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New Acyloxymethyl Ketones: Useful Probes for Cysteine Protease Profiling

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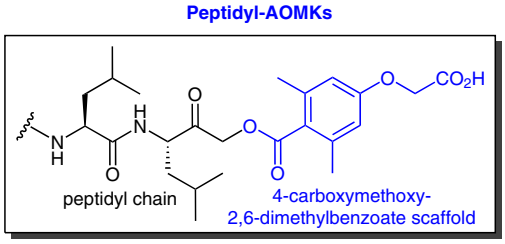

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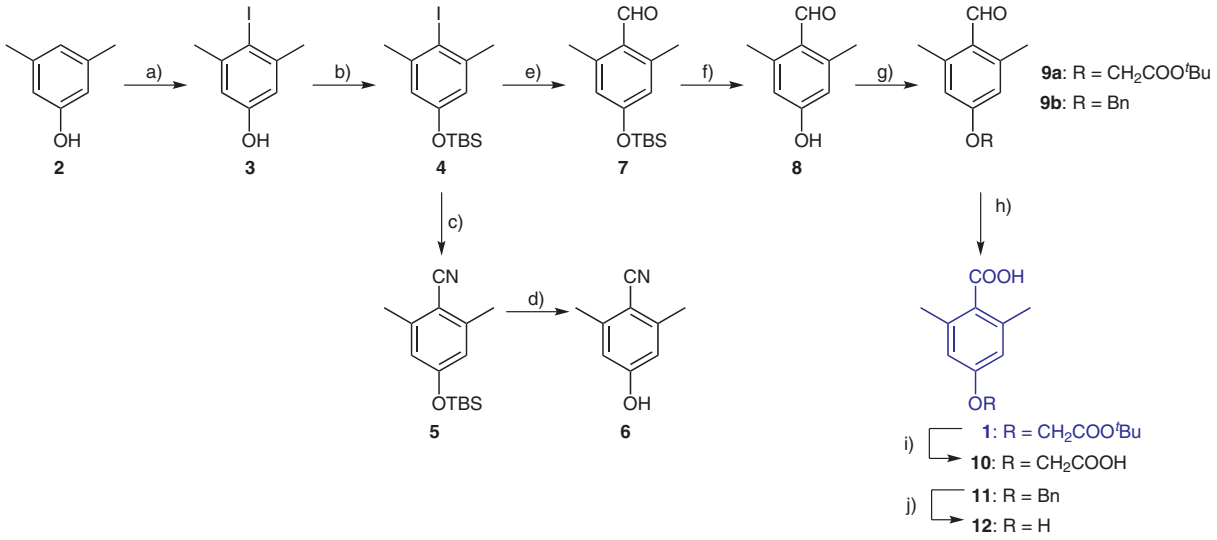
Abstract Peptidyl-acyloxymethyl ketones (AOMKs) belong to a class of selective, irreversible inhibitors (activity-based probes) widely used as chemical tools of investigating proteins, for example, in activity-based protein profiling. The synthesis of the AOMKs has always been challenging and current methodologies involve both solution and solid-phase synthesis. Herein, the synthesis of a new scaffold useful for the preparation of peptidyl-AOMKs is reported and it is demonstrated that the new synthetic probes bearing a 4-functionalized 2,6-dimethylbenzoate efficiently inhibit cysteine proteases like cathepsin B.

Key words acyloxymethyl ketones, activity-based probes, peptidyl-AOMKs, 2,6-dimethylbenzoates, solid-phase peptide synthesis

Activity-based protein profiling (ABPP)[1] is a powerful tool for protein investigation that makes use of synthetic small molecules (ABPs: activity-based probes) able to specifically and covalently bind target proteins in a complex biological mixture. The reactive group of an ABP is usually an electrophilic moiety that is susceptible to the attack of amino acids nucleophilic groups within the protein active site.[2]

The acyloxymethyl ketones (AOMKs), in particular peptidyl-AOMKs, represent a class of compounds that have found great applicability in ABPP, especially due to their selectivity for cysteine proteases versus serine proteases.[2,3] The inactivation mechanism of the cysteine-proteases by peptidyl-AOMK follow a nucleophilic substitution (the thiol group of the active site cysteine attacks the electrophilic carbon of the ketone yielding a S–C covalent bond[2c]).

Dipetidyl-AOMKs were first developed by the group of Krantz[4] as an alternative to the aldehyde- or the halomethyl ketone-based inhibitors for cathepsin B and calpains. These studies established the selectivity of the dipeptidyl-AOMKs for cathepsin B as well as dependency of the inhibition rates on the pK_a of the carboxylate leaving group. At first, the synthesis of the peptidyl-AOMKs was accomplished in solution for short sequences of only two amino acids, by transformation of the carboxyl group into the corresponding diazomethyl ketone, halomethyl ketone and, finally, the acyloxymethyl ketone.[4a] Demand for longer and more complex peptide sequences led to development of solid-phase strategies (SPPS). First method was reported by Mujica and Jung,[5] who developed the synthesis of the aspartyl acyloxymethyl ketone, on carboxy-functionalized resins. This strategy is very convenient for amino acids bearing side-chain functional groups. Later, Ellman et al.[6] developed a solid-phase methodology, which uses the ketone carbonyl group of the halomethyl ketone for direct attachment, through a hydrazone bond, onto a carbazate-linked solid support and further growth of the peptide sequence. Another strategy that proved to be very useful for synthesizing longer peptidyl-AOMKs was developed by Bogyo et al.[3j] namely synthesis of the C-terminal target peptide by SPPS and subsequent solution transformation into the peptidyl-diazomethyl ketone, bromomethyl ketone and peptidyl-AOMK, respectively. Thus, to the best of our knowledge, a general synthetic method for peptidyl-AOMKs is not available. Fmoc-solid-phase synthesis of the peptidyl-AOMKs has always been challenging, on one hand because the benzoate moiety lacks a functional group for the attachment to the solid support and, on the other hand, as a result of the susceptibility to hydrolysis of the ester moiety to the basic conditions required for Fmoc deprotection. However, this latter difficulty has been solved by using diethylamine as deprotecting reagent.[3k]



Scheme 1 Synthesis of compound **1**. *Reagents and conditions*: a) KI, KIO_3, HCl, MeOH, 0 °C, 30 min, r.t., 20 h, 60%; b) *tert*-butyldimethylchlorosilane (TBSCl, 1.1 equiv), imidazole (2.2 equiv), DMF, 0 °C, argon, 30 min, 96%; c) CuCN (1.12 equiv), DMF, reflux, 2 h, 85%; d) i) NaOH (25 equiv), H_2O/EtOH, 1 h, reflux, 80% or ii) HCl, 3 h, reflux, 84%; e) *n*-BuLi (1.4 equiv), THF, –78 °C, DMF (2 equiv), argon, 3 h, 72%; f) KF (1.5 equiv), MeCN/H_2O, 88%; g) *tert*-butyl bromoacetate or benzyl bromide (1.1 equiv), KI (1.1 equiv), K_2CO_3 (1.1 equiv), acetone, r.t., overnight, 70% for **9a** and 42% for **9b**; h) $KMnO_4$ (1.5 equiv), 1,4-dioxane/H_2O, r.t., 4 h, 47% for **1** and 41% for **11**; i) TFA 25% in CH_2Cl_2, quant; j) HBr (33 wt% in AcOH), 40%.

In this context, we report herein the synthesis of 4-substituted 2,6-dimethylbenzoic acids as new scaffolds useful for the preparation of peptidyl-AOMKs and their assays as inhibitors of cysteine proteases. These probes can be synthesized using both solution and SPSS strategies since the attachment to the solid support was designed to occur through derivatization in position 4 of the benzoate moiety. Enzyme inhibition experiments using cathepsin B[7] were performed in order to investigate whether this modification of the benzoate moiety influence the interaction with the active site of the protein.

Our design for a new AOMK scaffold that derives from 4-hydroxy-2,6-dimethylbenzoic acid was based on the hypothesis that modification of the 2,6-disubstituted benzoate moiety will not affect the inhibition efficiency.[4a] Therefore, we designed compound **1** (Scheme 1) to contain a *tert*-butyl protected carboxyl group which, after grafting on any amino acid and deprotection, enables covalent attachment on the solid-support and the subsequent possibility to grow any peptide sequence.

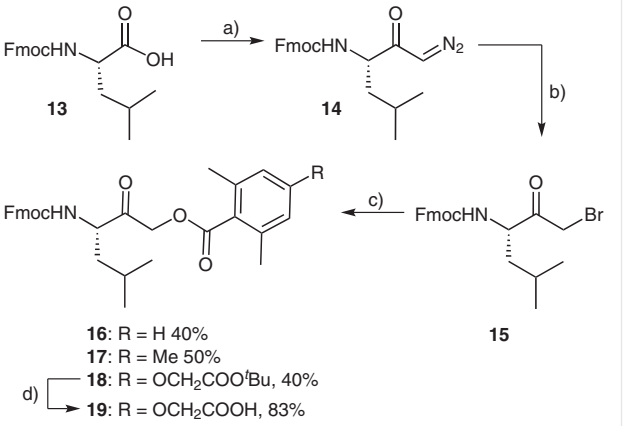
Synthesis of compound **1** was achieved through a six-step strategy, starting from 3,5-dimethylphenol (Scheme 1). Iodination[8] of the phenol **2** led to the corresponding iodo derivative **3** in 60% yield that was further treated[9] with *tert*-butyldimethylchlorosilane to provide the protected phenol **4** in 96% yield. Initially, we attempted to introduce the carboxyl group through an *ipso* substitution[10] of the iodine with the cyano group (compound **5**, 85%) and further hydrolysis of the nitrile. All efforts to hydrolyze the nitrile group failed either under basic or acidic conditions. In all cases, the reaction occurred only with the deprotection of the TBS group and the 2,6-dimethyl-4-hydroxybenzonitrile (**6**) was recovered (see the Supporting Information).

Therefore, our attention was turned towards introduction of the formyl group, taking into account that TBS acts as an efficient protecting group for phenol group in both lithiation and formylation.[9] Unlike previous reported procedures, which starts from the corresponding bromo derivative, in the presence of *s*-BuLi or MeLi-LiBr complex, the preparation of the aldehyde **8** was optimized to successfully use *n*-BuLi. Thus, use of the readily available iodo derivative **4** affords employment of more convenient *n*-BuLi as lithiation reagent (1.4 equiv) to enable formation of the product **7** in very good yields, without detection of any by-products.[9] Deprotection of the TBS group provided the desired aldehyde **8** in 88% yield.

In order to avoid selectivity issues, aldehyde **8** was first reacted with *tert*-butyl bromoacetate or benzyl bromide, leading to the esters **9**, which were further oxidized to the carboxylic derivatives **1** and **11**. Screening through the well-known oxidizing agents for efficient oxidation of the aldehydes to carboxylic acids indicated as the optimum choice the 'classical' potassium permanganate (1.5 equiv) in a water/1,4-dioxane mixture. This procedure provided the target compounds in 47% (for **1**) and 41% (for **11**) and complete recovery of the unreacted aldehydes. No oxidation of the methyl groups was detected when the reaction was performed at room temperature for 4 hours.

The attempt to perform the oxidation reaction of **9a** using Oxone[11] as the oxidizing agent led to the formate ester (see the Supporting Information), while the same reaction

performed with silver(I) oxide resulted in the deprotection of the *tert*-butyl ester. Diacid **10** was obtained by stirring compound **1** with a solution of 25% TFA in dichloromethane in 83% yield, while the benzyl group in compound **11** was removed using HBr 33 wt% solution in acetic acid.



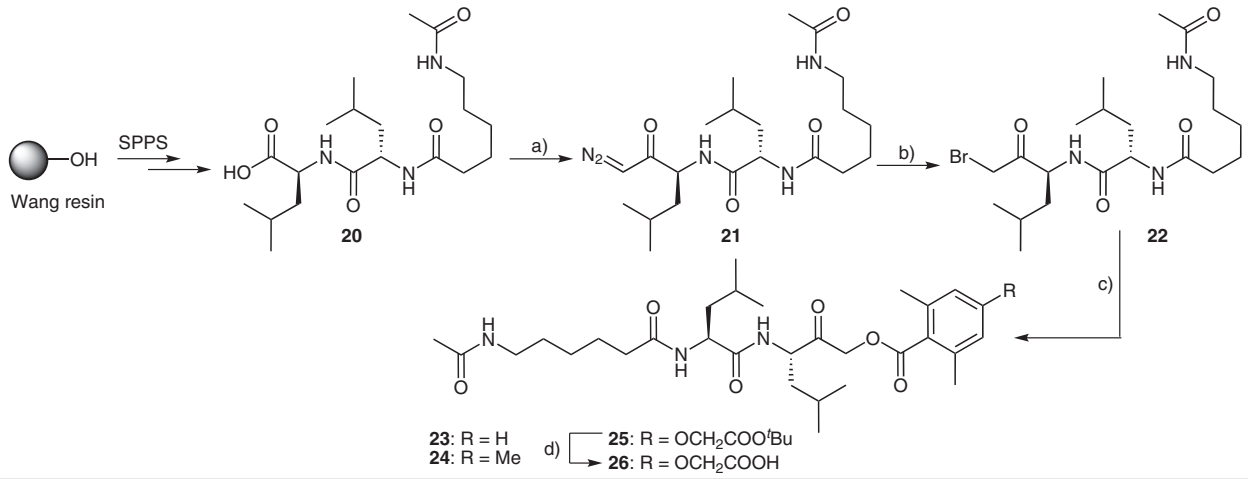
Scheme 2 Synthesis of compounds **16**–**19**. *Reagents and conditions*: a) i) isobutyl chloroformate, *N*-methylmorpholine, –10 °C, 30 min; ii) CH_2N_2/Et_2O (in situ), 0 °C, 3 h, 70%; b) HBr (33 wt% in AcOH), THF, 0 °C, 15 min, 88%; c) KF, MeCN, r.t., 12 h and i) for compound **16**: 2,6-dimethylbenzoic acid, 40%, ii) for compound **17**: 2,4,6-trimethylbenzoic acid, 50%, iii) for compound **18**: acid **1**, 40%; d) TFA (25% in CH_2Cl_2), 83%.

Next, Fmoc-Leu-OH (**13**) (Scheme 2) was chosen as model amino acid to perform the transformation of the carboxyl group into the AOMK moieties through a previously described three-step strategy.[4a] Preparation of the diazomethyl ketone **14** was followed by conversion into the bromo derivative **15** and subsequent synthesis of the acyloxymethyl ketones **16**–**18**. Compound **18** was treated with TFA to enable the formation of **19** in 83% yield. Compounds **16**, **17**, and **19** were tested for their capacity to inhibit cathepsin B activity at high concentration (1 mM) indicating a similar inhibitory activity (data not shown).

Further, in order to improve the efficiency of the cathepsin B inhibition, the peptidyl-AOMK probes **23**, **24**, and **26** were synthesized (Scheme 3). Thus, the probes were obtained in solution by reaction between 2,6-dimethylbenzoic acid, 2,4,6-trimethylbenzoic acid, or compound **1**, respectively, and the peptidyl bromomethyl ketone **22**. Peptide **22** was prepared from the corresponding diazomethyl ketone **21**, which was, in its turn, obtained from peptide **20**. The N-acetylated peptide **20** was synthesized by classical SPPS protocol, using Wang resin, in 90% yield (Scheme 3).

To confirm our hypothesis that the modification in position 4 of the benzoate moiety does not have negative impact on the inhibition efficiency, the IC50 values were determined for the probes **20**, **23**, **24**, and **26** against cathepsin B. The biochemical assays were performed as described in the experimental section by fluorescence measurements, using the fluorogenic substrate N^{α}-CBZ-Arg-Arg-7-amido-4-methylcoumarin. As expected, probe **20** had no inhibitory activity at the maximum concentration tested (20 µM), while the IC50 of probe **23** was found to be 7 µM (Table 1). The inhibitory efficiency significantly improved for probes **24** and **26** down to 0.7 µM. Thus, we have demonstrated that substitution in position 4 of the 2,4-dimethylbenzoate scaffold with a carboxymethyloxy residue does not affect the biological activity of the probe.

We recently reported[12] a solid-phase strategy for the synthesis of fluoromethyl ketones (FMKs) based on the functionalization of the FMK amino acid, with a bifunctional linker, through a ketalization reaction of the ketone carbonyl that enables attachment to the solid support and sub-



Scheme 3 Synthesis of acyloxymethyl ketones **23**–**26**; yield of precursor **20** by SPPS: 90%. *Reagents and conditions*: a) i) isobutyl chloroformate, *N*-methylmorpholine, –10 °C, 30 min; ii) CH_2N_2/Et_2O (in situ), 0 °C, 3 h, 47%; b) HBr (33 wt% in AcOH), THF, 0 °C, 15 min, 30%; c) 2,6-dimethylbenzoic acid or 2,4,6-trimethylbenzoic acid or compound **1**, respectively, KF, MeCN, r.t., 12 h, 91% for **23**, 85% for **24**, and 50% for **25**; d) TFA 25% in CH_2Cl_2, 57%.

Table 1 Inhibition of Cathepsin B by AOMKs **20**, **23**, **24**, **26**

Compound	IC50
20	>20 μM
23	7 μM
24	0.7 μM
26	0.7 μM

sequent growth of the required peptide sequence. Hence, we approached this strategy for the preparation of the peptidyl-AOMKs and attempted to synthesize a similar derivative of **16** through use of the carbonyl ketone and a bifunctional linker (see Scheme S1 in the Supporting Information). We used 2,2-bis(hydroxymethyl)propanoic acid as the starting material, transformed the hydroxyl groups into silyl ethers (compound **28** in the Supporting Information) and attempted to perform the ketalization reaction, in the presence of TMSOTf, under anhydrous and inert conditions. The reaction failed most probably due to the sterical hindrance brought by the 2,6-dimethylbenzoate scaffold or the amino acid bulky side-chain moiety. To clarify this point, the corresponding Gly-AOMK was synthesized (compound **27** in the Supporting Information) and subjected to the ketalization reaction with the 2,2-bis(hydroxymethyl)propanoic acid silyl ether in the same conditions. Failure of this reaction indicated that the bulky AOMK residue prevents closure of the 1,3-dioxane ring, unlike the much smaller fluorine atom in the case the fluoromethyl ketone.

In conclusion, we have described synthesis of a new functionalized 2,6-dimethylbenzoate scaffold useful to prepare peptidyl-AOMKs. The synthetic strategy starts from simple and commercially available reagents and reactants and furnishes the *tert*-butyl ester of the 4-substituted 2,6-dimethylbenzoate moiety that is acid sensitive and can be easily deprotected. Graft of this scaffold on amino acids or peptidyl sequences was further performed and the probes were assayed against cathepsin B to evaluate the inhibitory efficiency. The IC50 value of the new probe is similar to the well encountered C-terminal peptidyl derived 2,6-dimethylbenzoate or 2,4,6-trimethylbenzoate, providing, thus, evidence that our new 4-functionalized 2,6-dimethylbenzoate moiety does not interfere in the reaction.

The air- and water-sensitive reactions were performed using anhyd solvents and inert atmosphere. Anhyd THF was distilled over Na and benzophenone. All other commercial solvents and reagents were used without further purification. NMR spectra were recorded on Bruker NMR spectrometers operating at 300 MHz or 500 MHz for ^{1}H and 75 MHz or 125 MHz for ^{13}C. Chemical shifts (δ) are reported in parts per million (ppm) using residual solvent peak as internal reference. High-resolution mass spectra were recorded on a ThermoScientific (LTQ XL Orbitrap) spectrometer using APCI or ESI technique and Orbital Ion Trap mass analyzer. TLC was performed on silica gel 60 coated aluminum F254 plates. All plates were visualized by UV irradiation at 254 nm. Preparative column chromatography was performed on Merck silica gel 60 (0.040–0.063 mm).

For the preparation of aldehyde **8**, see the Supporting Information.

tert-Butyl 2-(4-Formyl-3,5-dimethylphenoxy)acetate (9a)

Compound **8** (1.5 g, 10 mmol) was dissolved in acetone (30 mL), and K_2CO_3 (1.52 g, 11 mmol), KI (1.83 g, 11 mmol), and *tert*-butyl bromoacetate (1.63 mL, 11 mmol) were added. The reaction mixture was allowed to stir at r.t. overnight and the solvent was removed in vacuo. The residue was partitioned between H_2O and EtOAc. The organic layer was washed with H_2O, brine and dried (anhyd $MgSO_4$). Column chromatography yielded 1.8 g (70%) of the pure product **9a** as a colorless oil; R_f = 0.53 (silica gel, EtOAc/hexane = 1:4).

^{1}H NMR (300 MHz, $CDCl_3$): δ = 10.47 (s, 1 H, CHO), 6.57 (s, 2 H, H_{Ar}), 4.55 (s, 2 H, CH_2), 2.59 (s, 6 H, CH_3), 1.48 (s, 9 H, *t*-C_4H_9).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 191.8, 167.5, 161.1, 144.7, 126.8, 115.4, 82.9, 65.4, 28.2, 21.2.

HRMS (APCI+): *m*/*z* [M + H]$^+$ calcd for $C_{15}H_{21}O_4$: 265.1434; found: 265.1433.

4-(Benzyloxy)-2,6-dimethylbenzaldehyde (9b)

Compound **9b** was synthesized starting from aldehyde **8** (240 mg, 1.6 mmol) using benzyl bromide (2 mL, 1.68 mmol) following the same experimental procedure as described for **9a**, and purified by column chromatography; yield: 160 mg (42%); white solid; mp 68–70 °C; R_f = 0.57 (silica gel, EtOAc/PE = 1:4).

^{1}H NMR (300 MHz, $CDCl_3$): δ = 10.48 (s, 1 H, CHO), 7.40–7.42 (overlapped peaks, 3 H, H_{Ar}), 7.35–7.38 (m, 2 H, H_{Ar}), 6.68 (s, 2 H, H_{Ar}), 5.11 (s, 2 H, CH_2), 2.61 (s, 6 H, CH_3).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 191.8, 162.0, 144.6, 136.4, 128.84, 128.4, 127.6, 126.3, 115.8, 70.0, 21.3.

HRMS (APCI+): *m*/*z* [M + H]$^+$ calcd for $C_{16}H_{17}O_2$: 241.1223; found: 241.1239.

4-[2-(*tert*-Butoxy)-2-oxoethoxy)-2,6-dimethylbenzoic Acid (1)

Aldehyde **9a** (500 mg, 1.9 mmol) was dissolved in 1,4-dioxane (1 mL), and $KMnO_4$ (360 mg, 2.27 mmol) in H_2O (10 mL) was added. The reaction mixture was allowed to stir at r.t. for 3 h while monitoring the reaction by TLC. The mixture was filtered, the brown solid thoroughly washed with H_2O, and acidified. The aqueous phase was extracted with EtOAc, the organic layer was washed with brine, and dried (anhyd $MgSO_4$). Evaporation of the solvent in vacuo afforded 200 mg (47%) of pure white solid; mp 102–104 °C; R_f = 0.51 (silica gel, EtOAc/PE = 1:1).

^{1}H NMR (300 MHz, $CDCl_3$): δ = 6.58 (s, 2 H, H_{Ar}), 4.51 (s, 2 H, CH_2), 2.42 (s, 6 H, CH_3), 1.48 (s, 9 H, *t*-C_4H_9).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 173.8, 167.9, 159.0, 139.3, 125.1, 114.3, 82.7, 65.6, 28.1, 21.2.

HRMS (ESI–): *m*/*z* [M – H]$^-$ calcd for $C_{15}H_{19}O_5$: 279.1227; found: 279.1234.

4-(Carboxymethoxy)-2,6-dimethylbenzoic Acid (10)

Compound **1** (0.48 g, 1.71 mmol) was dissolved in 25% TFA in CH_2Cl_2 (1 mL) and the solution was stirred at r.t. for 5 h. The resulting white precipitate was filtered, washed with CH_2Cl_2, and dried; yield: 375 mg (quant) as a white solid; mp 203–205 °C; R_f = 0.2 (silica gel, EtOAc/PE = 1:1).

^{1}H NMR (500 MHz, $CDCl_3$): δ = 6.62 (s, 2 H, H_{Ar}), 4.67 (s, 2 H, CH_2), 2.24 (s, 6 H, CH_3).

^{13}C NMR (125 MHz, $CDCl_3$): δ = 170.5, 170.1, 157.6, 136.0, 128.2, 113.3, 64.2, 19.8.

HRMS (ESI–): *m*/*z* [M – H]$^-$ calcd for $C_{11}H_{11}O_5$: 223.0601; found: 223.0613.

4-(Benzyloxy)-2,6-dimethylbenzoic Acid (11)

Compound **11** was synthesized from **9b** (140 mg, 0.58 mmol) using the same procedure as described for acid **1** and was obtained in 41% yield (50 mg); white solid; mp 109–110 °C; R_f = 0.13 (silica gel, EtOAc/PE = 1:2).

^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.34–7.44 (overlapped peaks, 5 H, H_{Ar}), 6.69 (s, 2 H, H_{Ar}), 5.07 (s, 2 H, CH_2), 2.45 (s, 6 H, CH_3).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 174.6, 159.9, 139.4, 136.7, 128.8, 128.2, 127.6, 124.6, 114.6, 69.9, 21.3.

HRMS (ESI–) *m*/*z* [M – H]$^-$ calcd for $C_{16}H_{15}O_3$: 255.1016; found: 255.1023.

4-Hydroxy-2,6-dimethylbenzoic Acid (12)

Compound **11** (180 mg, 1.08 mmol) was dissolved in HBr (33 wt% in AcOH, 3.5 mL) and left to react at r.t. for 1 h. H_2O was added and the aqueous phase was extracted with EtOAc (3 × 10 mL). The combined organic phases were washed with brine, dried (anhyd $MgSO_4$), and evaporated in vacuo. The residue was chromatographed (using gradient of 25% of PE in EtOAc until 100% EtOAc to yield 70 mg (40%) of **12**; white solid; mp 168–170 °C; R_f = 0.41 (silica gel, EtOAc/PE = 1:2).

^{1}H NMR (500 MHz, CD_3OD): δ = 6.45 (s, 2 H, H_{Ar}), 2.28 (s, 6 H, CH_3).

^{13}C NMR (125 MHz, CD_3OD): δ = 174.1, 159.2, 138.2, 127.2, 115.4, 20.3.

HRMS (ESI–): *m*/*z* [M – H]$^-$ calcd for $C_9H_9O_3$: 165.0546; found: 165.0559.

(*S*)-(9*H*-Fluoren-9-yl)methyl (1-Diazo-5-methyl-2-oxohexan-3-yl)carbamate (14)

To a solution of Fmoc-Leu-OH (**13**; 5.3 g, 15 mmol) in anhyd THF (50 mL) at –10 °C were added *N*-methylmorpholine (2.1 mL, 18.75 mmol) and isobutyl chloroformate (2.25 mL, 17.25 mmol). The mixture was stirred at –10 °C for 30 min, then a solution of diazomethane in Et_2O (15–20 mmol, generated in situ from ethereal α-nitrozomethylurea[13] and dried over solid KOH) was added at 0 °C. The mixture was further stirred for 3 h at r.t. and quenched with H_2O (15 mL) and AcOH (1.5 mL). The mixture was diluted with EtOAc (50 mL), washed with H_2O, brine, and sat. aq $NaHCO_3$. The organic layer was dried (anhyd $MgSO_4$) and the solvent removed in vacuo. Column chromatography on silica gel furnished pure product **14** as a light-yellow oil in 70% yield (2.8 g); R_f = 0.26 (EtOAc/pentane = 1:3).

^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.77 (d, ^3J = 8.0 Hz, 2 H, H_{Ar}-Fmoc), 7.59 (dd, $^3J_1$ = 8.0 Hz, 2 H, H_{Ar}-Fmoc), 7.40 (t, ^3J = 8.0 Hz, 2 H, H_{Ar}-Fmoc), 7.31 (t, ^3J = 8.0 Hz, 2 H, H_{Ar}-Fmoc), 5.31 (s, 1 H, CH=N_2), 4.40–4.52 (m, 2 H, CH_2-Fmoc), 4.17–4.25 (overlapped peaks, 2 H, C*H*-Fmoc, NHC*H*), 1.41–1.74 [overlapped peaks, 3 H, C*H*(CH_3), CH_2CH(CH_3)$_2$], 0.94 [d, ^3J = 8.0 Hz, 6 H, (CH_3)$_2$CH].

^{13}C NMR (75 MHz, $CDCl_3$): δ = 194.4, 156.1, 143.8, 141.4, 127.8, 127.2, 125.2, 125.1, 120.1, 66.8, 54.5, 47.4, 41.4, 24.8, 23.2.

HRMS (ESI+): *m*/*z* [M + H – N_2]$^+$ calcd for $C_{22}H_{24}N_3O_3$: 350.1751; found: 350.1740.

(*S*)-(9*H*-Fluoren-9-yl)methyl 1-(2-Bromoacetyl)-3-methylbutylcarbamate (15)

Diazomethyl ketone **14** (3.2 g, 8.5 mmol) was dissolved in THF (25.5 mL) and the solution was cooled to 0 °C. A mixture of HBr (33 wt% in AcOH and H_2O in a 1:2 volumetric ratio) was added dropwise. The reaction mixture was allowed to stir until no evolution of gas was observed (10–15 min) and diluted with EtOAc (20 mL). The organic layer was washed with H_2O, brine, and sat. aq $NaHCO_3$. The organic layer was dried (anhyd $MgSO_4$) and the solvent removed in vacuo to yield the product as a pure white solid in 88% yield (3.2 g); mp 112–113 °C; R_f = 0.65 (silica gel, EtOAc/pentane = 1:3).

^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.77 (d, ^3J = 8.0 Hz, 2 H, H_{Ar}-Fmoc), 7.59 (d, ^3J = 8.0 Hz, 2 H, H_{Ar}-Fmoc), 7.41 (t, ^3J = 8.0 Hz, 2 H, H_{Ar}-Fmoc), 7.32 (t, ^3J = 8.0 Hz, 2 H, H_{Ar}-Fmoc), 5.13 (s, 1 H, CONH), 4.59–4.64 (m, 1 H, NHC*H*), 4.42–4.53 (m, 2 H, CH_2-Fmoc), 4.21 (t, ^3J = 8.0 Hz, 1 H, C*H*-Fmoc), 4.02 (d, ^2J = 12.0 Hz, 1 H, C*H*HBr), 3.96 (d, ^2J = 12.0 Hz, 1 H, C*H*HBr), 1.60–1.66 [overlapped peaks, 2 H, C*HH*CH(CH_3)$_2$], 1.40–1.48 [m, 1 H, C*H*(CH_3)], 0.97 [d, ^3J = 8.0 Hz, 3 H, (CH_3)$_2$CH], 0.95 [d, ^3J = 8.0 Hz, 3 H, (CH_3)$_2$CH].

^{13}C NMR (100 MHz, $CDCl_3$): δ = 201.4, 158.5, 143.6, 141,4, 127.8, 127.1, 124.9, 120.1, 66.8, 56.4, 47.3, 40.7, 30.9, 24.9, 23.2.

HRMS (ESI+): *m*/*z* [M + H]$^+$ calcd for $C_{22}H_{25}BrNO_3$: 430.1012; found: 430.1002.

(*S*)-3-({[(9*H*-Fluoren-9-yl)methoxy]carbonyl}amino)-5-methyl-2-oxohexyl 2,6-Dimethylbenzoate (16)

Bromomethyl ketone **15** (617 mg, 1.4 mmol) was dissolved in MeCN (10 mL) and the solution was cooled in an ice bath. KF (244 mg, 4.2 mmol) and 2,6-dimethylbenzoic acid (258 mg, 1.72 mmol) were added and the mixture was stirred overnight at r.t. The solvent was removed and the residue was dissolved in EtOAc. The organic layer was washed with H_2O, brine, and sat. aq $NaHCO_3$, dried (anhyd $MgSO_4$), and the solvent removed in vacuo. Column chromatography furnished the pure product as a white solid in 40% yield (292 mg); mp 104–105 °C; R_f = 0.43 (silica gel, EtOAc/pentane = 1:5).

^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.96 [d, ^3J = 3.6 Hz, 6 H, (CH_3)$_2$CH], 1.47–1.54 [m, 1 H, C*H*(CH_3)], 1.66–1.72 [overlapped peaks, 2 H, C*H*-*H*CH(CH_3)$_2$], 2.40 (s, 6 H, CH_3-AOMK), 4.23 (t, ^3J = 6.4 Hz, 1 H, C*H*-Fmoc), 4.41–4.52 (overlapped peaks, 2 H, C*HH*-Fmoc), 4.56–4.70 (m, 1 H, NHC*H*), 4.96 (d, ^2J = 17.0 Hz, 1 H, CH*H*OCO), 5.05 (d, ^2J = 17.0 Hz, 1 H, CH*H*OCO), 5.15 (d, ^3J = 8.1 Hz, CONH), 7.05 (d, ^3J = 7.6 Hz, 2 H, H_{Ar}-AOMK), 7.21 (t, ^3J = 7.6 Hz, 1 H, H_{Ar}-AOMK), 7,32 (t, ^3J = 7.6 Hz, 2 H, H_{Ar}-Fmoc), 7,40 (t, ^3J = 7.6 Hz, 2 H, H_{Ar}-Fmoc), 7.60 (d, ^3J = 7.6 Hz, 2 H, H_{Ar}-Fmoc), 7.77 (d, ^3J = 7.6 Hz, 2 H, H_{Ar}-Fmoc).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 202.8, 169.1, 156.2, 143.8, 141.5, 135.8, 132.6, 129.8, 127.9, 127.8, 127.2, 125.2, 125.1, 120.2, 67.1, 66.7, 55.9, 47.4, 40.4, 24.7, 23.2, 20.1.

HRMS (ESI+): *m*/*z* [M + Na]$^+$ calcd for $C_{31}H_{33}NO_5Na$: 522.2251; found: 522.2235.

(*S*)-3-({[(9*H*-Fluoren-9-yl)methoxy]carbonyl}amino)-5-methyl-2-oxohexyl 2,4,6-Trimethylbenzoate (17)

Compound **17** was synthesized following the same experimental procedure as described for **16**, using bromomethyl ketone **15** (30 mg, 70 μmol) and 2,4,6-trimethylbenzoic acid (14 mg, 84 μmol) to yield 17 mg (50%) of **17** as a white solid; R_f = 0.34 (silica gel, EtOAc/PE = 1:4).

^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.76 (d, ^3J = 7.2 Hz, 2 H, H_{Ar}-Fmoc), 7.60 (d, ^3J = 7.2 Hz, 2 H, H_{Ar}-Fmoc), 7.40 (t, ^3J = 7.2 Hz, 2 H, H_{Ar}-Fmoc), 7.32 (t, ^3J = 7.2 Hz, 2 H, H_{Ar}-Fmoc), 6.87 (s, 2 H, H_{Ar}-AOMK), 5.15 (d, ^3J = 7.7 Hz, CONH), 5.03 (d, ^2J = 17.0 Hz, 1 H, CH*H*OCO), 4.94 (d, ^2J = 17.0 Hz, 1

H, CHHOCO), 4.43–4.51 (m, 3 H, CH_2-Fmoc, NHCH), 4.22 (t, ^3J = 6.5 Hz, 1 H, CH-Fmoc), 2.40 (s, 6 H, CH_3-AOMK), 2.29 (s, 3 H, CH_3-AOMK), 1.67–1.72 [m, 2 H, $CH_2CH(CH_3)_2$], 1.49–1.54 [m, 1 H, $CH(CH_3)$], 0.96 [d, ^3J = 2.5 Hz, 6 H, $(CH_3)_2CH$].

^{13}C NMR (125 MHz, $CDCl_3$): δ = 202.9, 169.2, 156.2, 143.9, 141.5, 139.9, 136.0, 129.6, 128.6, 127.9, 127.3, 127.2, 125.2, 125.1, 120.1, 67.0, 66.7, 55.9, 47.4, 40.5, 24.8, 23.4, 21.3, 20.1.

HRMS (ESI+): *m*/*z* [M + Na]$^+$ calcd for $C_{32}H_{35}NO_5Na$:536.2407; found: 536.2418.

(*S*)-3-({[(9*H*-Fluoren-9-yl)methoxy]carbonyl}amino)-5-methyl-2-oxohexyl 4-[2-(*tert*-Butoxy)-2-oxoethoxy]-2,6-dimethylbenzoate (18)

Compound **18** was synthesized following the same experimental procedure as described for **16**, using bromomethyl ketone **15** (100 mg, 0.23 mmol) and acid **1** (82 mg, 0.28 mmol) to yield 60 mg (40%) of a white solid; mp 179–180 °C; R_f = 0.5 (silica gel, EtOAc/PE = 1:4).

^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.76 (d, ^3J = 7.4 Hz, 2 H, H_{Ar}-Fmoc), 7.58 (d, ^3J = 7.4 Hz, 2 H, H_{Ar}-Fmoc), 7.40 (t, ^3J = 7.4 Hz, 2 H, H_{Ar}-Fmoc), 7.31 (t, ^3J = 7.4 Hz, 2 H, H_{Ar}-Fmoc), 6.57 (s, 2 H, H_{Ar}-AOMK), 5.17 (d, ^2J = 8.2 Hz, 1 H, NH), 5.03 (d, ^2J = 17.0 Hz, 1 H, CHHCOO), 4.93 (d, ^2J = 17.0 Hz, 1 H, CHHCOO), 4.49 (s, 2 H, $COCH_2OCO$), 4.44–4.65 [overlapped peaks, 3 H, CH_2-Fmoc, $CHCH_2CH(CH_3)_2$], 4.21 (t, ^2J = 6.4 Hz, 1 H, CH-Fmoc), 2.39 (s, 6 H, CH_3-AOMK), 1.65–1.71 (m, 2 H, $CH_2CH(CH_3)_2$), 1.49–1.60 [m, 1 H, $CH(CH_3)_2$], 1.49 (s, 9 H, *t*-C_4H_9), 0.95 [d, ^3J = 5.3 Hz, 6 H, $(CH_3)_2CH$].

^{13}C NMR (75 MHz, $CDCl_3$): δ = 202.9, 168.7, 167.8, 158.7, 156.2, 143.8, 141.5, 138.8, 127.8, 127.2, 125.7, 125.2, 125.1, 114.0, 82.6, 67.0, 66.7, 65.6, 55.7, 47.4, 40.5, 24.8, 23.4, 21.5, 20.7.

HRMS (ESI–): *m*/*z* [M – H – *t*-Bu]$^-$ calcd for $C_{37}H_{34}NO_8$: 572.2279; found: 572.2278.

(*S*)-2-[4-({[3-({[(9*H*-Fluoren-9-yl)methoxy]carbonyl}amino)-5-methyl-2-oxohexyl]oxy}carbonyl)-3,5-dimethylphenoxy]acetic Acid (19)

Compound **18** (50 mg, 0.08 mmol) was dissolved in 25% TFA in CH_2Cl_2 (3 mL). The reaction mixture was allowed to stir at r.t. until complete conversion of the substrate. The solvent was removed in vacuo and the product was passed through a short pad of silica gel (CH_2Cl_2/PE = 1:6) to yield the pure product as a white solid in 83% yield (34 mg).

^{1}H NMR (500 MHz, DMSO-d_6): δ = 7.89 (d, ^3J = 8.0 Hz, 2 H, H_{Ar}-Fmoc), 7.79 (d, ^3J = 8.0 Hz, 1 H, NH), 7.70 (d, ^3J = 8.0 Hz, 2 H, H_{Ar}-Fmoc), 7.41 (t, ^3J = 8.0 Hz, 2 H, H_{Ar}-Fmoc), 7.32 (t, ^3J = 8.0 Hz, 2 H, H_{Ar}-Fmoc), 6.53 (s, 2 H, H_{Ar}-AOMK), 5.01 (d, ^2J = 17.0 Hz, 1 H, CHHOCO), 4.93 d, ^2J = 17.0 Hz, 1 H, CHHOCO), 4.38–4.46 (overlapped peaks, 2 H, CHH-Fmoc), 4.24 (t, ^3J = 6.6 Hz, 1 H, CH-Fmoc), 4.13–4.17 (overlapped peaks, 3 H, CHNH, CH_2CO_2H), 2.26 (s, 6 H, CH_3-AOMK), 1.58–1.63 [m, 1 H, $CH(CH_3)$], 1.49–1.52 [overlapped peaks, 2 H, $CHHCH(CH_3)_2$], 0.88 (d, ^3J = 6.5 Hz, 3 H, $(CH_3)_2CH$], 0.84 [d, ^3J = 6.5 Hz, 3 H, $(CH_3)_2CH$].

^{13}C NMR (125 MHz, DMSO-d_6): δ = 203.8, 170.8, 168.2, 159.8, 156.3, 143.8, 140.9, 137.2, 127.7, 127.4, 127.1, 125.3, 124.1, 120.2, 113.8, 67.5, 66.6, 65.4, 56.3, 46.9, 38.2, 24.1, 23.2, 21.1.

HRMS (ESI–): *m*/*z* [M – H]$^-$ calcd for $C_{33}H_{34}NO_8$: 572.2279; found: 572.2279.

Peptide 20

Peptide **20** was synthesized following the standard SPPS procedure on Wang resin. 1.5 g Wang resin (loading 0.9 mmol/g, 1 equiv) swelled in DMF was followed by coupling of each Fmoc-protected amino acid (5.4 mmol, 4 equiv) using PyBOP (5.4 mmol, 4 equiv) and HOBt (5.4 mmol, 4 equiv) as coupling activating reagents and DIPEA (12 equiv) as base dissolved in DMF. Deprotection of Fmoc group was achieved using piperidine and the coupling efficiency was checked by Kaiser test. Cleavage of the peptide was performed using a standard cleavage cocktail (95% TFA, 2.5% H_2O, and 2.5% TIS). Obtained as a white solid; overall yield: 1.43 g (90%).

HRMS (APCI+): *m*/*z* [M + H]$^+$ calcd for $C_{20}H_{38}N_3O_5$: 400.2806; found: 400.2822.

Peptidyl-Diazomethyl Ketone 21

Peptidyl-diazomethyl ketone **21** was synthesized following the same experimental procedure as described for **14** from peptide **20** (200 mg, 0.5 mmol) to yield 100 mg (47%) of the pure product as a yellow oil after purification by column chromatography (silica gel, starting with EtOAc/MeOH = 99:1 and increasing the gradient up to 100% MeOH).

HRMS (ESI+): *m*/*z* [M + Na]$^+$ calcd for $C_{21}H_{37}N_5O_4Na$: 446.2738; found [M + Na]$^+$ 446.2767, [M – N_2]$^+$ 396.2857.

Peptidyl-Bromethyl Ketone 22

Peptidyl-bromomethyl ketone **22** was synthesized following the same experimental procedure as described for **15** from peptide **21** (100 mg, 0.23 mmol) to yield 32 mg (30%) of the pure product as a yellow oil.

HRMS (ESI+): *m*/*z* [M + H]$^+$ calcd for $C_{21}H_{39}BrN_3O_4$: 476.2118; found: 476.2120.

Peptidyl-Acyloxymethyl Ketone 23

Peptidyl-acyloxymethyl ketone **23** was synthesized starting from peptidyl-bromomethyl ketone **22** (5 mg, 10.5 µmol) using 2,6-dimethylbenzoic acid (2 mg, 12.6 mmol) following the same experimental procedure as described for **16** to yield 5.2 mg (91%) of the pure product as a colorless oil.

HRMS (ESI+): *m*/*z* [M + H]$^+$ calcd for $C_{30}H_{48}N_3O_6$: 546.3535; found: 546.3538.

Peptidyl-Acyloxymethyl Ketone 24

Peptidyl-acyloxymethyl ketone **24** was synthesized starting from peptidyl-bromomethyl ketone **22** (5 mg, 10.5 µmol) using 2,4,6-trimethylbenzoic acid (2 mg, 12.6 mmol) following the same experimental procedure as described for **16** to yield 5 mg (85%) of the pure product as a colorless oil.

HRMS (ESI+): *m*/*z* [M + H]$^+$ calcd for $C_{31}H_{50}N_3O_6$: 560.3694; found: 560.3709.

Peptidyl-Acyloxymethyl Ketone 25

Peptidyl-acyloxymethyl ketone **25** was synthesized starting from peptidyl-bromomethyl ketone 22 (10 mg, 21 µmol) using acid **1** (7.4 mg, 25.2 µmol) following the same experimental procedure as described for **16** to yield 7 mg (50%) of the pure product as a colorless oil.

HRMS (ESI+): *m*/*z* [M + H]$^+$ calcd for $C_{36}H_{58}N_3O_9$: 676.4168; found: 676.4139.

Peptidyl-Acyloxymethyl Ketone 26

Peptide **25** (7 mg, 10 µmol) was dissolved in a 25% solution of TFA in CH_2Cl_2 (1 mL). The reaction mixture was allowed to stir at r.t. overnight and the solvent was evaporated to yield 3.5 mg (57%) of the pure product as a colorless oil.

HRMS (ESI+): *m*/*z* [M + H]$^+$ calcd for $C_{32}H_{50}N_3O_9$: 620.3542; found: 620.3511.

Enzyme Inhibition Assays

Cathepsin B (0.1 U) from bovine spleen (Sigma) was incubated for 30 min at 37 °C with various concentrations of compounds **20**, **23**, **24**, and **26**, respectively, in the reaction buffer (NaOAc 50 mM, pH = 5, EDTA 2.5 mM, β-mercaptoethanol 10 mM, Tween 20 0.1%, DMSO 1%). Following compound incubation, the fluorogenic substrate N^{α}-CBZ-Arg-Arg-7-amido-4-methylcoumarin was added at a final concentration of 10 μM. The reaction was further incubated for 5 min at 37 °C. Fluorescence measurements (λ_{ex} = 360 nm, λ_{em} = 460 nm) were performed and the data were fitted to a sigmoidal 4 parameters curve. IC50 values (Table 1) were determined using the GraphPad Prism software.

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Supporting Information

Supporting information for this article is available online at http://dx.doi.org/10.1055/s-0035-1562781.

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