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ScienceDirect
Bioorganic & Medicinal Chemistry 16 (2008) 2385–2390
Bioorganic & Medicinal Chemistry

Bornyl (3,4,5-trihydroxy)-cinnamate - An optimized human neutrophil elastase inhibitor designed by free energy calculations

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Received 7 June 2007; revised 20 November 2007; accepted 23 November 2007
Available online 19 December 2007

Abstract—Human neutrophil elastase (HNE), a serine protease, is involved in the regulation of inflammatory processes and controlled by endogenous proteinase inhibitors. Abnormally high levels of HNE can cause degradation of healthy tissues contributing to inflammatory diseases such as rheumatoid arthritis, and also psoriasis and delayed wound healing. In continuation of our research on HNE inhibitors we have used the recently developed binding mode model for a group of cinnamic acid derivative elastase inhibitors and created bornyl (3,4,5-trihydroxy)-cinnamate. This ligand exhibited improved binding affinity predicted by means of free energy calculations. An organic synthesis scheme for the ligand was developed and its inhibitory activity was tested toward the isolated enzyme. Its IC_{50} value was found to be three times lower than that of similar compounds, which is in line with the computational result showing the high potential of free energy calculations as a tool in drug development.

1. Introduction

Human neutrophil elastase is a serine protease playing an important role in physiological functions of the immune system. It is produced and stored in the neutrophil granulocytes and has the ability to hydrolyze a wide variety of protein substrates, notably elastin, a protein that gives skin its elastic properties. HNE's specificity lies in the cleavage after small aliphatic amino acid residues, especially valine. The functional enzyme contains 218 amino acids, organized into two domains of β-barrels, and is stabilized by four disulfide bridges. Nineteen arginine residues on the protein surface and only nine acidic residues make it a highly basic protein. The activity of HNE is tightly regulated in vivo by several physiological inhibitors. However, in inflamed tissue an impaired balance between the enzyme and its natural inhibitors exists, leading to the degradation of healthy tissue.[1–3] Interestingly, HNE also has an important regulatory role in the local inflammatory response by proteolytic modification of chemokines and cytokines and has been implicated in wound healing.[4] Active HNE is detected in psoriatic lesions and induces keratinocyte hyperproliferation via the EGFR signaling pathway.[5,6] Thus, the involvement of HNE in such pathological processes makes it an interesting target for the development of anti-inflammatory drugs. Considering local inflammations, small molecules might be more advantageous compared to peptidic inhibitors because of their better penetration rate into the skin.

Recently, various natural compounds have been studied as inhibitors of HNE[7–9]; among them cinnamic acid ester derivatives were found to have IC_{50} values in the μM

Abbreviations: MD, molecular dynamics; HNE, human neutrophil elastase; TI, thermodynamic integration; vdW, van-der-Waals.
Keywords: Inflammation; Human neutrophil elastase; Free energy calculation; Drug design.
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doi:10.1016/j.bmc.2007.11.070

range. No X-ray crystal structure of HNE cocrystallized with an esterified cinnamic acid derivative has been reported up to now, but a putative binding mode for bornyl caffeate and similar compounds has been proposed and evaluated in our recent work.[10] Based on this complex structure, the binding modes of modified compounds can be simulated and their affinities for HNE can be predicted.

In this work, we report a binding affinity prediction, organic synthesis, and activity measurement for bornyl (3,4,5-trihydroxy)-cinnamate. Our combination of computer simulations with organic synthesis and pharmacological activity measurements shows that the advances in computer hardware and molecular simulation software in the last years have made free energy calculations a robust and efficient tool in biochemical and pharmaceutical studies. While still too time-consuming for routine employment as screening tools, they are very well suited for structural improvements of known ligands and accurate binding free energy predictions.

2. Results

2.1. Binding affinity prediction

Predictions of binding free energies were performed on a number of various derivatives of bornyl caffeate selected after visual inspection of the proposed protein-ligand complex. Among these, only bornyl (3,4,5-trihydroxy)-cinnamate resulted in a predicted higher binding affinity compared to bornyl caffeate. Therefore, only results for this compound will be reported here. To judge the binding strength of the proposed ligand to HNE, a TI calculation was set up that compares the binding free energy of bornyl (3,4,5-trihydroxy)-cinnamate with that of bornyl caffeate.

Since the free energy difference between two thermodynamic states is only a physically meaningful quantity if both states have the same number and types of atoms, a simple thermodynamic cycle was used to predict binding free energies: in this cycle the two states are equal to the two ligands that are compared ($\lambda = 0$ was chosen to represent bornyl (3,4,5-trihydroxy)-cinnamate and $\lambda = 1$ equals bornyl caffeate) and two systems are set up representing those ligands bound to the receptor or simply solvated in water. Simulating the transition between both states, a free energy change, as calculated by Eq. 1, is determined for both the solvated ligands and the ligand-protein complexes.

The ligand transformations were simulated in two steps, first all atomic partial charges were transformed from their initial ($\lambda = 0$) to their final ($\lambda = 1$) values and in a subsequent second step the MD atom types and vdW parameters were transformed. This was done for reasons of simulation stability, because changing partial charges and vdW radii simultaneously can result in vanishing atoms with non-zero charges getting very close to non-bonded neighboring atoms, leading to very high electrostatic energies. Each of the transformations was simulated by performing MD simulations of the equilibrated systems at seven λ values chosen after a Gaussian numerical integration scheme (Eq. 2[11]). Each 'λ-window' consisted of a 50 ps equilibration phase and a 100 ps data collection phase, performed under the NPT ensemble using a 2 fs time step. The simulations yielded a $\Delta A^0_{\text{Unbound}}$ of 47.1 kcal/mol for the unbound (48.1 kcal/mol from the partial charge and −1.0 kcal/mol from the atom type and vdW transformation) and a $\Delta A^0_{\text{Bound}}$ of 50.8 kcal/mol for the bound states (51.0 and −0.2 kcal/mol from partial charge and atom type transformations, respectively). This results in a $\Delta\Delta A^0_{\text{Bind}}$ of +3.7 kcal/mol, meaning that bornyl (3,4,5-trihydroxy)-cinnamate is predicted to inhibit HNE with a binding constant about 500 times lower than that of bornyl caffeate, placing it in the low nanomolar inhibitor range.

From the two separate steps of the transformation the contributions of different interaction types to the relative binding strengths can be seen. The change of electrostatic interactions (step 1) resulted in a $\Delta\Delta A^0_{\text{Bound}}$ of 2.9 kcal/mol and the change of vdW types (step 2) resulted in a $\Delta\Delta A^0_{\text{Bound}}$ of 0.8 kcal/mol. From this it can be inferred that the new ligand's improved binding strength is mostly, but not completely, owed to stronger electrostatic interactions with the receptor. The promising prediction of bornyl (3,4,5-trihydroxy)-cinnamate's binding properties prompted the organic synthesis and pharmacological testing of the compound.

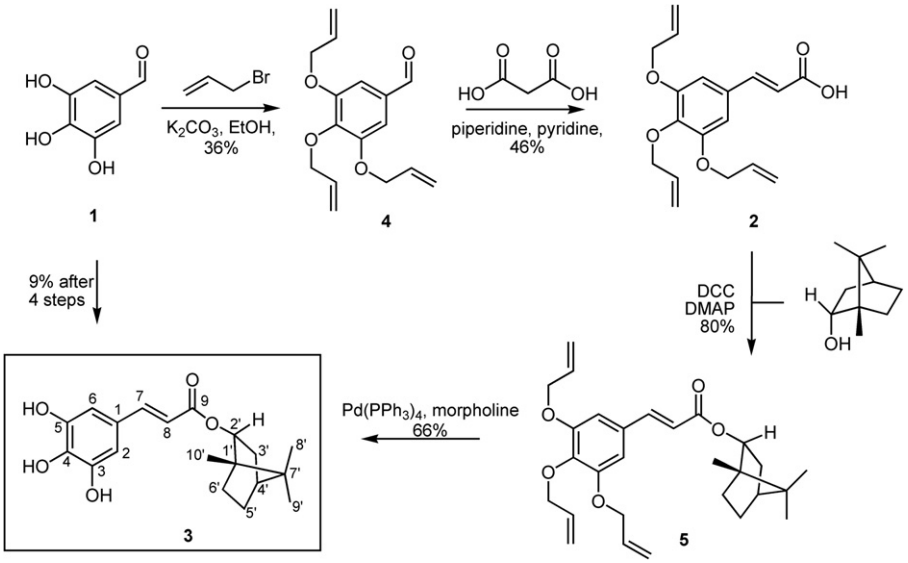
2.2. Organic synthesis

The synthesis of bornyl (3,4,5-trihydroxy)-cinnamate was performed as outlined in Scheme 1. The cinnamic acid **2** was prepared by *O*-allylation of trihydroxybenzaldehyde (**1**) and subsequent Knoevenagel condensation of the *O*-allyl-protected aldehyde with malonic acid. Dicyclohexylcarbodiimide/4-dimethylaminopyridine-induced condensation of the cinnamic acid **2** with (*R*)-(+)-borneol afforded the allyl-protected bornylic ester which was deallylated by means of morpholine/$Pd(PPh_3)_4$ to yield the trihydroxyester **3**.

A colorless crystalline solid in about 10% overall yield was obtained. The final product had a purity of 98% as proved by NMR to be directly used in the HNE activity assay. The product was found to be very susceptible to oxidation when exposed to air, so handling under inert gas conditions was necessary (Fig. 1).

2.3. Elastase inhibition assay

Bornyl (3,4,5-trihydroxy)-cinnamate and bornyl caffeate were studied for their inhibitory effect on HNE using the isolated enzyme (see Table 1).[12,13] The recently reported IC_{50} value of 1.6 μM of the latter one could be confirmed,[12] whereas an IC_{50} value of 0.54 μM was obtained for the newly synthesized compound. As α1-antitrypsin from human plasma is the physiological inhibitor of the elastase[14] and widely used[15,16] this compound should be very specific. We obtained an IC_{50} va-



Scheme 1.

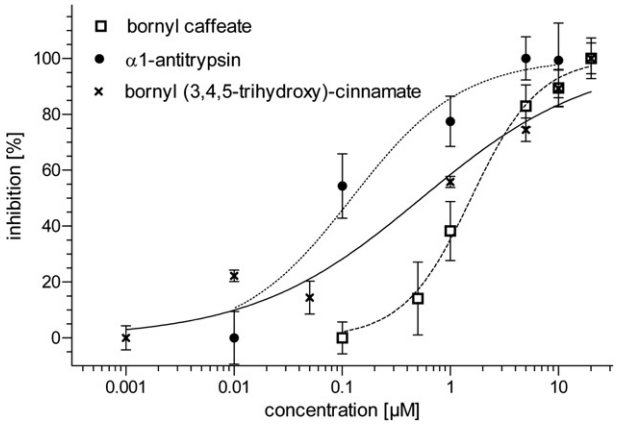


Figure 1. Comparison of the three compounds regarding their inhibitory activity against HNE.

Table 1. IC_{50} values and 95% confidence intervals of the tested compounds, calculated by GraphPad Prism 4

Compound	IC_{50} in μM	95% Confidence interval
Bornyl (3,4,5-trihydroxy)-cinnamate	0.54	0.33–0.89
Bornyl caffeate	1.56	1.05–2.30
α1-Antitrypsin	0.12	0.05–0.29

lue of 0.12 μM. Hernandez et al.[15] reported that 90 μg/mL of this physiological compound inhibited HNE to 84%.

3. Discussion

Free energy calculations predicted that the ligand would inhibit HNE with an IC_{50} value in the nanomolar range. This prediction turned out to be correct with a measured IC_{50} of 540 nM making bornyl (3,4,5-trihydroxy)-cinnamate the strongest binding cinnamic acid derivative known up to date. Considering that a binding constant was predicted about 500 times lower than that of bornyl caffeate, an IC_{50} value of about 3.1 nM should be obtained. This is not the case. The measured IC_{50} value is higher, which means that the calculated $\Delta\Delta A^0$ overestimated the binding strength by ca. 3.1 kcal/mol. A conformational bias resulting from an induced fit of the binding site to the starting ligand structure can be excluded as a cause of this error, because this could only have led to an underestimation of the new ligand's strength. Bornyl caffeate lacks the additional hydroxyl group of bornyl (3,4,5-trihydroxy)-cinnamate and could thus exhibit a more flexible bound state. It is possible that sampling of the conformational space of bound bornyl caffeate was therefore less thorough than that of bornyl (3,4,5-trihydroxy)-cinnamate, possibly leading to the observed deviation. However, while very accurate free energies have been reported in TI applications[17] in very long simulations for test cases, the authors believe an error of 1–2 kcal/mol to be typical of biochemical TI applications, caused by insufficient sampling and inaccuracies of the used non-polarizable force fields. Advances both in computer power and quality of MD models will certainly improve the precision of such calculations in the future, but applications as the one described here are already sufficiently predictive to provide at least qualitative and sometimes quantitative[10] predictions of ligand binding strengths superior to those that could be obtained by using docking scoring functions or other simple empirical models.

The necessity to divide the TI transformation into two separate steps of changing electrostatic and vdW interactions led to stable simulations and easily integrateable free energy curves. This however came with the cost of doubling the necessary simulation time. A more efficient mixing function for the two potential energies than Eq. 3 could be employed to improve sampling and simulation

convergence. In this respect, a 'softcore' potential similar to the one described in Refs. 17 and 18 seems like one promising way to improve the efficiency of free energy calculations in the future.

Notably, bornyl (3,4,5-trihydroxy)-cinnamate lacks the catechol group that was a feature of previously known good binders to HNE like bornyl caffeate or fucinolic acid. However, vicinal di-hydroxy functional groups are present. Substances rich in hydroxyl groups have been studied as promising candidates of HNE inhibition before[7] and the structure of the newly synthesized ligand gives rise to the assumption that more available hydroxyl functionalities tend to improve the affinity of a compound to HNE. Nevertheless, our results show that prediction of binding constants is a valuable tool to improve the inhibitory activity of ligands. Finally, in vivo studies have to demonstrate whether these small molecule inhibitors are suitable for the treat-ment of inflammatory diseases, especially for local inflammations.

4. Methods

4.1. Thermodynamic integration calculations

Free energies were computed by performing thermodynamic integration calculations[10,19,20] in which the free energy difference between two states is computed by coupling their potential functions V_0 and V_1 via a parameter λ and simulating a transition of λ from zero to one. Statistical mechanics allows for the calculation of the free energy difference between the states, ΔA^0:

$$\Delta A^0 = A_1^0 - A_0^0 = \int_{\lambda=0}^{1} \left\langle \frac{\partial V(\lambda)}{\partial \lambda} \right\rangle_\lambda \mathrm{d}\lambda \qquad (1)$$

where A_1^0 and A_0^0 refer to the free energies of the perturbed and unperturbed states, respectively. The angular brackets denote a statistically weighted sampling of conformational space that can (at least approximately) be accomplished by performing molecular dynamics simulations on the system at varying values of λ. Then, the integral can be solved numerically by summing up the weighted average contributions of different 'λ-windows'.[11]

$$\Delta A^0 \approx \sum_{i=1}^{n} w_i \left\langle \frac{\partial V}{\partial \lambda} \right\rangle_{\lambda_i} \qquad (2)$$

The mixed potential function $V(\lambda)$ was set to:

$$V(\lambda) = (1-\lambda)^4 V_0 + [1-(1-\lambda)^4] V_1 \qquad (3)$$

All transformations were set up so that atoms disappeared when changing λ from zero to one. In combination with the potential mixing function in Eq. 3, this prevents the 'origin singularity' effect[21] from occurring, by making the divergence of the potential energy difference at large λ values integrateable. For predictions of binding free energy changes, the transformation of a ligand into another is simulated both in the receptor bound and unbound state. The difference between these transformation free energies is equal to the difference in binding free energy of the compared ligands A and B:

$$\begin{aligned} \Delta\Delta A^0_{\mathrm{Bind}} &= \Delta A^0_{\mathrm{Bound}}(A \rightarrow B) - \Delta A^0_{\mathrm{Unbound}}(A \rightarrow B) \\ &\approx \Delta\Delta G^0_{\mathrm{Bind}} \end{aligned} \qquad (4)$$

Note that in our definition $\Delta\Delta A^0{}_{\mathrm{Bind}}$ is positive if A binds stronger to the receptor than B. Since all simulations were performed under conditions of constant pressure and temperature (the NPT ensemble), we interpret the result as approximately equal to $\Delta\Delta G^0{}_{\mathrm{Bind}}$, the binding free enthalpy difference of the ligands obtained by an experiment.

4.2. Molecular dynamics simulations

Molecular dynamics simulations were carried out using the *Amber8* molecular modeling suite.[22] Two different sets of force field parameters were used, namely *ff03*[23] for the protein parts of the system and *gaff*[24] for the ligands. The TIP3P water model[25] was used for solvation. Ligand partial charges were derived by the RESP methodology,[26] based on quantum mechanical calculations on the HF/6-31G* level using Gaussian98.[27] As the starting geometry for the protein–ligand complex the recently proposed binding model for cinnamic acid derivative bornyl esters was taken from,[10] namely from the equilibrated structure of the #2 placement group of bornyl caffeate as determined by ligand docking and MM-PBSA calculations. The bound bornyl caffeate was changed to bornyl (3,4,5-trihydroxy)-cinnamate, the complex was solvated in a 12 Å deep solvation layer and neutralized using Cl^- counterions.

The systems were equilibrated under periodic boundary conditions according to the following procedure: after 1000 steps of preliminary minimization to remove close vdW contacts, the system temperature was brought to 300 K during 50 ps of constant volume dynamics by a Berendsen-type[28] temperature coupling algorithm. In doing so, all solute atoms had 10 kcal/mol^2 harmonic positional restraints applied. Finally, a volume equilibration was performed during 150 ps of constant pressure dynamics. A time step of 2 fs in combination with the SHAKE[29] algorithm to constrain bond lengths involving hydrogen atoms was used for all simulations. All equilibration was performed using the starting state hamiltonian ($\lambda = 0$).

4.3. Materials and test compounds

Bornyl caffeate was isolated from *Verbesina turbacensis* Kunth.[12] Purity was evaluated by TLC to be 97%. α_1-Antitrypsin from human plasma was bought from Sigma. Enzyme substrate *N*-methoxysuccinyl-L-Ala-L-Ala-L-Pro-L-Val-*p*-nitroanilide (MeO-Suc-Ala-Ala-Pro-Val-*p*NA), trypsin inhibitor from soybean, and DMSO as well as elastase from human leukocytes (EC 3.4.21.37) were purchased from Sigma. TRIS (tris-(hydroxymethyl)-amino methane) and HCl 25% were from Merck.

Ten millimolar of stock solutions were prepared in DMSO and then diluted with DMSO and 50 μM Tris–HCl buffer, pH 7.5, to the final concentration. DMSO was restricted to 1% for the HNE assay.

The absorbance was measured using a Uvikon 933 UV–vis double-beam spectrometer.

4.4. Synthesis of bornyl (3,4,5-trihydroxy)-cinnamate

The synthesis of bornyl (3,4,5-trihydroxy)-cinnamate was performed by modifying the synthetic strategy for the similar natural product (+)-rosmarinic acid worked out by Eicher et al.[30] (Scheme 1). Educts were obtained in highest commercially available purity. The enantiomeric excess of **3** can be assumed to be the unchanged *ee* of the (*R*)-(+)-borneol used (*ee* > 98% after GC).

(a) Allyl bromide (3.3 equiv), K_2CO_3 (3.3 equiv), EtOH, 2 h, 90 °C, 36%; (b) malonic acid (2.0 equiv), piperidine (0.3 equiv), pyridine, 4 h, 80 °C, 46%; (c) dicyclohexylcarbodiimide (1.1 equiv), 4-(dimethylamino)-pyridine (0.2 equiv), (*R*)-(+)-borneol (2.3 equiv, *ee* > 98%), tetrahydrofurane, 16 h, room temperature, 80%; (d) $Pd(PPh_3)_4$ (5 mol-%), morpholine (2.9 equiv), tetrahydrofurane, room temperature, 6 h, 66%.

4.4.1. (3,4,5-Triallyloxy)benzylaldehyde (4). Allyl bromide (810 μL, 1.2 g, 9.6 mmol, 3.3 equiv) and K_2CO_3 (1.33 g, 9.59 mmol, 3.3 equiv) were added to a solution of trihydroxybenzaldehyde·monohydrate (**1**, 500 mg, 2.9 mmol) in EtOH (10 mL) at room temperature. The reaction mixture was stirred at 90 °C for 2 h. Buffer, pH 7 (15 mL) and *tert*-butyl-methylether (TBME) (15 mL) were added after cooling to room temperature. After extraction with TBME (2× 15 mL) the combined organics were dried ($MgSO_4$), evaporated, and chromatographed (EtOAc/cyclohexane 10:1) to give pure **4** (295 mg, 36%). ^{1}H NMR (300.1 MHz, $CDCl_3$/TMS): δ = 4.63–4.67 (m, 6H), 5.20 (d, J = 10.3, 1H), 5.30 (d, J = 9.2, 2H), 5.35 (d, J = 15.8, 1H), 5.44 (d, J = 17.0, 2H), 6.01–6.14 (m, 3H), 7.11 (s, 2H), 9.82 (s, 1H). IR (film) ν = 2980, 2935, 2870, 1695, 1585, 1440, 1385, 1325, 1110, 920, 735 cm^{-1}.

4.4.2. (3,4,5-Triallyloxy)-cinnamic acid (2). Piperidine (30 μL, 26 mg, 0.30 mmol, 0.3 equiv) and malonic acid (220 mg, 2.1 mmol, 2.0 equiv) were added to a solution of aldehyde **4** (290 mg, 1.1 mmol) in pyridine (3 mL) at room temperature. The reaction mixture was stirred at 88 °C for 4.5 h and then acidified with HCl (3 M). After extraction with EtOAc (2× 15 mL) the combined organics were dried ($MgSO_4$) and evaporated to give pure **2** (150 mg, 46%). ^{1}H NMR (300.1 MHz, $CDCl_3$): δ = 4.60–4.62 (m, 6 H), 5.19 (d, J = 10.4, 1H), 5.30 (d, J = 9.2, 2H), 5.34 (d, J = 14.4, 1H), 5.43 (d, J = 17.1, 2H), 6.02–6.11 (m, 3H), 6.30 (d, J = 16.0, 1H), 6.77 (s, 2H), 7.65 (d, J = 16.0, 1H). IR (film) ν = 2980, 2930, 2865, 1670, 1625, 1495, 1385, 1285, 1120, 930, 735 cm^{-1}.

4.4.3. Bornyl (3,4,5-triallyloxy)-cinnamate (5). To a solution of acid **2** (80 mg, 0.25 mmol) in THF (3 mL) dicyclohexylcarbodiimide (57 mg, 0.28 mmol, 1.1 equiv), dimethylamino pyridine (5 mg, 0.04 mmol, 0.2 equiv), were added, and (+)-borneol (90 mg, 0.58 mmol, 2.3 equiv), and the solution was stirred at room temperature for 16 h. The solvents were evaporated, and the crude mixture chromatographed (EtOAc/cyclohexane 5:1) to give pure **5** (92 mg, 80%). ^{1}H NMR (300.1 MHz, $CDCl_3$): δ = 0.88 (s, 3H), 0.90 (s, 3H), 0.95 (s, 3H), 1.05 (dd, J = 13.8, J = 3.7, 1H), 1.24–1.49 (m, 2H), 1.71 (m_c, 1H), 1.79 (m_c, 1H), 2.05 (m_c, 1H), 2.41 (m_c, 1H), 4.59 (m, 6 H), 5.02 (d, J = 8.6, 1H), 5.19 (dq, J = 10.4, J = 1.6, 1H), 5.29 (dq, J = 10.5, J = 1.4, 2H), 5.33 (dq, J = 15.1, J = 1.5, 1H), 5.43 (dq, J = 17.3, J = 1.6, 2H), 6.01–6.12 (m, 3H), 6.31 (d, J = 16.0, 1H), 6.76 (s, 2H), 7.53 (d, J = 16.0, 1H). IR (film) ν = 2980, 2935, 2870, 1710, 1445, 1385, 1275, 1150, 1115, 1075, 920, 735 cm^{-1}.

4.4.4. Bornyl (3,4,5-trihydroxy)-cinnamate (3). $Pd(PPh_3)_4$ (11 mg, 9.5 μmol, 5 mol-%) and morpholine (45 μL, 45 mg, 0.51 mmol, 2.9 equiv) were added to a solution of **5** (80 mg, 0.18 mmol) in THF (4 mL). The reaction mixture was stirred under Argon at room temperature for 6 h. Et_2O (10 mL) and HCl (1 M, 10 mL) were added. After phase separation the obtained phase was extracted with Et_2O (2× 10 mL), washed with HCl (1 M, 2× 5 mL), dried ($MgSO_4$), and evaporated. Flash chromatography (cyclohexane/acetone 2:1) gave pure **3** (39 mg, 66%). ^{1}H NMR (300.1 MHz, $CDCl_3$): δ = 0.88 (CH_3-10′, s), 0.90 (CH_3-9′, s), 0.95 (CH_3-8′, s), 1.06 (H-3′α, d, J = 14.9), 1.26 (H-5′α, m), 1.32 (H-6′β, m), 1.71 (H-4′, m), 1.77 (H-5′β, m), 2.02 (H-6′α, m), 2.41 (H-3′β, m), 5.00 (H-2′β, d, J = 8.1), 6.29 (H-8, d, J = 15.6), 6.74 (H-2 and 6, s), 7.50 (H-7, d, J = 15.6); ^{13}C NMR (100.6 MHz, $CDCl_3$): δ = 13.7 (C-10′), 19.0 (C-8′), 19.8 (C-9′), 27.3 (C-6′), 28.1 (C-5′), 36.9 (C-3′), 45.0 (C-4′), 48.0 (C-7′), 49.1 (C-1′), 80.8 (C-2′), 108.4 (C-2 and 6), 116.2 (C-8), 126.6 (C-1), 134.5 (C-4), 144.4 (C-7), 145.4 (C-3 and 5), 168.9 (C-9); MS (EI): *m/z* (%) = 332 (M^+), 179. HRMS (EI) Calcd for $C_{19}H_{24}O_5$ 332.1624, found: 332.1625; IR (film): υ = 2980, 2935, 2870, 1445, 1385, 1115, 920, 735 cm^{-1}; assignment of the NMR data according to Refs. 12 and 31.

4.5. Human neutrophil elastase activity assay

Determination of the HNE activity was performed according to Refs. 12 and 13 with slight modifications. Briefly, 250 μL of substrate solution (700 μM MeO-Suc-Ala-Ala-Pro-Val-*p*NA in Tris–HCl buffer, 50 μM, pH 7.5) was mixed with 100 μL of test solution (test substance solubilized in Tris–HCl buffer, pH 7.5) and vortexed. The solution was prewarmed to 37 °C. After addition of 250 μL enzyme solution (approximately 25 mU) and vortexing, the samples were incubated for 1 h at 37 °C in a shaker water bath. The positive control consisted of buffer, substrate, and enzyme. The reaction was stopped by addition of 500 μL soybean trypsin inhibitor solution (0.2 mg/mL in Tris–HCl buffer, pH 7.5). The samples were vortexed and placed in an ice bath. The absorbance of the released *p*-nitroaniline was measured at 405 nm.

4.6. Statistical analysis

All assays were performed at least in three separated experiments. Inhibition rates were calculated relative to the enzyme activity without inhibitor in percent. Statistical analysis and non-linear regression were performed using GraphPad Prism 4 software. The results

are expressed as IC_{50} values with 95% confidence interval.

Acknowledgments

The authors thank Prof. Reinhard Brückner for supporting and funding the organic synthesis part of this work. Birgit Schneider greatly assisted in synthesizing the compounds. We thank Prof. David A. Case for his help with the preparation of the manuscript and the Amber user community for countless helpful discussions and support.

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