupled with 3-phenylpropylamine (135 mg, 1.0 mmol) and then hydrogenated as reported for **7**: yield 134 mg (80%) over the two steps; TLC ($CHCl_3$/MeOH, 9:1) R_f 0.5; mp 148 °C; FAB-MS *m/z* 339.2 [M + H^+]; M_r = 339.1 calcd for $C_{20}H_{22}N_2O_3$; ^{1}H NMR (DMSO-d_6) δ 10.53 (s, 1H, N*H*OH), 8.79 (s, 1H, NHO*H*), 7.62 (dd, 1H, N*H*−CH$_2$CH$_2$-CH$_2$−Ph), 7.07−7.30 (m, 9H, C_6H_4 + C_6H_5), 3.42 (s, 4H, 2 CH_2 (Ind)), 3.06 (quart, 2H, NH−C*H*$_2$CH$_2$CH$_2$−Ph), 2.49 (dd, 2H, NH−CH$_2$CH$_2$C*H*$_2$−Ph), 1.67 (q, 2H, NH−CH$_2$C*H*$_2$CH$_2$−Ph).

BnONH-Mal(Dbch)-OH (23). 6,7-Dihydro-5*H*-dibenzo[*a,f*]-cycloheptene-6,6-dicarboxylic acid diethyl ester[25] (3.36 g, 10 mmol) was saponified to the monoethyl ester as described for **3** and then reacted with (benzyloxy)amine hydrochloride following the procedure for **4**. Finally, BnONH-Mal(Dbch)-OEt was saponified as described for **6**: yield 1.73 g (45%) of a colorless powder over the three steps; TLC ($CHCl_3$/MeOH, 4:1) R_f 0.7; FAB-MS *m/z* 388.0 [M + H^+]; M_r = 387.1 calcd for $C_{24}H_{21}NO_4$; ^{1}H NMR (DMSO-d_6) δ 13.0 (br, 1H, COOH), 11.43 (s, 1H, NH), 7.15−7.45 (m, 13H, arom), 4.66 (s, 2H, C*H*$_2$C$_6$H$_5$), 3.35 (m, 4H, 2 CH_2 (Dbch)).

HONH-Mal(Dbch)-NH-CH$_2$CH$_2$CH$_2$-Ph (24). Compound **23** (193 mg, 0.5 mmol) was coupled with 3-phenylpropylamine (135 mg, 1.0 mmol) and then hydrogenated as described for **7**: yield 136 mg (66%) over the two steps; TLC ($CHCl_3$/MeOH, 9:1) R_f 0.6; mp 199 °C; FAB-MS *m/z* 415.3 [M + H^+]; M_r = 414.2 calcd for $C_{26}H_{26}N_2O_3$; ^{1}H NMR (DMSO-d_6) δ 10.52 (s, 1H, N*H*OH), 8.80 (s, 1H, NHO*H*), 7.62 (dd, 1H, N*H*−CH$_2$CH$_2$-CH$_2$−Ph), 7.10−7.40 (m, 13H, arom), 3.29 (s, 4H, 2 CH_2 (Dbch)), 3.10 (ddd, 2H, NH−C*H*$_2$CH$_2$CH$_2$−Ph), 2.50 (t, 2H, NH−CH$_2$CH$_2$C*H*$_2$−Ph), 1.70 (ddd, 2H, NH−CH$_2$C*H*$_2$CH$_2$−Ph).

Abbreviations: MMP, matrix metalloproteinase; MMP8, human neutrophil collagenase; EDCI, 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide hydrochloride; HOBT, 1-hydroxybenzotriazole; Mal(R,R′), 2,2-bis-substituted malonyl; *i*-Bu, isobutyl; Me, methyl; Bn, benzyl, Ph, phenyl; Ind, indan-2,2-dicarboxy; Dbch, 6,7-dihydro-5*H*-dibenzo[*a,f*]cycloheptene-6,6-dicarboxy; NMM, *N*-methylmorpholine.

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