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Article

Synthesis and Evaluation of Non-peptidic Cysteine Protease Inhibitors of *P. falciparum* Derived from Etacrynic Acid

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Abstract: A series of etacrynic acid derivatives was synthesized and screened for their *in vitro* activity against *Plasmodium falciparum*, as well as their activity against recombinantly expressed falcipain-2 and -3. The two most active compounds of the series displayed IC₅₀ values of 9.0 and 18.8 μM against *Plasmodia*.

Keywords: Malaria; Cysteine protease inhibitor; Etacrynic acid.

Introduction

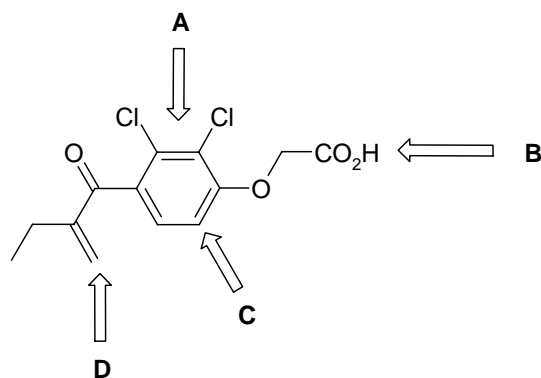
Malaria is still a major cause of global human morbidity and mortality, with *Plasmodium falciparum* being the deadliest among the four *Plasmodium* species that infect humans. Annually 350–500 million clinical malaria episodes occur, causing more than 1 million deaths each year [1]. The

increasing spread of *P. falciparum* due to inadequate vector control, increasing drug resistance, and the lack of effective vaccine strengthens the need for the development of novel drugs for the treatment of malaria [2, 3]. New therapeutic strategies are therefore urgently needed. Cysteine proteases of *P. falciparum* represent attractive antiplasmodial drug targets due to their essential functions for the parasite erythrocytic cycle [4, 5, 6]. Among them are falcipain-2 and falcipain-3, which are located in the acidic food vacuole of the parasite, play a pivotal role in hemoglobin hydrolysis, and may also participate in erythrocyte rupture [5, 7]. Disruption of the hemoglobin degradation pathway is lethal for the parasite. Development of compounds targeting falcipain-2 and falcipain-3 is therefore currently a focus of research. A recent study has further shown that individual cysteine protease inhibitors significantly reduce microgametogenesis in *P. falciparum*, pointing to a potential role of these inhibitors for transmission blocking strategies [8]. So far several peptidic and peptidomimetic as well as non-peptidic cysteine protease inhibitors have been synthesized and tested against malaria parasites [9-12]. In the present work a set of non-peptidic cysteine protease inhibitors derived from the loop diuretic etacrynic acid as a lead compound were evaluated for their potency to inhibit recombinantly expressed falcipain-2 and falcipain-3 [13-15]. Furthermore, activities against chloroquine-sensitive *P. falciparum* strain 3D7 and the chloroquine-resistant strain W2 were investigated. Several of these compounds have initially been tested against papain, the prototype cysteine protease of the CAC1 family, and against the SARS coronavirus main protease M^{pro}, as described elsewhere [16, 17].

Results and Discussion

In these previous studies compounds possessing an activated double bond revealed covalent, but reversible binding to the cysteine residue of the respective protease. In addition to the structural modifications implemented in previous work [16, 17], namely within the compounds **1**, **2**, **8-21**, we now included fluoro-substituted compounds **3-7**, several analogues without an activated double bond **25-32**, as well as derivatives with polar side chains **22-23**, and finally, a biotin-labeled inhibitor **24**. In summary, the structure of etacrynic acid was modified as follows (Scheme 1): **A**: substitution pattern of the aromatic ring, **B**: esters and amides, **C**: ortho-position of the double bond containing side chain (cpd. **2**), **D**: removal of the double bond.

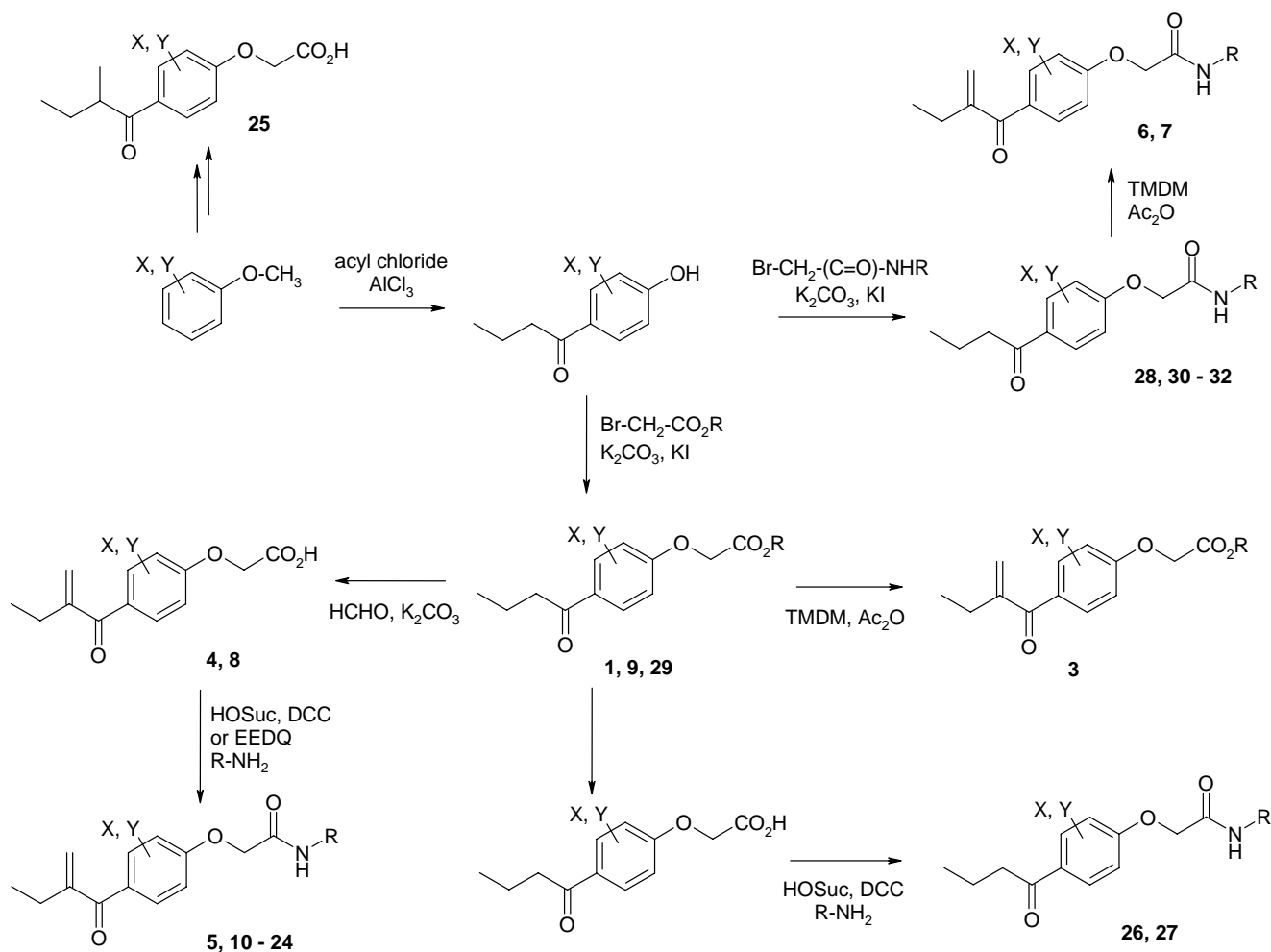
Scheme 1. Sites of modification of the etacrynic acid lead.



A: substitution pattern of the aromatic ring; **B**: esters, amides, acids; **C**: ortho-position; **D**: removal of the double bond.

The inhibitors were synthesized according to previously described pathways [16,17] which are summarized in Scheme 2. Halogen substituted anisoles were subjected to Friedel-Crafts acylation yielding the corresponding phenolic ketones. Further alkylation of the phenolic hydroxyl functions yielded amides **28**, **30** – **32**, and esters **1**, **9** and **29**. Introduction of the double bond was performed either by Mannich reaction with TMDM (\rightarrow **3**, **6**, **7**) or by aldol condensation with formaldehyde (\rightarrow **4**, **8**). The latter yielded the free acids **4** and **8** due to concurrent hydrolysis of the ester function. The free acids were coupled to various amides to give the amides **5**, **10** – **24**. Amides **26** and **27** without activated double bond were also synthesized by standard amide coupling methods.

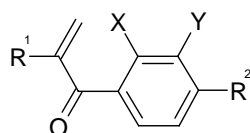
Scheme 2. Synthetic pathways to the etacrynic acid derivatives. HOSuc, *N*-hydroxysuccinimide; DCC, dicyclohexylcarbodiimide, TMDM, *N,N,N',N'* tetramethyldiaminomethane; EEDQ, ethyl 1,2-dihydro-2-ethoxyquinoline-1-carboxylate.



Recombinant falcipain-2 and falcipain-3 were produced as previously described [14, 18]. Inhibitory activities against recombinant falcipain-2 and falcipain-3 were evaluated in fluorometric microplate assays using the substrates Cbz-Phe-Arg-AMC and Cbz-Leu-Arg-AMC (AMC , 7-amino-4-methylcoumarin) [19]. The cysteine protease inhibitor E-64 was used as a positive control [20]. The solvent DMSO was used as negative control. Compounds **1**–**6**, **8**–**11** and **13**–**28** were tested *in vitro* against the CQ-sensitive 3D7 *P. falciparum* strain or the CQ-resistant W2 *P. falciparum* strain. The corresponding

IC₅₀ values are shown in Table 1. The *in vitro* data for the etacrynic acid derivatives are compared to those of the well known drug chloroquine and to E-64. In addition, the cytotoxicity of the inhibitor **23** was studied on human kidney epithelium cell-line 293T, as described previously [21, 22], resulting in an IC₅₀ value of >160 μM.

Table 1. Inhibition of falcipain-2 / -3 (FP-2 / -3) as well as antiplasmodial activity of non-peptidic Michael-acceptors derived from etacrynic acid.



Cpd.	R ¹	X	Y	R ²	FP-2 IC ₅₀ , (μM)	FP-3 IC ₅₀ , (μM)	P. f. 3D7/W2, IC ₅₀ (μM)
1	H ₅ C ₂	H	Cl	O-CH ₂ -CO ₂ Et	498±8	346±14	ni ^c
2	H ₅ C ₂	O-CH ₂ -CO ₂ Et	H	Cl	110±5	381±21	ni ^c
3	H ₅ C ₂	H	F		ni	nd	142±5 ^b
4	H ₅ C ₂	H	F	O-CH ₂ CO ₂ H	nd	nd	79.8±6 ^b
5	H ₅ C ₂	H	F		80±5	nd	205±9 ^b
6	H ₅ C ₂	H	F		ni	nd	141±4 ^b
7	H ₅ C ₂	H	F		ni	nd	nd
8	H ₅ C ₂	Cl	Cl	O-CH ₂ -CO ₂ H	443±17	ni	ni ^c
9	H ₅ C ₂	Cl	Cl	O-CH ₂ -CO ₂ Et	60.6±4.2	163±5.6	ni ^c
10	H ₅ C ₂	Cl	Cl		178±14	56.7±5.7	ni ^c
11	H ₅ C ₂	Cl	Cl		333±5	158±11	ni ^c
12	H ₅ C ₂	Cl	Cl		165±12	nd	nd

Table 1. Cont.

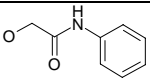
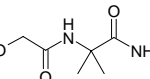
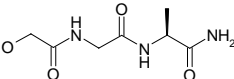
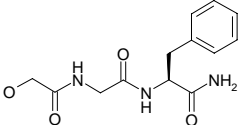
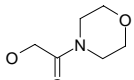
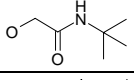
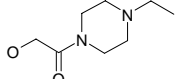
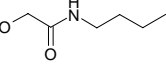
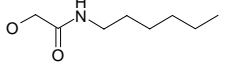
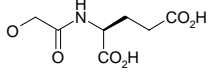
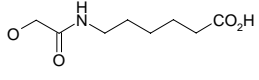
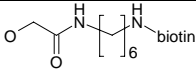
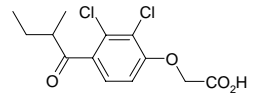
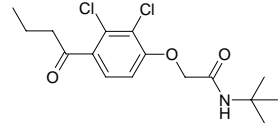
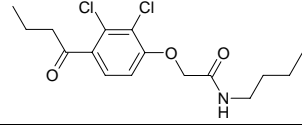
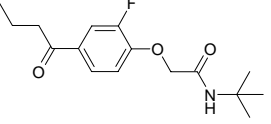
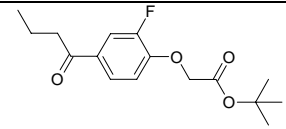
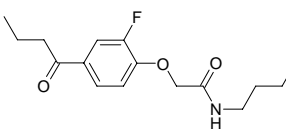
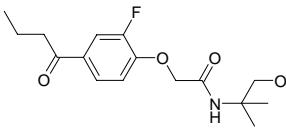
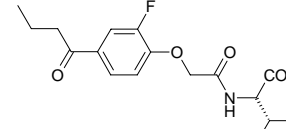
13	H ₅ C ₂	Cl	Cl		269±21	87.2±6	ni ^c
14	H ₅ C ₂	Cl	Cl		318±14	ni	29.3±3.4 ^c
15	H ₅ C ₂	Cl	Cl		212±17	ni	ni ^c
16	H ₅ C ₂	Cl	Cl		242±19	ni	ni ^c
17	H ₅ C ₂	Cl	Cl		305±30	479±23	ni ^c
18	H ₅ C ₂	Cl	Cl		255±4	153±16	ni ^c
19	H ₅ C ₂	Cl	Cl		144±11	557±23	27.4±4.1 ^c
20	H ₅ C ₂	Cl	Cl		184±17	158±17	ni ^c
21	H ₅ C ₂	Cl	Cl		182±9	123±8	ni ^c
22	H ₅ C ₂	Cl	Cl		ni	ni	ni ^c
23	H ₅ C ₂	Cl	Cl		57.1±13	96.5±0.6	18.8±0.9 ^c
24	H ₅ C ₂	Cl	Cl		3.0±1.1	11.9±1.1	9.0±0.4 ^c
25					531 ^a	ni	ni ^c
26					484 ^a	ni	ni ^c
27					713 ^a	ni	ni ^c
28					80±6	nd	66.4±2.2 ^c

Table 1. Cont.

29		80±6	nd	nd
30		ni	nd	nd
31		ni	nd	nd
32		ni	nd	nd
E-64		0.015±0.008	0.075±0.02	5.3±1.05 ^c
CQ (W2)		nd	nd	0.24[19]
CQ (3D7)		nd	nd	0.01±0.0048

^a Only one experiment; ni, no inhibition; nd, not determined; CQ, chloroquine; ^b 3D7 strain;

^c W2 strain.

Inspection of the data in Table 1 allows the following conclusions to be drawn: in general, the etacrynic acid derivatives are weak or moderate inhibitors of falcipains and *P. falciparum*. Nevertheless, some structure-activity relationship can be found:

The ethyl ester derivative (**9**) is more potent than the etacrynic acid (**8**), both against falcipain-2 and -3. This is in accordance with the results for papain and the SARS-CoV M^{pro}. The dichloro-substituted compounds (e.g. **9**, **20**) are better inhibitors than the mono-chloro- (e.g. **1**) or fluoro-substituted (**6**) compounds, with one exception, namely inhibitor **5** (compared to **18**). The α,β -unsaturated system appears to be favourable for activity against falcipains when comparing the analogous dichloro-compounds **20** and **27** or compounds **18** and **26**. Short voluminous moieties such as the *tert*butyl moiety of compounds **5**, **28**, **29**, seem to be advantageous if combined with the fluoro-substituted aromate. In these cases, the activated double bond is apparently not essential for inhibition.

Nearly all etacrynic acid amides show better inhibition properties than the free acid (**8**). A longer acidic side-chain significantly enhances the activity (**23**, compared to **20**, **21**), making **23** the most active inhibitor of falcipains and *P. falciparum* within the series. However, insertion of an additional acidic group (e.g. **22**) diminishes the inhibiting activity. The cytotoxicity/antiplasmodial ratio for the most active compound **23** is >8.5, indicating selectivity against the parasite. As the data against the target enzymes and the parasites do not correlate in all cases (e.g. **14**), the question arises whether there are additional or other targets. In order to allow further affinity binding studies the biotinylated dichloro-substituted etacrynic acid amide **24** was included and synthesized according to the methods

recently described [17,23] (Scheme 2). Notably, this compound emerged as the most potent inhibitor of falcipains and *P. falciparum* within the series.

Conclusions

In summary, this paper describes a comprehensive screening of non-peptidic Michael acceptors using etacrynic acid as lead structure. The best inhibition against recombinantly synthesized falcipain-2 and falcipain-3 revealed the compound **24**. Moreover, this etacrynic acid amide as well as compound **23** displayed modest antiplasmodial activity *in vitro* with IC₅₀ values of 9 and 18.8 μM, respectively, which are in the range of the standard cysteine protease inhibitor E-64. In addition, the high IC₅₀ value of >160 μM for compound **23** obtained from cytotoxicity assays using the human kidney epithelium cell-line 293T indicates selectivity against the parasite. These results provide basic information for the development of further non-peptidic irreversible cysteine protease inhibitors with etacrynic acid amides as lead compounds. In addition, the good inhibitory properties of **24** allow further affinity binding studies.

Experimental

General

Melting points were determined in open capillary on a melting point apparatus, model 530, from Büchi, Switzerland. NMR spectra were recorded on an AVANCE 400 MHz spectrometer from Bruker Biospin GmbH, Germany [solvent CDCl₃ (unless otherwise noted); ¹H-NMR, 400.13 MHz; ¹³C-NMR, 100.61 MHz]. The optical rotation values were determined on a Perkin-Elmer 241 polarimeter. ESI mass spectra were recorded on an Agilent 1100 ion trap equipped with an Agilent HPLC system. Hydrostatic column chromatography was performed with silica gel 60 (0.063-0.2 mm). All solvents were purified and dried prior to use according to standard literature procedures. Chloroquine, E64 and DMSO (dimethyl sulfoxide) were obtained from Sigma-Aldrich, Deisenhofen, Germany. Alamar Blue[®] was purchased from Trinova Biochem, Giessen, Germany. DMEM (Dulbecco's Modified Eagle's Medium) high glucose was delivered from Gibco/Sigma-Aldrich, Deisenhofen, Germany. Hygromycin was obtained from Merck, Darmstadt, Germany.

Syntheses of inhibitors

The syntheses of compounds **1**, **2**, **8-21**, and **25** are described in [17].

Method A: Introduction of the double bond. To substances **29**, **30** or **32** (1 equiv.) and TMDM (*N,N,N',N'*-tetramethyldiaminomethane, 20 equiv.), acetic anhydride (20 equiv.) was added slowly. The mixture was heated under reflux at 85 °C. The reaction is followed by ¹H-NMR spectroscopy. After completion of the reaction the mixture was cooled to room temperature and saturated K₂CO₃ solution was added until gas evolution stopped. The product was extracted with Et₂O, washed with water and brine and dried with Na₂SO₄. The solvent was removed *in vacuo* and the product purified by column chromatography.

Method B: Syntheses of bromoacetamides. Bromoacetyl bromide (1 equiv.) in absolute CH_2Cl_2 was cooled to $-30\text{ }^\circ\text{C}$. Amine (1 equiv.) and triethylamine (1 equiv.) in absolute CH_2Cl_2 were added dropwise. After the addition, the reaction mixture was warmed up to room temperature and stirred for 1–2 h further. The solvent was removed *in vacuo*, the product was dissolved in acetone and filtered off. The bromoacetamides were used directly without further purification.

Method C: Coupling of the bromoacetamide or bromoacetic acid ester with 1-(3-Fluoro-4-hydroxyphenyl)butan-1-one. 1-(3-Fluoro-4-hydroxyphenyl)butan-1-one (1 equiv.), K_2CO_3 (1.5 equiv.) and KI (0.1 equiv.) were refluxed in dry acetone for 1 h. Bromoacetamide (method B) or bromoacetic acid ester (2 equiv.) were added and the mixture heated to reflux for a further 4 h. The mixture was cooled to room temperature, filtered and the solvent was removed *in vacuo*. The product was dissolved in Et_2O , washed with brine, KOH solution (5%) and water, and dried with Na_2SO_4 . The solvent was removed *in vacuo* and the product was purified by column chromatography.

Tert-butyl 2-(2-fluoro-4-(2-methylenebutanoyl)phenoxy)acetate (3). Method A: Tert-butyl-2-(4-butyryl-2-fluorophenoxy)acetate (**29**, 558 mg, 1.89 mmol), TMDM (5.14 mL, 37.7 mmol), acetic anhydride (3.56 mL, 37.7 mmol). R_f : 0.73 (cyclohexane/ethyl acetate: 1/1); yield 180 mg (0.58 mmol, 31 %); yellow oil; LOOP-ESI-MS: calcd. for $\text{C}_{16}\text{H}_{21}\text{FO}_4$ 308.35, found $[\text{M}+\text{H}]^+$ 309.2; LC-MS: R_t = 38.2 min, purity 100 %; $^1\text{H-NMR}$: δ 1.09 (t, 3H, $^3J = 7.5\text{ Hz}$, $\text{CH}_2\text{CH}_2\text{CH}_3$), 1.47 (s, 9H, $\text{C}_q(\text{CH}_3)_3$), 2.45 (q, 2H, $^3J = 7.4\text{ Hz}$, $\text{CH}_2\text{CH}_2\text{CH}_3$), 4.64 (s, 2H, $\text{OCH}_2\text{C}=\text{O}$), 5.50 (s, 1H, $\text{C}_q=\text{CH}_2$), 5.74 (s, 1H, $\text{C}_q=\text{CH}_2$), 6.88 (dd, 1H, $J = 8.4\text{ Hz}$, $\text{CH}_{\text{arom.}}$), 7.54–7.59 (m, 2H, $\text{CH}_{\text{arom.}}$); $^{13}\text{C-NMR}$: δ 12.30 ($\text{C}_q\text{CH}_2\text{CH}_3$), 25.51 ($\text{C}_q\text{CH}_2\text{CH}_3$), 28.01 ($\text{C}_q(\text{CH}_3)_3$), 66.48 ($\text{OCH}_2\text{C}=\text{O}$), 82.95 ($\text{C}_q(\text{CH}_3)_3$), 114.09 ($\text{CH}_{\text{arom.}}$), 117.98 ($\text{CH}_{\text{arom.}}$), 122.82 ($\text{C}_q=\text{CH}_2$), 126.52 ($\text{CH}_{\text{arom.}}$), 131.64 ($\text{C}_q\text{C}=\text{O}$), 149.39 ($\text{C}_q=\text{CH}_2$), 150.58 (C_qF or $\text{C}_q\text{OCH}_2\text{C}=\text{O}$), 153.06 (C_qF or $\text{C}_q\text{OCH}_2\text{C}=\text{O}$), 166.96 ($\text{OCH}_2\text{C}=\text{O}$), 196.12 ($\text{C}_q\text{C}=\text{O}$).

2-(2-fluoro-4-(2-methylenebutanoyl)phenoxy)acetic acid (4): 1-(3-fluoro-4-hydroxyphenyl)butan-1-one. 2-Fluoroanisole (4.00 g, 31.7 mmol) and butyric acid chloride (5.07 g, 47.6 mmol) were dissolved under a N_2 atmosphere in absolute CH_2Cl_2 (50 mL) and the mixture was cooled to $0\text{--}10\text{ }^\circ\text{C}$. AlCl_3 (6.35 g, 47.6 mmol) was added within 30 minutes, and the mixture was stirred for 2–3 h. An additional amount of AlCl_3 (6.35 g, 47.6 mmol) was added, and the mixture was heated under reflux for 2 h. The mixture was poured on ice and acidified with concentrated HCl to pH 1. Tartaric acid was added for complexation of aluminum until the solution was clear. The solution was extracted with Et_2O and the organic layer was washed with KOH solution (10%) and brine. The organic layer was dried with Na_2SO_4 and solvent was removed *in vacuo*. Yield 4.97 g (27.3 mmol, 86 %); white solid; mp. $91\text{ }^\circ\text{C}$ (water); $^1\text{H-NMR}$: δ 0.98 (t, 3H, $^3J = 7.3\text{ Hz}$, $\text{CH}_2\text{CH}_2\text{CH}_3$), 1.74 (sext, 2H, $^3J = 7.3\text{ Hz}$, $\text{CH}_2\text{CH}_2\text{CH}_3$), 2.87 (t, 2H, $^3J = 7.3\text{ Hz}$, $\text{CH}_2\text{CH}_2\text{CH}_3$), 6.04 (bs, 1H, OH), 7.04 (dd, 1H, $J = 8.5\text{ Hz}$, $\text{CH}_{\text{arom.}}$), 7.67–7.73 (m, 2H, $\text{CH}_{\text{arom.}}$).

Ethyl-2-(4-butyryl-2-fluorophenoxy)acetate. 1-(3-Fluoro-4-hydroxyphenyl)butan-1-one (2.00 g, 11.0 mmol), bromoacetic acid ethyl ester (3.67 g, 22.0 mmol), K_2CO_3 (2.28 g, 16.5 mmol) and KI (183 mg, 1.10 mmol) were refluxed in dry acetone for 5–6 h. The reaction mixture was cooled to room temperature and filtered off. The solvent was removed *in vacuo* and the product was extracted with

Et₂O, washed with NaOH solution (10%), water and brine, dried with Na₂SO₄, and the solvent was removed *in vacuo*. Yield 2.30 g (8.60 mmol, 78%); white solid; mp. 79–80 °C (Et₂O). ¹H-NMR (DMSO-d₆): δ 0.90 (t, 3H, ³J = 7.5 Hz, CH₂CH₂CH₃), 1.20 (t, 3H, ³J = 7.2 Hz, OCH₂CH₃), 1.60 (sext, 2H, ³J = 7.3 Hz, CH₂CH₂CH₃), 2.93 (t, 2H, ³J = 7.1 Hz, CH₂CH₂CH₃), 4.17 (q, 2H, ³J = 7.1 Hz, OCH₂CH₃), 4.99 (s, 2H, OCH₂C=O), 7.20 (dd, 1H, J = 8.5, CH_{arom.}), 7.74–7.78 (m, 2H, CH_{arom.}).

2-(2-fluoro-4-(2-methylenebutanoyl)phenoxy)acetic acid (**4**). Ethyl-2-(4-butyryl-2-fluorophenoxy)-acetate (2.11 g, 7.87 mmol), formaldehyde solution (40%, 1.08 mL, 15.7 mmol) and K₂CO₃ (2.17 g, 15.7 mmol, dissolved in water) were refluxed in ethanol for 24 h. The reaction mixture was cooled to room temperature, acidified with concentrated HCl to pH 1 and the product was extracted with Et₂O. The solvent was removed *in vacuo*. Yield 556 mg (2.20 mmol, 28%); white solid; mp 99–101 °C (Et₂O); LOOP-ESI-MS: calcd. for C₁₃H₁₃FO₄ 252.24, found [M+H]⁺ 253.1; LC-MS: R_t = 23.8 min, purity 100 %; ¹H-NMR: δ 1.10 (t, 3H, ³J = 7.5 Hz, C_qCH₂CH₃), 2.46 (q, 2H, ³J = 7.4 Hz, C_qCH₂CH₃), 4.81 (s, 2H, OCH₂C=O), 5.52 (s, 1H, C_q=CH₂), 5.78 (s, 1H, C_q=CH₂), 6.95 (dd, 1H, J = 8.4 Hz, CH_{arom.}), 7.56–7.60 (m, 2H, CH_{arom.}); ¹³C-NMR: δ 12.30 (C_qCH₂CH₃), 25.44 (C_qCH₂CH₃), 65.65 (OCH₂C=O), 114.50 (CH_{arom.}), 118.19 (CH_{arom.}), 123.35 (C_q=CH₂), 126.59 (CH_{arom.}), 132.35 (C_qC=O), 148.87 (C_q=CH₂), 149.33 (C_qF or C_qOCH₂C=O), 153.11 (C_qF or C_qOCH₂C=O), 172.29 (OCH₂C=O), 196.16 (C_qC=O).

N-tert-butyl-2-(2-fluoro-4-(2-methylenebutanoyl)phenoxy)acetamide (**5**). 2-(2-Fluoro-4-(2-methylenebutanoyl)phenoxy)acetic acid (**4**, 767 mg, 3.00 mmol) and *N*-hydroxysuccinimide (336 mg, 3.00 mmol) were dissolved under a N₂ atmosphere in absolute CH₂Cl₂ (10 mL). Dicyclohexylcarbodiimide (DCC) (619 mg, 3.00 mmol) was added in small portions and the mixture was stirred overnight. The mixture was filtered into a flask with *tert*-butylamine (219 mg, 3.00 mmol) in CH₂Cl₂ (10 mL), stirred for 3 h at room temperature and filtered. The reaction mixture was washed with brine and KOH solution (5%), dried with Na₂SO₄ and the solvent was removed *in vacuo*. The product was purified by column chromatography. R_f: 0.43 (cyclohexane/ethyl acetate: 2/1); yield 314 mg (1.02 mmol, 34 %); white solid; mp 85 °C (cyclohexane/ethyl acetate); LOOP-ESI-MS: calcd. for C₁₇H₂₂FNO₃ 307.37, found [M+H]⁺ 308.4; LC-MS: R_t = 18.0 min, purity 100 %; ¹H-NMR: δ 1.10 (t, 3H, ³J = 7.5 Hz, C_qCH₂CH₃), 1.40 (s, 9H, C_q(CH₃)₃), 2.46 (q, 2H, ³J = 7.4 Hz, C_qCH₂CH₃), 4.46 (s, 2H, OCH₂C=O), 5.51 (s, 1H, C_q=CH₂), 5.78 (s, 1H, C_q=CH₂), 6.43 (bs, 1H, NH), 6.96 (dd, 1H, J = 8.2 Hz, CH_{arom.}), 7.56–7.62 (m, 2H, CH_{arom.}); ¹³C-NMR: δ 12.30 (C_qCH₂CH₃), 25.45 (C_qCH₂CH₃), 28.71 (C_q(CH₃)₃), 51.46 (C_q(CH₃)₃), 68.50 (OCH₂C=O), 114.05 (CH_{arom.}), 117.73 (CH_{arom.}), 123.23 (C_q=CH₂), 126.92 (CH_{arom.}), 132.23 (C_qC=O), 146.73 (C_q=CH₂), 148.87 (C_qF or C_qOCH₂C=O), 149.35 (C_qF or C_qOCH₂C=O), 165.95 (OCH₂C=O), 196.01 (C_qC=O).

N-butyl-2-(2-fluoro-4-(2-methylenebutanoyl)phenoxy)acetamide (**6**). Method A: *N*-butyl-2-(4-butyryl-2-fluorophenoxy)acetamide (**30**) (105 mg, 0.36 mmol), TMDM (0.97 mL, 7.11 mmol), acetic anhydride (0.67 mL, 7.11 mmol). R_f: 0.59 (cyclohexane/ethyl acetate: 1/2); yield 55.5 mg (0.18 mmol, 51 %); white solid; mp 73 °C (cyclohexane/ethyl acetate); LOOP-ESI-MS: calcd. for C₁₇H₂₂FNO₃ 307.37; found [M+H]⁺ 308.4; LC-MS: R_t = 15.8 min, purity 100 %; ¹H-NMR: δ 0.93 (t, 3H, ³J = 7.4 Hz, NHCH₂CH₂CH₂CH₃), 1.11 (t, 3H, ³J = 7.4 Hz, C_qCH₂CH₃), 1.36 (sext, 2H, ³J = 7.4 Hz,

NHCH₂CH₂CH₂CH₃), 1.53 (sext, ³J = 7.3 Hz, NHCH₂CH₂CH₂CH₃), 2.46 (q, 2H, ³J = 7.5 Hz, C_qCH₂CH₃), 3.37 (q, 2H, ³J = 6.7 Hz, NHCH₂CH₂CH₂CH₃), 4.57 (s, 2H, OCH₂C=O), 5.51 (s, C_q=CH₂), 5.78 (s, C_q=CH₂), 6.60 (s, 1H, NH), 6.96 (dd, 1H, J = 8.1 Hz, CH_{arom.}), 7.57–7.62 (m, 2H, CH_{arom.}); ¹³C-NMR: δ 12.31 (C_qCH₂CH₃), 13.69 (NHCH₂CH₂CH₂CH₃), 20.00 (NHCH₂CH₂CH₂CH₃), 25.45 (C_qCH₂CH₃), 31.53 (NHCH₂CH₂CH₂CH₃), 38.90 (NHCH₂CH₂CH₂CH₃), 68.21 (OCH₂C=O), 113.90 (CH_{arom.}), 117.77 (CH_{arom.}), 123.22 (C_q=CH₂), 126.92 (CH_{arom.}), 149 (C_qF or C_qOCH₂C=O), 166.77 (OCH₂C=O).

Methyl-2-(2-(2-fluoro-4-(2-methylenebutanoyl)phenoxy)acetamido)-3-methylpentanoate (7). Method A: Methyl-2-(2-(4-butyryl-2-fluorophenoxy)acetamido)-3-methylpentanoate (**32**, 58.0 mg, 0.16 mmol), TMDM (0.43 mL, 3.16 mmol), acetic anhydride (0.30 mL, 3.16 mmol). R_f: 0.75 (cyclohexane/ethyl acetate: 1/1); yield 41.5 mg (0.11 mmol, 68 %); colorless oil; LOOP-ESI-MS: calcd. for C₂₀H₂₆FNO₅ 379.43; found [M+H]⁺ 380.4; LC-MS: R_t = 18.9 min, purity 100 %; [α]_D²²: 61.1 (CHCl₃, c = 0.1). ¹H-NMR: δ 0.90–0.94 (m, 6H, Ile-CH₂CH₃ and Ile-CHCH₃), 1.11 (t, 3H, ³J = 7.5 Hz, C_qCH₂CH₃), 1.14–1.22 (m, 2H, Ile-CH₂CH₃), 1.40–1.48 (m, 1H, Ile-C=O), 1.93–1.99 (m, 1H, Ile-CHCH₃), 2.47 (t, 2H, ³J = 7.3 Hz, C_qCH₂CH₃), 3.74 (s, 3H, OCH₃), 4.62 (s, 2H, OCH₂C=O), 5.51 (s, C_q=CH₂), 5.78 (s, C_q=CH₂), 6.98 (dd, 1H, ³J = 8.2 Hz, CH_{arom.}), 7.08 (d, 1H, J = 8.5 Hz, NH), 7.57–7.63 (m, 2H, CH_{arom.}); ¹³C-NMR: δ 11.51 (Ile-CH₂CH₃ or Ile-CHCH₃), 12.31 (C_qCH₂CH₃), 15.50 (Ile-CH₂CH₃ or Ile-CHCH₃), 25.08 (C_qCH₂CH₃ or Ile-CHC=O), 25.43 (C_qCH₂CH₃ or Ile-NCHC=O), 37.79 (Ile-CHCH₃), 52.23 (OCH₃), 68.32 (OCH₂C=O), 114.25 (CH_{arom.}), 117.86 (CH_{arom.}), 123.26 (C_q=CH₂), 126.81 (CH_{arom.}), 132.46 (C_qC=O), 149.37 (C_qOCH₂C=O), 150.66 (C_q=CH₂), 153.14 (C_qF), 166.83 (OCH₂C=O), 171.68 (C_qOCH₃), 195.98 (C_qC=O).

2-{2-[2,3-Dichloro-4-(2-methylene-butyl)-phenoxy]acetylamino}pentanedioic acid (22). Compound **22** was synthesized according to [17] starting from etacrynic acid (240 mg, 0.79 mmol), *N*-hydroxy-succinimide (91 mg, 0.79 mmol), DCC (163 mg, 0.79 mmol) in THF. The solvent was removed and a solution of glutamic acid (116 mg, 0.79 mmol) and KOH (133 mg, 2.37 mmol) in water was added. After stirring 7 days at room temperature the mixture was acidified with conc. HCl and extracted with dichloromethane. The crude product, which still contained etacrynic acid, was suspended in hot benzene and filtered. The solid residue yielded the pure amide as a white powder. Yield: 55 mg (0.127 mmol, 16 %). ¹H-NMR (*d*₄-MeOH): δ = 1.14 (t, 3H, ³J = 8.0 Hz, CH₂CH₃), 2.05 (m, 1H), 2.27 (m, 1H), 2.38–2.47 (m, 4H, 2 CH₂), 4.57 (m, 1H, NHCH), 4.75 (s, 2H, OCH₂CO), 5.60 (s, 1H, C=CH), 6.02 (s, 1H, C=CH), 7.10 (d, 1H, ³J = 8.0 Hz, Ar-H), 7.24 (d, 1H, ³J = 8.0 Hz, Ar-H); LC-MS (negative mode): *m/z*: 431.2 (100) [M-H]⁻, R_t = 2.3 min.

6-{2-[2,3-Dichloro-4-(2-methylene-butyl)-phenoxy]acetylamino}-hexanoic acid (23). Compound **23** was synthesized according to [17] starting from etacrynic acid (201 mg, 0.66 mmol), *N*-hydroxy-succinimide (76 mg, 0.66 mmol), DCC (137 mg, 0.66 mmol) in dichloromethane (20 mL). The solvent was removed and the residue taken up in THF. This solution was added to a solution of 6-amino-hexanoic acid (130 mg, 0.99 mmol) and KOH (111 mg, 1.98 mmol) in water (10 mL). After 3 days stirring at room temperature the mixture was acidified with conc. HCl and extracted with dichloromethane. After removal of the organic solvent the crude product yielded a yellow oil which was

purified by column chromatography (MeOH/CHCl₃ 10:2). Product: colourless oil, which solidified to a white solid. Yield: 93 mg (0.22 mmol, 34 %); ¹H-NMR (*d*₄-MeOH): δ = 1.14 (t, 3H, ³*J* = 8.0 Hz, CH₂CH₃), 1.33–1.40 (m, 2H, CH₂), 1.53–1.66 (m, 4H, 2 CH₂), 2.28 (t, 2H, ³*J* = 8.0 Hz, CH₂), 2.44 (q, 2H, ³*J* = 8.0 Hz, CH₂CH₃), 3.30 (m, 2H, NHCH₂), 4.68 (s, 2H, OCH₂CO), 5.59 (s, 1H, C=CH), 6.03 (s, 1H, C=CH), 7.07 (d, 1H, ³*J* = 8.0 Hz, Ar-H), 7.24 (d, 1H, ³*J* = 8.0 Hz, Ar-H); ¹³C-NMR (*d*₄-MeOH): δ = 13.0 (CH₃), 24.5 (CH₂), 25.7 (CH₂), 27.4 (CH₂), 30.1 (CH₂), 34.9 (CH₂), 40.0 (CH₂), 69.5 (OCH₂CO), 112.9 (Ar-CH), 124.0 (Ar-qC), 128.5 (Ar-CH), 129.9 (C=CH₂), 131.8 (Ar-qC), 135.0 (Ar-qC), 151.7 (qC, C=CH₂), 156.9 (Ar-qC), 169.7 (qC, NHCONH), 177.6 (qC, COOH), 197.3 (qC, C=O); LC-MS: *m/z*: 418.0 (15), R_t = 0.5 min.

5-(2-Oxo-hexahydro-thieno[3,4-d]imidazol-4-yl)pentanoic acid (6-{2-[2,3-dichloro-4-(2-methylenebutyryl)-phenoxy]acetylamino}-hexyl)-amide (24)

(A) *Coupling of Boc-diaminohexane and D-biotin*: Boc-diaminohexane (443 mg, 2.05 mmol) and D-biotin (500 mg, 2.05 mmol) were suspended in absolute DMF (45 mL) and cooled to 0 °C. DPPA (620 mg, 2.25 mmol) and triethylamine (229 mg, 2.25 mmol) were added and the mixture was stirred at 4 °C for 8 days. Dichloromethane (25 mL) was added and the organic phase was washed twice with citric acid (10%). Upon addition of water to the organic phase the amide precipitated as a white solid which was filtered off.

(B) *Removal of the Boc-protecting group*: The solid obtained in step (A) was suspended in dichloromethane (9 mL) and treated with TFA (3 mL) at 0 °C. Excess TFA was repeatedly removed *in vacuo*. For better evaporation dichloromethane was added to the residue. Quantitative removal of the protecting group was verified by ¹H-NMR.

(C) *Amide coupling to etacrynic acid*: Biotin-(6-amino)-hexylamide (TFA-salt) (175 mg, 0.383 mmol), triethylamine (39 mg, 0.383 mmol) and etacrynic acid (232 mg, 0.767 mmol) were dissolved in DMF (2 mL). EEDQ (190 mg, 0.767 mmol) was added and the mixture was stirred at room temperature for 14 days. Ethyl acetate (50 mL) was added. Treatment of the organic phase with Na₂CO₃-solution (2%, 25 mL) yielded the desired product as a white precipitate which was filtered off. Overall yield: 62 mg (0.0988 mmol) as a white solid; ¹H-NMR (DMSO-*d*₆): δ = 1.07 (t, 3H, ³*J* = 7.4 Hz, CH₂CH₃), 1.23–1.57 (m, 14H, CH₂), 2.03 (t, 2H, ³*J* = 7.2 Hz, CH₂), 2.36 (q, 2H, ³*J* = 7.4 Hz, CH₂CH₃), 2.56 (d, 1H, ³*J* = 12.3 Hz, SCH), 2.80 (dd, 1H, ³*J* = 12.4 Hz, ²*J* = 4.8 Hz, SCH), 2.97–3.12 (m, 5H, 2 NHCH₂, SCH), 4.11 (m, 1H, NHCH), 4.29 (m, 1H, NHCH), 4.70 (s, 2H, OCH₂CO), 5.55 (s, 1H, C=CH), 6.06 (s, 1H, C=CH), 6.33 (s, 1H, biotin-NH), 6.39 (s, 1H, biotin-NH), 7.07 (d, 1H, ³*J* = 8.6 Hz, Ar-H), 7.32 (d, 1H, ³*J* = 8.6 Hz, Ar-H), 7.70 (br s, 1H, CONH), 8.01 (br s, 1H, CONH); ¹³C-NMR (DMSO-*d*₆): δ = 12.3 (CH₃), 22.9 (CH₂CH₃), 25.3 (CH₂), 26.0 (CH₂), 26.0 (CH₂), 28.0 (CH₂), 28.2 (CH₂), 28.9 (CH₂), 29.1 (CH₂), 35.2 (CH₂), 38.2 (CH₂), 38.3 (CH₂), 39.8 (SCH₂), 55.4 (SCH), 59.2 (NHCH), 61.0 (NHCH), 67.9 (OCH₂CO), 111.9 (Ar-CH), 121.2 (Ar-qC), 127.4 (Ar-CH), 129.3 (C=CH₂), 129.9 (Ar-qC), 132.4 (Ar-qC), 149.3 (qC, C=CH₂), 155.5 (Ar-qC), 162.6 (qC, NHCONH), 166.3 (qC, CONH), 171.7 (qC, CONH), 195.1 (qC, C=O); LC-MS: *m/z* 628.0 (100) [M+H]⁺, R_t = 16.3 min.

N-tert-butyl-2-(4-butyryl-2,3-dichloro-phenoxy)-acetamide (26). Compound **26** was synthesized according to [17] starting from 2,3-dichloro-4-butyrylphenoxyacetic acid (500 mg, 1.72 mmol), *N*-hydroxysuccinimide (198 mg, 1.72 mmol), DCC (354 mg, 1.72 mmol) and *t*-butylamine (251 mg, 3.43 mmol) in dichloromethane (15 mL). Reaction time: 21 d stirring at room temperature. The crude product was purified by column chromatography (SiO₂, Cy/EtOAc 1:1). Yield: 374 mg (1.08 mmol, 63 %) as white solid; ¹H-NMR: δ = 0.97 (t, 3H, ³J = 7.4 Hz, CH₂CH₃), 1.41 (s, 9H, (CH₃)₃), 1.72 (sext, 2H, ³J = 7.3 Hz, CH₂CH₃), 2.88 (t, 2H, ³J = 7.2 Hz, COCH₂CH₂CH₃), 4.44 (s, 2H, OCH₂), 6.62 (br s, 1H, NH), 6.84 (d, 1H, ³J = 8.6 Hz, Ar-H), 7.38 (d, 1H, ³J = 8.6 Hz, Ar-H). ¹³C-NMR: δ = 13.7 (CH₂CH₃), 17.8 (CH₂CH₃), 28.7 (C(CH₃)₃), 44.7 (COCH₂), 51.5 (C(CH₃)₃), 68.4 (OCH₂), 111.1 (Ar-CH), 123.2 (Ar-qC), 127.6 (Ar-CH), 131.4 (Ar-qC), 134.9 (Ar-qC), 155.2 (Ar-qC), 165.5 (qC, CONH), 201.8 (C=O).

N-butyl-2-(4-butyryl-2,3-dichloro-phenoxy)-acetamide (27). Compound **27** was synthesized according to [17] starting from 2,3-dichloro-4-butyrylphenoxyacetic acid (500 mg, 1.72 mmol), *N*-hydroxysuccinimide (198 mg, 1.72 mmol), DCC (354 mg, 1.72 mmol) and *n*-butylamine (251 mg, 3.43 mmol) in dichloromethane (15 mL). Reaction time: 21 d stirring at room temperature. The crude product was purified by column chromatography (SiO₂, Cy/EtOAc 1:1). Yield: 270 mg (0.880 mmol, 45 %) as a white solid; ¹H-NMR: δ = 0.92–0.98 (m, 6H, NH(CH₂)₃CH₃, COCH₂CH₂CH₃), 1.38 (sext, 2H, ³J = 7.4 Hz, NHCH₂CH₂CH₂CH₃), 1.55 (quint, 2H, ³J = 7.3 Hz, NHCH₂CH₂CH₂CH₃), 1.72 (sextett, 2H, ³J = 7.3 Hz, COCH₂CH₂CH₃), 2.88 (t, 2H, ³J = 7.3 Hz, COCH₂CH₂CH₃), 3.37 (q, 2H, ³J = 6.7 Hz, NHCH₂), 4.55 (s, 2H, OCH₂), 6.72 (br s, 1H, NH), 6.85 (d, 1H, ³J = 8.8 Hz, Ar-H), 7.38 (d, 1H, ³J = 8.6 Hz, Ar-H); ¹³C-NMR: δ = 13.7 (*n*-butyl-CH₃, CH₂CH₃), 17.8 (CH₂CH₃), 20.0 (NHCH₂CH₂CH₂CH₃), 31.5 (NHCH₂CH₂CH₂CH₃), 38.9 (NHCH₂), 44.8 (COCH₂CH₂CH₃), 68.2 (OCH₂), 111.0 (Ar-CH), 123.3 (Ar-qC), 127.6 (Ar-CH), 131.4 (Ar-qC), 135.0 (Ar-qC), 155.1 (Ar-qC), 166.4 (qC, CONH), 201.8 (C=O).

N-tert-butyl-2-(4-butyryl-2-fluorophenoxy)acetamide (28). Method B: Bromoacetyl bromide (2.94 g, 14.6 mmol), *tert*-butylamine (1.07 mg, 14.6 mmol), triethylamine (2.05 mL, 14.6 mmol); Method C: 1-(3-fluoro-4-hydroxyphenyl)butan-1-one (856 mg, 4.70 mmol), K₂CO₃ (974 mg, 7.05 mmol), KI (78.0 mg, 0.47 mmol), 2-bromo-*N-tert*-butylacetamide (see method B). R_f: 0.31 (cyclohexane/ethyl acetate: 2/1); yield 1.00 g (3.38 mmol, 72%); white solid; mp 80 °C (cyclohexane/ethyl acetate). ¹H-NMR: δ 0.99 (t, 3H, ³J = 7.5 Hz, CH₂CH₂CH₃), 1.37 (s, 9H, C_q(CH₃)₃), 1.75 (sext, 2H, ³J = 7.3 Hz, CH₂CH₂CH₃), 2.88 (t, 2H, ³J = 7.2 Hz, CH₂CH₂CH₃), 4.46 (s, 2H, OCH₂C=O), 6.41 (bs, 1H, NH), 6.97 (dd, 1H, J = 8.4 Hz, CH_{arom.}), 7.72–7.76 (m, 2H, CH_{arom.}).

Tert-butyl-2-(4-butyryl-2-fluorophenoxy)acetate (29). Method C: 1-(3-Fluoro-4-hydroxyphenyl)butan-1-one (1.00 g, 5.49 mmol), K₂CO₃ (1.19 g, 8.64 mmol), KI (91.0 mg, 0.55 mmol), bromoacetic acid *tert*-butylester (1.50 mL, 10.2 mmol); R_f: 0.69 (cyclohexane/ethyl acetate: 1/1); yield 1.46 g (4.93 mmol, 90 %); white solid; mp 62 °C (cyclohexane/ethyl acetate); ¹H-NMR: δ 0.98 (t, 3H, ³J = 7.3 Hz, CH₂CH₂CH₃), 1.47 (s, 9H, C_q(CH₃)₃), 1.74 (sext, 2H, ³J = 7.3 Hz, CH₂CH₂CH₃), 2.86 (t, 2H, ³J = 7.2 Hz, CH₂CH₂CH₃), 4.64 (s, 2H, OCH₂C=O), 6.89 (dd, 1H, J = 8.5 Hz, CH_{arom.}), 7.68–7.72 (m, 2H, CH_{arom.}).

N-butyl-2-(4-butyryl-2-fluorophenoxy)acetamide (**30**). Method B: Bromoacetyl bromide (2.08 g, 10.3 mmol), *n*-butylamine (753 mg, 10.3 mmol), triethylamine (1.45 mL, 10.3 mmol). Method C: 1-(3-Fluoro-4-hydroxyphenyl)butan-1-one (750 mg, 4.12 mmol), K₂CO₃ (853 mg, 6.17 mmol), KI (68.4 mg, 0.41 mmol), 2-bromo-*N*-*n*-butylacetamide (see method B). R_f: 0.32 (cyclohexane/ethyl acetate: 1/2); yield 1.00 g (3.39 mmol, 82 %); white solid; mp 75 °C (cyclohexane/ethyl acetate); ¹H-NMR: δ 0.93 (m, 3H, NHCH₂CH₂CH₂CH₃), 0.99 (t, 3H, ³J = 7.4 Hz, CH₂CH₂CH₃), 1.36 (sext, 2H, ³J = 7.3 Hz, NHCH₂CH₂CH₂CH₃), 1.53 (sext, ³J = 7.3 Hz, NHCH₂CH₂CH₂CH₃), 1.75 (sext, 2H, ³J = 7.3 Hz, CH₂CH₂CH₃), 2.88 (t, 2H, ³J = 7.3 Hz, CH₂CH₂CH₃), 3.36 (q, 2H, ³J = 6.8 Hz, NHCH₂CH₂CH₂CH₃), 4.57 (s, 2H, OCH₂C=O), 6.59 (s, 1H, NH), 6.97 (dd, 1H, J = 8.3 Hz, CH_{arom.}), 7.72–7.76 (m, 2H, CH_{arom.}); ¹³C-NMR: δ 13.68 und 13.82 (CH₂CH₂CH₃ und NHCH₂CH₂CH₂CH₃), 17.76 (CH₂CH₂CH₃), 19.98 (NHCH₂CH₂CH₂CH₃), 31.52 (NHCH₂CH₂CH₂CH₃), 38.89 (NHCH₂CH₂CH₂CH₃), 40.24 (CH₂CH₂CH₃), 68.15 (OCH₂C=O), 114.04 (CH_{arom.}), 116.03 (CH_{arom.}), 125.31 (CH_{arom.}), 131.22 (C_qC=O), 149.12 (C_qF or C_qOCH₂C=O), 153.30 (C_qF or C_qOCH₂C=O), 166.69 (OCH₂C=O), 197.84 (C_qC=O).

2-(4-Butyryl-2-fluorophenoxy)-*N*-(1-hydroxy-2-methylpropan-2-yl)acetamide (**31**). Method B: Bromoacetyl bromide (0.76 mL, 8.75 mmol), 2-amino-2-methyl-1-propanol (780 mg, 8.75 mmol), triethylamine (1.23 mL, 8.75 mmol). Method C: 1-(3-Fluoro-4-hydroxyphenyl)butan-1-one (495 mg, 2.72 mmol), K₂CO₃ (563 mg, 4.08 mmol), KI (45.2 mg, 0.27 mmol), 2-bromo-*N*-(1-hydroxy-2-methylpropan-2-yl)acetamide (see method B). R_f: 0.28 (cyclohexane/ethyl acetate: 1/2); yield 455 mg (1.46 mmol, 54 %); white solid; mp 84 °C (cyclohexane/ethyl acetate); ¹H-NMR: δ 0.99 (t, 3H, ³J = 7.5 Hz, CH₂CH₂CH₃), 1.35 (s, 6H, C_q(CH₃)₂), 1.75 (sext, 2H, ³J = 7.4 Hz, CH₂CH₂CH₃), 2.88 (t, 2H, ³J = 7.3 Hz, CH₂CH₂CH₃), 3.65 (s, 2H, C_qCH₂OH), 4.52 (s, 2H, OCH₂C=O), 6.66 (s, 1H, NH), 6.98 (dd, 1H, J = 8.1 Hz, CH_{arom.}), 7.73–7.76 (m, 2H, CH_{arom.}); ¹³C-NMR: δ 13.83 (CH₂CH₂CH₃), 17.74 (CH₂CH₂CH₃), 24.66 (C_q(CH₃)₂), 40.26 (CH₂CH₂CH₃), 56.50 (C_q(CH₃)₂), 68.24 (OCH₂C=O), 70.17 (C_qCH₂OH), 114.38 (CH_{arom.}), 116.30 (CH_{arom.}), 125.28 (CH_{arom.}), 132.21 (C_qC=O), 148.87 (C_qF or C_qOCH₂C=O), 153.34 (C_qF or C_qOCH₂C=O), 167.42 (OCH₂C=O), 197.85 (C_qC=O).

Methyl 2-(2-(4-butyryl-2-fluorophenoxy)acetamido)-3-methylpentanoate (**32**). Method B: Bromoacetyl bromide (2.00 g, 9.92 mmol), isoleucine methylester HCl (1.80 mg, 9.92 mmol), triethylamine (2.79 mL, 19.8 mmol). Method C: 1-(3-Fluoro-4-hydroxyphenyl)butan-1-one (600 mg, 3.29 mmol), K₂CO₃ (683 mg, 4.94 mmol) KI (54.6 mg, 0.33 mmol), methyl-2-(2-bromoacetamido)-3-methyl-pentanoate (see method B). R_f: 0.65 (cyclohexane/ethyl acetate: 1/1); yield 616 mg (1.68 mmol, 51 %); white solid; mp 74 °C (cyclohexane/ethyl acetate); [α]_D²² = 61.1 (CHCl₃, c = 0.1); ¹H-NMR: δ 0.90–0.94 (m, 6H, Ile-CH₂CH₃ and Ile-CHCH₃), 0.99 (t, 3H, ³J = 7.5 Hz, CH₂CH₂CH₃), 1.12–1.23 (m, 2H, Ile-CH₂CH₃), 1.40–1.49 (m, 1H, Ile-CHC=O), 1.75 (sext, ³J = 7.3 Hz, CH₂CH₂CH₃), 1.92–1.99 (m, 1H, Ile-CHCH₃), 2.88 (t, 2H, ³J = 7.3 Hz, CH₂CH₂CH₃), 3.74 (s, 3H, OCH₃), 4.62 (s, 2H, OCH₂C=O), 6.99 (dd, 1H, ³J = 8.5 Hz, CH_{arom.}), 7.08 (d, 1H, J = 8.5 Hz, NH), 7.72–7.77 (m, 2H, CH_{arom.}).

Protein expression and purification

Falcipain-2 and falcipain-3 were recombinantly expressed in *E. coli* and refolded to active enzyme as previously described [13,24].

Enzyme assays, in-vitro assays

IC₅₀ values for inhibition of falcipain-2 and falcipain-3 were determined as described previously using the fluorogenic substrates Cbz-Phe-Arg-AMC and Cbz-Leu-Arg-AMC, respectively (AMC, 7-amino-4 methylcoumarin) [7, 13, 14]. As a positive control the well-known cysteine protease inhibitor E-64 [7, 25, 26] and as a negative control the solvent (DMSO) was used. Dose-dependent effects of compounds on parasite development (*P. falciparum* strain W2) were quantified by flow cytometry according to a previously published method [27]. First, a screening with inhibitor concentrations of 100, 10 and 1 mM was performed and the percentage activity of infected red blood cells (RBCs) relative to the negative control was determined. Compounds showing concentration dependent inhibition in these assays were selected for determination of IC₅₀ values (Table 1). Chloroquine [20] and E-64 were used as positive controls, and the solvent DMSO was used as a negative control. For selected compounds assays with the *P. falciparum* strain 3D7 were performed using a fluorometric assay with Hoechst-33258 [28], or the microculture tetrazolium test measuring parasite lactate dehydrogenase activity [12, 29]. The parasites were cultivated *in vitro* as described previously [30]. Compounds were screened at concentrations between 1 nM and 100 μM. Synchronized ring stages were plated in 96-well plates at a parasitemia of 1%, in the presence of the compounds (dissolved in DMSO). Incubation of parasites with DMSO alone at a concentration of 0.5% v/v was used as a negative control. IC₅₀ values were calculated by non-linear regression analyses using the programs GaphPad[®] Prism 4 and GraFit[®] [31, 32].

In vitro cytotoxicity assay

The human kidney epithelium-cell line 293T was cultured in DMEM medium supplemented with 20% fetal bovine serum and 1% l-glutamine (200 mM) at 37 °C in a humidified atmosphere containing 5% CO₂. For the experimental procedures, cells were detached from the flasks with a rubber policeman, washed twice with PBS (phosphate-buffer saline) containing 1 mL trypsin-EDTA (1 x), and suspended at 2 x 10⁶ cells mL⁻¹ in complete medium. Inhibitor assays were carried out as described elsewhere except that the cell concentration used in this study was 2 x 10⁴ mL⁻¹ well⁻¹ [33].

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Sample Availability: Samples of compound **24** are available from the authors.

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