A Levulinoyl Ester-Based Cleavable Linker for Activity-Based Protein Profiling


7.1 Introduction

Activity-based protein profiling has become an attractive strategy to report on enzyme activity in the context of complex biological samples (see Chapter 1).\textsuperscript{1,2} An activity-based profiling probe (ABP) normally consists of a recognition element targeted at a specific enzyme (family), and is equipped with a reactive group through which the targeted enzymes are covalently and irreversibly modified. Enzymes that use an amino acid side-chain nucleophile in their catalytic process can be made to react with an electrophilic trap, or suicide substrate,\textsuperscript{3,4} whereas activity-based profiling strategies of enzymes that do not employ such active site residues normally rely on the use of photoactivatable groups.\textsuperscript{5-7} A third essential element of ABPs is the affinity/identification tag, which can be either incorporated into the ABP directly or alternatively installed via a bioorthogonal process in two-step activity-based protein profiling strategies.\textsuperscript{8-10} Visualization/identification tags come in several flavours, including fluorophores and immune epitope tags. The most popular tag in this context is the biotin group. It is relatively small, easy to install in a wide variety of differently functionalized activity-based probes and has strong binding affinity to (strept)avidin. As a consequence a range of biochemical tools have become available over the decades for either the visualization of biotinylated biomolecules or their pull-down. As such, biotin is both an identification tag and visualization tag in one.

The strong affinity of streptavidin for biotin makes that the release of biotinylated proteins captured by, for instance, streptavidin-coated beads can be difficult. Most
effective procedures rely on relatively harsh conditions, such as boiling of the sample in
denaturing conditions and often in the presence of unmodified biotin. As a
consequence, next to (denatured) streptavidin, the eluted protein pool is often
contaminated with endogenously biotinylated biomolecules, present in the initial
biological sample.

The introduction of a specific linker between biotin and the ABP reactive group,
which can be cleaved chemoselectively so that only the ABP bound material is released,
is an attractive strategy to circumvent this problem (Figure 1A). Examples of such
cleavable linkers (CL) are the disulfide linkage, enzymatically (Tev) cleavable, acid
cleavable, diazobenzene derived (cleavable with Na₂S₂O₄), and hydrazone-based
linkers. An ideal cleavable linker is stable towards the various conditions (acidic,
basic, reductive, including generally applied buffer systems) to which the biological
sample may be exposed, depending on the nature of the experiment, and can also
withstand the reactive (nucleophilic) species that are present in a cell extract. At the
same time, the linker should be susceptible to mild cleavage conditions which are
selective with respect to functional groups inherent to the biological sample. With this
reasoning in mind, attention was focussed on the levulinoyl ester, a versatile protective
group often applied in synthetic organic chemistry. It is acid stable and can be removed
selectively with respect to other esters by treatment with hydrazine. The levulinoyl ester
thus meets to considerable extend the demands outlined above. A shortcoming is its
intrinsic base-lability and it was reasoned that this drawback can be rectified by
choosing the alcohol, with which the levulinolate group is condensed, such that it is
both electron-rich (to reduce its leaving group properties) and sterically congested (to
minimize intermolecular nucleophilic attack). Altogether, epoxomicin based, levulinolate modified activity-based proteasome probe 1 (Figure 1B) was designed. This
chapter describes its synthesis and application in the activity-based enrichment and
identification of the proteasome active subunits from cell lysate.

![Figure 1](image)

**Figure 1.** (A) Schematic representation of an activity-based enrichment experiment using the levulinoyl ester based cleavable linker. After labelling of the target enzyme and pull-down the linker is (chemo)selectively cleaved with hydrazine. (B) Structure of target proteasome probe 1 containing the levulinoyl ester based cleavable linker.
7.2 Results and Discussion

The synthesis of ABP 1 is shown in Scheme 1. Commercially available 2,6-diisopropylphenol 2 was converted into benzaldehyde 3 in a Duff reaction and the alcohol was subsequently protected as the benzyl ether (4). The ensuing Horner-Wadsworth-Emmons reaction with phosphonate 5 proceeded smoothly to give 6. Hydrogenation and concomitant benzyl deprotection led to alcohol 7. Next, the ketone in diethyl 4-oxopimelate (8) was protected as ketal (9) and one of the ethyl esters was selectively saponificated. The obtained carboxylate was reduced to the alcohol (10), which was converted into azide 12 via its tosylate (11). Saponification followed by acidic hydrolysis gave compound 14, which was condensed with 7 to produce levulinoyl ester 15. The tert-butyl ester was removed and the resulting carboxylic acid (16) was transformed into its activated NHS ester (17). Condensation with the epoxomicin peptide sequence 18 gave compound 19, which was reacted with alkyne 20 to give target compound 1.


Reagents and conditions: (a) hexamine, AcOH, H₂O, reflux, 97%; (b) BnBr, K₂CO₃, acetone, 93%; (c) 5, NaH, THF, 0 °C, quant.; (d) Pd/C, H₂, MeOH, 94%; (e) ethylene glycol, PPTS, toluene, reflux, quant.; (f) i) KOH, EtOH, 50 °C; ii) ethyl chloroformate, Et₃N, THF; iii) NaBH₄, H₂O, 37%; (g) TsCl, Et₃N, DMAP, DCM, 85%; (h) NaN₃, DMF, 75 °C, 99%; (i) NaOH, MeOH, 89%; (j) HCl, H₂O, THF, quant.; (k) 7, DIC, DMAP, DCM, 73%; (l) TFA, DCM, quant.; (m) HOSu, EDC, DCM, 95%; (n) 18, DiPEA, DMF, 63%; (o) 20, CuSO₄, sodium ascorbate, DMF, 41%.

The linker system present in 1 was designed after conducting a number of studies (see Scheme 2). Initially, compound 23 was synthesized in order to test the possibility of hydrazine mediated cleavage in a Tris buffer in general. Cleavage of the ester
appeared to be fast (within 30 minutes at room temperature), however the ester linkage was also easily hydrolyzed in a Tris buffer system at pH 7.5. In addition, the synthesis from 5-aminolevulinic acid hydrochloric acid (22) is cumbersome due to its lability under basic conditions. Next, a panel of compounds derived from levulinic acid coupled to different alcohols (primary, secondary, tertiary alcohols and phenols) or secondary amines (Scheme 2) was studied for their synthetic viability, cleavage properties and stability towards hydrolysis. The primary and secondary alcohols derived esters proved to be substantially susceptible towards hydrolysis. Both the tertiary alcohol and 2,6-di-tert-butylphenol derived esters could not be constructed under the commonly applied condensation conditions. Furthermore, the secondary amides, although being stable to hydrolysis, could not be cleaved with hydrazine. The optimal results, in terms of synthesis, cleavage and stability, were obtained for the 2,6-dimethyl- and 2,6-diisopropyl phenols.

Scheme 2. Design of the levulinoyl ester-based cleavable linker system.

Based on these results compound 24 was created, in which the \( \alpha \)-amine functionality (in 23) was replaced by an azide. Although this compound showed improved properties, compared to 23, it still suffered from two issues. The ester linkage was still a little prone to hydrolysis in Tris buffer and the \( \alpha \)-azide moiety was too labile to allow for a clean ‘click’ reaction. The third generation designed was compound 25. The isopropyl groups *ortho* to the ester appeared sterically sufficiently bulky to avoid hydrolysis under basic conditions. Yet they allow intramolecular cyclization of the
A levulinoyl ester-based cleavable linker

Hydrazone, which is formed after condensation of the ketone with hydrazine. The 4-alkyl substituent proved to be favourable compared to a 4-carbonyl substituent in that this more electron rich phenol makes the ester less prone to hydrolysis. Also, the additional ethylene moiety introduced between azide and ketone allowed for a smooth click reaction with biotin propargyl amide. Although the ester linkage was completely optimized, a preliminary pull-down experiment showed that hydrazine mediated cleavage of the captured construct from streptavidin-coated beads was marginal. It was reasoned that the cleavable linker was in too close proximity to the streptavidin-bound biotin and hence inaccessible for hydrazine. Therefore in the final design (I) an additional Ahx spacer was placed between the cleavable linker and biotin, which allowed an easier release from the beads (*vide infra*).

The stability and cleavability of the linker system under several conditions was studied by LC-MS analysis of a test-substrate containing the linker. The results are shown in Table 1.

**Table 1.** Stability/cleavage of the indicated test-substrate (10 mM) under different conditions.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Buffer (pH)</th>
<th>H₂NNH₂</th>
<th>Temp. (°C)</th>
<th>Time (h)</th>
<th>Additive</th>
<th>Cleavage</th>
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<tbody>
<tr>
<td>1</td>
<td>Tris (7.5)</td>
<td>–</td>
<td>23</td>
<td>15</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2</td>
<td>Tris (7.5)</td>
<td>–</td>
<td>37</td>
<td>15</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>Tris (7.5)</td>
<td>+</td>
<td>23</td>
<td>15</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>Tris (7.5)</td>
<td>+</td>
<td>37</td>
<td>1</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>Tris (7.5)</td>
<td>+</td>
<td>37</td>
<td>15</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>Tris (7.5)</td>
<td>NH₂OH</td>
<td>37</td>
<td>15</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>7</td>
<td>Tris (7.5)</td>
<td>–</td>
<td>23</td>
<td>15</td>
<td>0.4% SDS</td>
<td>–</td>
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<tr>
<td>8</td>
<td>Tris (7.5)</td>
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<td>100</td>
<td>5 min.</td>
<td>4×SB</td>
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<td>23</td>
<td>15</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>11</td>
<td>MI (3.0)</td>
<td>–</td>
<td>23</td>
<td>15</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>12</td>
<td>MI (4.0)</td>
<td>–</td>
<td>23</td>
<td>15</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>13</td>
<td>MI (5.0)</td>
<td>–</td>
<td>23</td>
<td>15</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>14</td>
<td>MI (6.0)</td>
<td>–</td>
<td>23</td>
<td>15</td>
<td>–</td>
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*Concentrations used: [H₂NNH₂] = 100 mM; [NH₂OH] = 100 mM; [Tris] = 100 mM; [HEPES] = 50 mM. PBS (phosphate buffered saline): 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 0.14 M NaCl, 2.7 mM KCl. MI (Mcllvaine’s buffer): combination of 0.1 M citric acid and 0.2 M Na₂HPO₄, SB (sample buffer): 20 mM Tris, 1% SDS, 10% glycerol, 0.05% BPB, 0.25 M β-mercaptoethanol.*
From this, it can be seen that the cleavable linker is stable towards a wide variety of conditions to which a biological sample may be exposed, including different buffer systems, strong denaturing conditions and pH 3-7.5. Only upon addition of hydrazine the linker is cleaved (entries 3-5) into the two expected products. Hydroxyl amine was unable to cleave the linkage (entry 6).

The efficacy of compound I to select and identify proteasome catalytic activities from cell extracts in an activity-based labelling experiment followed by hydrazine mediated linker cleavage was investigated as follows. Incubation of HEK-293T cell lysate with I in a Tris buffer followed by treatment with or without hydrazine under different conditions and visualization by streptavidin Western blotting revealed that all three active subunits (β1, β2 and β5) are labelled (see Figure 2A). Cleavage of the linker appears to be hydrazine concentration dependent (lanes 3-7) and full cleavage was achieved after exposure to 50 mM hydrazine for 15 h. A second experiment, in which samples of HEK-293T cell lysate, incubated with I, were denatured and exposed to 100 mM hydrazine for different amounts of time, revealed that full cleavage was achieved within three to five hours (Figure 2B).

![Figure 2](image-url)

**Figure 2.** Optimal cleavage conditions in a biological environment. HEK-293T cell lysate was incubated with probe I (2.5 μM final concentration) for 1 h at 37 °C followed by denaturation. (A) Samples were treated with the indicated hydrazine concentration for 15 h, resolved by SDS-PAGE and all biotinylated proteins were visualized by anti-biotin Western blotting. Cleavage of the linker is shown by disappearance of the bands. PS: prestained marker low range. Lane 2: sample was pre-boiled with 1% SDS prior to incubation as a negative control. Lane 8: 20 mM SDS without denaturing. Lane 9: pH 8.5 is the pH value for a combination of 100 mM Tris and 100 mM hydrazine and was reached by addition of 1 μL 1 M Tris to the sample. (B) Samples were treated with 100 mM hydrazine for the indicated amounts of time and subsequently analyzed as described above. Lane 1: compound 21 (vide infra). BM: Biotinylated marker low range.

To evaluate the use of I for protein capture and release, HEK-293T cell lysate was treated with this compound followed by denaturation, cysteine bridge reduction (DTT) and capping (iodoacetamide), and capturing with streptavidin coated magnetic beads.
The beads were divided into equal aliquots and the elutes from each sample after treatment with or without hydrazine under different conditions were resolved by SDS-PAGE and visualized by silver stain (see Figure 3A). As a control experiment, biotinylated probe AdaLys(biotin)Ahx3Leu3VS (21) was added to the lysate which was then treated with either standard (non-selective) elution conditions (1% SDS, 10 µM biotin, 100 °C, lanes 1 and 7) or hydrazine (lanes 2 and 8). These results show that the captured proteins from 1 can be released chemoselectively with hydrazine both at RT and at 37 °C (lanes 4 and 10), whereas the captured construct derived from 21 is resistant towards these conditions (lanes 2 and 8). The streptavidin blots of the same samples in Figure 3B show a complete absence of biotin for the with compound 1 captured proteins, which proves that the cleavable linker is indeed cleaved. In addition it appears that a small amount of SDS (0.05%) is necessary during cleavage (compare lanes 3 and 9 with 4 and 10), whereas SDS at this concentration solely does not sustain cleavage (lane 5 and 11). Cleavage in HEPES buffer at pH 5.8 occurs as well, but is accompanied by a higher release of undesired material (compare lane 10 with 12). The cleavage efficiency was determined by extensive washing of the beads from Figure 3, eluting all captured leftovers with SDS boiling and silver staining after SDS-PAGE (see Figure 4). For the samples of lanes 6, 10 and 12 (Figure 2) the cleavage efficiency appears to be (near) quantitative (no proteasome characteristic bands are present) and for that of lane 4 only a small amount of active proteasome subunits is visible.

<table>
<thead>
<tr>
<th>Lane</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>PS</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
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<td>PS</td>
<td>21</td>
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<td>H</td>
<td>SB</td>
<td>PD</td>
<td>PD</td>
<td>PD</td>
<td>PD</td>
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<tr>
<td>H2NNH2 (100 mM)</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
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<td>+</td>
<td>−</td>
<td>+</td>
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<tr>
<td>SDS (0.05%)</td>
<td>++b</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>++b</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
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<td></td>
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<tr>
<td>T (°C)</td>
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<td>23</td>
<td>23</td>
<td>23</td>
<td>23</td>
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<td>37</td>
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</tr>
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</table>

Figure 3. Enriched proteins after pull-down with ABPs 1 and 21 and cleavage from streptavidin beads under the indicated conditions for 15 h. (A) Silver stain. (B) Streptavidin Western blot from the same samples. PS = prestained marker low range. a The applied buffers were: SB (30 mM Tris-HCl pH 6.8, 1% SDS, 9% glycerol, bromophenolblue (BPP), 0.25 M β-mercaptoethanol, 10 µM biotin); PD (50 mM Tris-HCl pH 7.5, 150 mM NaCl); H (50 mM HEPES pH 5.8). b ++ refers to the 1% SDS present in SB. The arrows indicate the excised bands analyzed by LC-MS/MS after in-gel tryptic digest.
Figure 4. Determination of the cleavage efficiency. The beads from the samples in Figure 3 after hydrazine mediated cleavage were washed extensively and boiled for 5 min. with sample buffer (20 mM Tris, 1% SDS, 10% glycerol, 0.05% BPP, 0.25 M β-mercaptoethanol) containing 10 μM biotin. Samples were resolved by SDS-PAGE and all proteins were visualized by silver stain. a The numbers correspond to the same samples in the lanes in Figure 3.

To establish that the visualized bands indeed correspond to the targeted proteasome subunits the indicated bands (arrows in Figure 3) were cut from the gel and analyzed by LC-MS/MS after in-gel tryptic digest. In this analysis multiple characteristic peptides for each of the indicated subunits were identified (see Figure 5A). Moreover, the expected modified active site fragment peptides derived from β2 and β5 were identified (see Figure 5B showing the MS/MS spectrum for the modified β5 active site).

Finally, the stability of compound 1 in serum and plasma was investigated. To this end, compound 1 was added to samples containing PBS, plasma or serum and incubated at 37 °C for 15 hours. LC-MS analysis (see Figure 6) indicated that compound 1 was completely stable in these environments, as evidenced by the fact that no products resulting from ester cleavage (hydrolysis) could be detected. These last results demonstrate the viability of the levulinoyl linker for ABP profiling of enzymes in vivo.

<table>
<thead>
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<th>Band</th>
<th>Subunit</th>
<th>np</th>
<th>Seq. Coverage</th>
</tr>
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<tr>
<td>1</td>
<td>β2</td>
<td>6</td>
<td>42.7%</td>
</tr>
<tr>
<td>2</td>
<td>β1</td>
<td>5</td>
<td>39.0%</td>
</tr>
<tr>
<td>3</td>
<td>β5</td>
<td>7</td>
<td>54.4%</td>
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</table>

Figure 5. (A) Table showing the proteasome active subunits identified from the indicated bands in Figure 3 after in-gel tryptic digest and LC-MS/MS analysis. np= number of identified peptides. (B) MS/MS spectrum of the β5 active site fragment attached to the expected cleaved probe. Insert: part of the LC-MS run showing the parent ion m/z (calc. m/z = 756.4704 for z = 2) for the peptide-inhibitor construct as shown at the bottom.
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Figure 6. LC-MS traces of samples containing PBS, plasma or serum and treated with compound 1 for 15 hours at 37 °C. The MS spectrum is shown for the peak at 8.47 minutes in the serum sample, indicating that compound 1 is intact. No products resulting from hydrolysis of the ester linkage could be detected.

7.3 Conclusion

In summary, a levulinoyl ester-based linker system for use in activity-based protein profiling was developed. Tuning the nature of the levulinoyl ester has produced a linker system that is robust enough to survive conditions commonly applied to cell extracts in biochemical experiments. The linker withstands aqueous, acidic and basic media (including the widely used Tris buffer) and is resistant towards disulfide reducing conditions. It is thus believed that the linker has some important advantages over the reported linker systems. The versatility of the linker in the ABP-mediated enrichment of the proteasome active sites was demonstrated and it was shown that the expected cleavage product is indeed found back on the active site peptides obtained after tryptic digestion. These results thus form the basis for the development of new activity-based profiling strategies, for instance two-step labelling strategies that include bio-orthogonal chemistry. From the synthetic scheme it is apparent that the linker is compatible with ‘click’ chemistry. In a preliminary experiment HEK-293T cell lysates were exposed to azide-containing probe 19. Ensuing treatment with biotinylated Staudinger phosphane, SDS-PAGE resolving of the protein contain by SDS-PAGE and streptavidin blotting revealed a pattern characteristic for the proteasome catalytic activities. This last result demonstrates the viability of the levulinoyl linker for two-step ABP profiling of enzymes in general.

Experimental section

General

Tetrahydrofuran was distilled over LiAlH₄ prior to use. Acetonitrile (ACN), dichloromethane (DCM), N,N-dimethylformamide (DMF), methanol (MeOH), disopropylethylamine (DIPEA) and trifluoroacetic acid (TFA) were of peptide synthesis grade, purchased at Biosolve, and used as received. All general chemicals (Fluka, Acros, Merck, Aldrich, Sigma) were used as received. O-(1H-6-chlorobenzotriazolyl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HCTU) was purchased...
at Iris Biotech (Marktrewitz, Germany). Traces of water were removed from reagents used in reactions that require anhydrous conditions by co-evaporation with toluene. Solvents that were used in reactions were stored over 4 Å molecular sieves, except methanol and acetonitrile which were stored over 3 Å molecular sieves. Column chromatography was performed on Screening Devices b.v. silica gel, with a particle size of 40-63 μm and pore diameter of 60 Å. The eluents toluene, ethyl acetate (EtOAc) and petroleum ether (PE) (40-60 °C boiling range) were distilled prior to use. TLC analysis was conducted on Merck aluminium sheets (Silica gel 60 F254). Compounds were visualized by UV absorption (254 nm), by spraying with a solution of (NH₄)₆Mo₇O₂⁴·₄H₂O (25 g/L) and (NH₄)₄Ce(SO₄)₂·2H₂O (10 g/L) in 10% sulfuric acid, a solution of KMnO₄ (20 g/L) and K₂CO₃ (10 g/L) in water, or ninhydrin (0.75 g/L) and acetic acid (12.5 mL/L) in ethanol, where appropriate, followed by charring at ca. 150 °C. ¹H- and ¹³C-NMR spectra were recorded on a Bruker AV-400 (400 MHz) spectrometer. Chemical shifts are given in ppm (δ) relative to tetramethylsilane, CD₃OD or CDCl₃ as internal standard. IR spectra were recorded on a Shimadzu FTIR-8300 and absorptions are given in cm⁻¹. High resolution mass spectra were recorded by direct injection (2 μL of a 2 μM solution in water/acetonitrile 50/50 (v/v) and 0.1% formic acid) on a mass spectrometer (Thermo Finnigan LTQ Orbitrap) equipped with an electrospray ion source in positive mode (source voltage 3.5 kV, sheath gas flow 10, capillary temperature 250 °C) with resolution R = 60,000 at m/z 400 (mass range m/z = 150-2000) and dioctylphthalate (m/z = 391.28428) as a "lock mass". The high resolution mass spectrometer was calibrated prior to measurements with a calibration mixture (Thermo Finnigan). Optical rotations [α]D were recorded on a Propol automatic polarimeter. LC-MS analysis was performed on a Jasco HPLC system with a Phenomenex Gemini 3 μm C18 50 × 4.60 mm column (detection simultaneously at 214 and 254 nm), coupled to a PE Sciex API 165 mass spectrometer with ESI (System A) or a Finnigan Surveyor HPLC system with a Gemini C18 50 × 4.60 mm column (detection at 200-600 nm), coupled to a Finnigan LCQ Advantage Max mass spectrometer with ESI (System B). The applied buffers were H₂O, ACN and 1.0% aq. TFA. Unless noted otherwise the gradient used was 10% → 90% ACN/0.1% aq. TFA. HPLC purifications were performed on a Gilson HPLC system coupled to a Phenomenex Gemini 5 μm 250 × 10 mm column and a GX281 fraction collector. The applied buffers were: 0.1% aq. TFA and ACN. Appropriate fractions were pooled, and concentrated.

4-hydroxy-3,5-diisopropylbenzaldehyde (3)¹⁹
2,6-diisopropylphenol (2, 18.4 g, 100 mmol) was dissolved in AcOH (83 mL) and H₂O (17 mL). To this was added hexamine (2 eq., 200 mmol, 28.0 g) and the mixture was heated to reflux for 5 min. After which a distillation head was installed and ca. 9 mL distillate was collected at 110 °C. The distillation head was removed again and the mixture was refluxed for another 2.5 h after which TLC analysis indicated complete consumption of the phenol starting compound. Next, the mixture was cooled to RT and H₂O (20 mL) was added. Upon further cooling to 0 °C a pale yellow solid precipitated. The mixture was allowed to stand at 0 °C for 1 h followed by filtration of the solid. The residue was washed two times with ice-cold water and dried at 60 °C under reduced pressure. The title compound was obtained without further purification as a pale yellow solid (yield: 20.1 g, 97.6 mmol, 97%). ¹H NMR (400 MHz, CDCl₃): δ = 9.79 (s, 1H), 7.62 (s, 2H), 4.14 (bs, 1H), 3.30 (p, J = 6.80 Hz, 2H), 1.28 (d, J = 6.80 Hz, 12H) ppm. ¹³C NMR (100 MHz, CDCl₃): δ = 192.35, 157.16, 135.26, 128.67, 126.01, 26.47, 22.24 ppm.

4-(benzyloxy)-3,5-diisopropylbenzaldehyde (4)
Phenol (3) (4.14 g, 20.0 mmol) was dissolved in acetone (100 mL) and to this were added benzylbromide (1.01 eq., 20.2 mmol, 3.46 g) and K₂CO₃ (2 eq., 40.0 mmol, 5.53 g). The suspension was stirred vigorously for 14 h after which TLC analysis revealed a completed reaction. The mixture was concentrated under reduced pressure, redissolved in EtOAc (100 mL) and extracted with H₂O and brine. After drying (MgSO₄) and concentration in vacuo of the organic layer the title
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compound was obtained as a colourless oil (yield: 5.50 g, 18.6 mmol, 93%). $^1$H NMR (400 MHz, CDCl$_3$): $\delta = 9.95$ (s, 1H), 7.70 (s, 2H), 7.48 (d, $J = 7.18$ Hz, 2H), 7.41 (t, $J = 7.30$ Hz, 2H), 7.35 (t, $J = 7.20$ Hz, 1H), 4.86 (s, 2H), 3.41 (sept., $J = 6.85$ Hz, 2H), 1.27 (d, $J = 6.96$ Hz, 12H) ppm. $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta = 191.68, 158.53, 158.13, 136.76, 133.13, 128.50, 128.07, 127.25, 126.13, 76.42, 26.66, 23.74$ ppm.

$^{(E)}$-tert-butyl 3-(4-(benzyloxy)-3,5-diisopropylphenyl)acrylate (6)

To a stirred solution of aldehyde 4 (0.23 g, 0.76 mmol) and tert-butyl 2-(diethoxyphosphoryl)acetate (5, 1.5 eq., 1.14 mmol, 0.29 g) in THF (10 mL) at 0 °C was added NaH (1.5 eq., 1.14 mmol, 46.0 mg). The reaction mixture was stirred for 1 h at RT after which TLC analysis indicated a completed reaction. EtOAc (10 mL) was added and the mixture was extracted with 0.1 M aq. HCl (2×), sat. aq. NaHCO$_3$ and brine. The organic layer was dried over MgSO$_4$ and concentrated under reduced pressure. The title compound was obtained after purification by column chromatography (100% PE $\rightarrow$ 5% EtOAc/PE) as a colourless oil (yield: 0.31 g, 0.77 mmol, quant.). $^1$H NMR (400 MHz, CDCl$_3$): $\delta = 7.60$ (d, $J = 15.94$ Hz, 1H), 7.47 (d, $J = 7.08$ Hz, 2H), 7.40 (t, $J = 7.31$ Hz, 2H), 7.33 (t, $J = 7.24$ Hz, 1H), 7.30 (s, 2H), 6.33 (d, $J = 15.93$ Hz, 1H), 4.81 (s, 2H), 3.38 (sept., $J = 6.89$ Hz, 2H), 1.54 (s, 9H), 1.24 (d, $J = 6.91$ Hz, 12H) ppm. $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta = 166.38, 154.87, 143.78, 142.42, 137.20, 130.97, 128.47, 127.94, 127.26, 124.02, 118.73, 80.14, 76.36, 28.13, 26.54, 23.88$ ppm. HRMS: calcd. for C$_{26}$H$_{34}$O$_3$ 395.25807 [M + H]$^+$; found 395.25797.

Tert-butyl 3-(4-hydroxy-3,5-diisopropylphenyl)propanoate (7)

Compound 6 (0.30 g, 0.76 mmol) was dissolved in MeOH (10 mL) and the solution was bubbled through with argon for 15 min. before Pd/C 10% w/w (10 mg) was added. The flask was charged with hydrogen for 1 h, after which TLC analysis indicated complete reduction. Argon was bubbled through for another 15 min. and all solids were removed by filtration over Celite. The title compound was obtained after evaporation of the solvent under reduced pressure as a colourless oil (yield: 0.22 g, 0.71 mmol, 94%). $^1$H NMR (400 MHz, CDCl$_3$): $\delta = 6.87$ (s, 2H), 5.09 (s, 1H), 3.17 (sept., $J = 6.80$ Hz, 2H), 2.85 (t, $J = 7.84$ Hz, 2H), 2.52 (t, $J = 7.86$ Hz, 2H), 1.42 (s, 9H), 1.24 (d, $J = 6.91$ Hz, 12H) ppm. $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta = 172.73, 154.87, 143.78, 142.42, 137.20, 130.97, 128.47, 127.94, 127.26, 124.02, 118.73, 80.14, 76.36, 28.13, 26.54, 23.88 ppm. HRMS: calcd. for C$_{19}$H$_{30}$O$_3$ 329.20872 [M + Na]$^+$; found 329.20871.

Diethyl 3-(4-hydroxy-3,5-diisopropylphenyl)propanoate (7)

Diethyl-4-oxopimelate (8, 11.4 g, 48.4 mmol), ethylene glycol (436 mmol, 24.0 mL) and PPTS (7.26 mmol, 1.82 g) were dissolved in toluene (50 mL) and the mixture was heated to reflux under Dean-Stark conditions for 2 h, after which TLC analysis indicated complete consumption of the ketone. The mixture was cooled to RT and extracted twice with sat. aq. NaHCO$_3$ (50 mL). The organic layer was dried over MgSO$_4$ and concentrated under reduced pressure. The title compound was obtained as a colourless liquid (yield: 13.2 g, 48.4 mmol, quant.). $^1$H NMR (300 MHz, CDCl$_3$): $\delta = 4.13$ (q, $J = 7.13$ Hz, 4H), 3.94 (s, 4H), 2.37 (t, $J = 7.65$ Hz, 4H), 1.98 (t, $J = 7.64$ Hz, 4H), 1.25 (t, $J = 7.14$ Hz, 6H) ppm. $^{13}$C NMR (75.5 MHz, CDCl$_3$): $\delta = 173.24, 109.84, 65.02, 60.20, 32.07, 28.78, 14.07$ ppm.

Ethyl 3-(2-(3-hydroxypropyl)-1,3-dioxolan-2-yl)propanoate (10)

A solution of KOH (1 eq., 47.7 mmol, 47.7 mL; 1 M in EtOH) was added dropwise to diethyl ester 9 (13.2 g, 47.7 mmol) at 50 °C in 4 h. The resulting mixture was stirred at 50 °C for 14 h after which all EtOH was evaporated under reduced pressure. The resulting residue was suspended in THF (250 mL) and Et$_3$N (0.5 eq., 23.8 mmol, 3.31 mL) and ethyl chloroformate (1.5 eq., 71.5 mmol, 6.84 mL) were added. After vigorously stirring for 2 h the mixture was added to a cooled (0 °C) solution of NaBH$_4$ (1.5 eq., 71.5 mmol, 2.71 g) in H$_2$O (250 mL) and the mixture was stirred at RT for 1 h.
The reaction was quenched by addition of 1 mM aq. HCl (100 mL) and the resulting mixture was extracted with Et₂O (3×). The combined organic layers were extracted with brine, dried over MgSO₄ and concentrated under reduced pressure. The title compound was obtained after purification by column chromatography (25% → 100% EtOAc/PE) as a colourless liquid (yield: 4.14 g, 17.8 mmol, 37%). ¹H NMR (300 MHz, CDCl₃): δ = 4.13 (q, J = 7.13 Hz, 2H), 3.96 (s, 4H), 3.64 (t, J = 5.83 Hz, 2H), 2.37 (t, J = 7.75 Hz, 2H), 2.01 (t, J = 7.65 Hz, 2H), 1.93 (bs, 1H), 1.76-1.62 (m, 4H), 1.26 (t, J = 7.13 Hz, 3H) ppm. ¹³C NMR (75.5 MHz, CDCl₃): δ = 208.84, 110.72, 65.00, 62.84, 60.34, 33.79, 31.90, 28.92, 26.89, 14.15 ppm. HRMS: calcd. for C₁₁H₂₀O₅ 255.12029 [M + Na]⁺; found 255.12036.

**Ethyl 3-(2-(3-(tosyloxy)propyl)-1,3-dioxolan-2-yl)propanoate (11)**

Alcohol 10 (4.14 g, 17.8 mmol) was dissolved in DCM (125 mL) and Et₃N (2.1 eq., 37.4 mmol, 5.18 mL), DMAP (0.25 eq., 4.45 mmol, 0.50 g) and TsCl (2.55 eq., 8.66 mmol, 45.4 g) were added. The mixture was stirred for 4 h after which TLC analysis indicated a complete consumption of the starting compound. DCM was evaporated under reduced pressure and the residue was dissolved in EtOAc, extracted with 1 mM aq. HCl (2×) and brine, dried over MgSO₄ and concentrated under reduced pressure. The title compound was obtained after purification by column chromatography (25% → 50% EtOAc/PE) as a colourless liquid (yield: 5.87 g, 15.2 mmol, 85%). ¹H NMR (400 MHz, CDCl₃): δ = 7.79 (d, J = 8.0 Hz, 2H), 7.34 (d, J = 8.4 Hz, 2H), 4.12 (q, J = 7.1 Hz, 2H), 4.04 (t, J = 6.4 Hz, 2H), 3.9-3.8 (m, 4H), 2.45 (s, 3H), 2.31 (t, J = 7.6 Hz, 2H), 1.92 (t, J = 7.6 Hz, 2H), 1.8-1.7 (m, 2H), 1.7-1.6 (m, 2H), 1.26 (t, J = 6.8 Hz, 3H) ppm. ¹³C NMR (100 MHz, CDCl₃): δ = 173.38, 144.80, 133.30, 129.80, 127.86, 110.15, 70.53, 65.06, 60.36, 33.01, 32.07, 28.83, 23.39, 21.60, 14.19 ppm.

**Ethyl 3-(2-(3-azidopropyl)-1,3-dioxolan-2-yl)propanoate (12)**

A solution of tosylate 11 (5.87 g, 15.2 mmol) and NaN₃ (1.2 eq., 18.2 mmol, 1.19 g) in DMF (120 mL) was stirred at 75 °C for 14 h, after which TLC analysis indicated a complete conversion. The mixture was concentrated under reduced pressure and the residue was dissolved in EtOAc, extracted with sat. aq. NaHCO₃ (2×), H₂O and brine, dried over MgSO₄ and concentrated under reduced pressure. The title compound was obtained as a colourless liquid (yield: 3.86 g, 15.0 mmol, 99%). ¹H NMR (400 MHz, CDCl₃): δ = 4.13 (q, J = 7.14 Hz, 2H), 3.94 (s, 4H), 3.32-3.26 (m, 2H), 2.36 (t, J = 7.60 Hz, 2H), 1.99 (t, J = 7.60 Hz, 2H), 1.71-1.65 (m, 4H), 1.26 (t, J = 7.15 Hz, 3H) ppm. ¹³C NMR (100 MHz, CDCl₃): δ = 173.23, 110.20, 64.93, 51.29, 34.07, 31.94, 28.73, 23.24, 14.04 ppm. HRMS: calcd. for C₁₁H₁₉N₃O₄ 258.14483 [M + H]⁺; found 258.14491. IR film (cm⁻¹) 2954.7, 2885.3, 2090.7, 1728.1, 1450.4, 1257.5, 1180.3, 1134.1, 1033.8, 948.9, 910.3, 864.1.

**3-(2-(3-azidopropyl)-1,3-dioxolan-2-yl)propanoic acid (13)**

Ethyl ester 12 (3.86 g, 15.0 mmol) was dissolved in MeOH (80 mL) and to this was added NaOH (4 eq., 60.0 mmol, 30 mL; 2 M in H₂O) at 0 °C. The mixture was allowed to slowly warm to RT and was stirred for 14 h after which TLC analysis revealed complete conversion. The mixture was concentrated under reduced pressure and the residue was dissolved in sat. aq. NaHCO₃/H₂O (3:1 v/v, 80 mL) and extracted twice with EtOAc. The latter organic layers were combined, dried over MgSO₄ and concentrated under reduced pressure, which yielded the title compound as a colourless liquid (yield: 3.05 g, 13.3 mmol, 89%). ¹H NMR (300 MHz, CDCl₃): δ = 10.20 (bs, 1H), 3.96 (s, 4H), 3.34-3.25 (m, 2H), 2.41 (t, J = 7.50 Hz, 2H), 2.00 (t, J = 7.51 Hz, 2H), 1.71-1.65 (m, 4H) ppm. ¹³C NMR (100 MHz, CDCl₃): δ = 179.31, 110.16, 64.95, 51.23, 34.07, 31.68, 28.46, 23.21 ppm.
7-azido-4-oxoheptanoic acid (14)
Concentrated aq. HCl (19 mL) was added to a solution of compound 13 (3.05 g, 13.3 mmol) in THF (60 mL) and the mixture was stirred for 3 h, after which TLC analysis showed complete consumption of starting material. Water (150 mL) was added carefully and the aqueous layer was extracted with EtOAc three times. The combined organic layers were extracted with brine, dried over MgSO\(_4\) and concentrated in vacuo. The title compound was obtained as a colourless oil (yield: 2.46 g, 13.3 mmol, quant.) without further purification necessary. \(^1\)H NMR (400 MHz, CDCl\(_3\)): \(\delta = 9.26\) (bs, 1H), 3.32 (t, \(J = 6.64\) Hz, 2H), 2.73 (t, \(J = 6.20\) Hz, 2H), 2.64 (t, \(J = 6.16\) Hz, 2H), 2.57 (t, \(J = 7.06\) Hz, 2H), 1.88 (p, \(J = 6.86\) Hz, 2H) ppm. \(^{13}\)C NMR (100 MHz, CDCl\(_3\)): \(\delta = 207.65, 178.28, 50.56, 39.10, 36.80, 27.68, 22.79\) ppm.

4-(3-(tert-butoxy)-3-oxopropyl)-2,6-diisopropylphenyl 7-azido-4-oxoheptanoate (15)
To a solution of alcohol 7 (1 eq., 13.3 mmol, 4.08 g), carboxylic acid 14 (1 eq., 13.3 mmol, 2.46 g) and DMAP (0.1 eq., 1.33 mmol, 0.16 g) in DCM (100 mL) was added DIC (1.2 eq., 16.0 mmol, 2.51 mL) and the mixture was stirred for 14 h. Next, the mixture was concentrated under reduced pressure, the residue dissolved in EtOAc (100 mL) and extracted with 1 M aq. HCl (2×), sat. aq. NaHCO\(_3\) and brine, dried over MgSO\(_4\) and concentrated under reduced pressure. The title compound was obtained after purification by column chromatography (100% Tol \(\rightarrow\) 10% EtOAc/Tol) as a colourless oil (yield: 4.41 g, 9.64 mmol, 73%). \(^1\)H NMR (400 MHz, CDCl\(_3\)): \(\delta = 6.96\) (s, 2H), 3.30 (t, \(J = 6.68\) Hz, 2H), 2.93-2.80 (m, 8H), 2.58 (t, \(J = 7.06\) Hz, 2H), 2.53 (t, \(J = 7.83\) Hz, 2H), 1.87 (p, \(J = 6.88\) Hz, 2H), 1.42 (s, 9H), 1.17 (d, \(J = 6.91\) Hz, 12H) ppm. \(^{13}\)C NMR (100 MHz, CDCl\(_3\)): \(\delta = 207.21, 172.21, 171.48, 143.67, 140.03, 138.61, 123.69, 80.15, 50.52, 39.19, 36.95, 36.88, 30.90, 27.95, 27.65, 27.32, 22.85 ppm. HRMS: calcd. for C\(_{26}\)H\(_{39}\)N\(_3\)O\(_5\) 496.27819 [M + Na\(^+\)]\(^\dagger\); found 496.27775.

3-(4-((7-azido-4-oxoheptanoyl)oxy)-3,5-diisopropylphenyl)propanoic acid (16)
TFA (10 mL) was added to a solution of tert-butyl ester 15 (1.40 g, 3.06 mmol) in DCM (10 mL) and this mixture was stirred for 30 min. after which TLC analysis showed complete consumption of starting material. Toluene (25 mL) was added and the mixture was concentrated under reduced pressure. In order to remove traces of TFA the mixture was coevaporated with toluene three times. The title compound was obtained as a colourless oil (yield: 1.28 g, 3.06 mmol, quant.). \(^1\)H NMR (400 MHz, CDCl\(_3\)): \(\delta = 9.40\) (s, 1H), 6.98 (s, 2H), 3.29 (t, \(J = 6.69\) Hz, 2H), 2.95-2.81 (m, 8H), 2.67 (t, \(J = 7.91\) Hz, 2H), 2.59 (t, \(J = 7.06\) Hz, 2H), 1.87 (p, \(J = 6.92\) Hz, 2H), 1.18 (d, \(J = 6.89\) Hz, 12H) ppm. \(^{13}\)C NMR (100 MHz, CDCl\(_3\)): \(\delta = 207.43, 178.29, 171.58, 169.03, 167.90, 144.16, 140.59, 137.15, 134.75, 140.18, 138.08, 123.64, 50.44, 39.14, 36.82, 35.59, 30.48, 27.59, 27.27, 22.79 ppm. HRMS: calcd. for C\(_{22}\)H\(_{31}\)N\(_3\)O\(_5\) 440.21559 [M + Na\(^+\)]\(^\dagger\); found 440.21539.

4-(3-((2,5-dioxopyrrolidin-1-yl)oxy)-3-oxopropyl)-2,6-diisopropylphenyl 7-azido-4-oxoheptanoate (17)
N-hydroxysuccinimide (1.5 eq., 1.30 mmol, 150 mg) and EDC (1.5 eq., 1.30 mmol, 249 mg) were added to a solution of carboxylic acid 16 (0.36 g, 0.87 mmol) in DCM (7 mL) and the mixture was stirred for 14 h, after which TLC analysis indicated complete consumption of starting material. The solvent was evaporated under reduced pressure and the residue was dissolved in EtOAc (20 mL). This was extracted with 1 M aq. HCl (2×) and brine, dried over MgSO\(_4\) and concentrated under reduced pressure. The title compound was obtained after purification by column chromatography (30% \(\rightarrow\) 60% EtOAc/PE) as a colourless oil (yield: 0.43 g, 0.83 mmol, 95%). \(^1\)H NMR (400 MHz, CDCl\(_3\)): \(\delta = 6.99\) (s, 2H), 3.31 (t, \(J = 6.67\) Hz, 2H), 3.06-3.00 (m, 2H), 2.95-2.81 (m, 12H), 2.60 (t, \(J = 7.06\) Hz, 2H), 1.88 (p, \(J = 6.90\) Hz, 2H), 1.18 (d, \(J = 6.89\) Hz, 12H) ppm. \(^{13}\)C NMR (100 MHz, CDCl\(_3\)): \(\delta = 207.26, 171.53, 169.03, 167.90, 144.16, 140.59, 137.15, 123.75, 50.60, 39.28, 36.95, 32.70, 30.35, 27.70, 27.44, 25.54, 22.91 ppm. LC-MS (System A): R\(_t\) (min): 9.38 (ESI-MS (m/z): 515.4 (M + H\(^+\))). HRMS: calcd. for C\(_{28}\)H\(_{33}\)N\(_4\)O\(_7\) 515.25003 [M + H\(^+\)]\(^\dagger\); found 515.24963.

Reagents and conditions: (a) HCTU, DiPEA, DCM, 89%; (b) i) DBU, DMF; ii) HOBr; iii) HCTU, DiPEA; iv) NH₂NNH₂·H₂O, MeOH, 72%; (c) i) tBuONO, HCl, DCM, DMF, -30 °C; ii) TFA·H₂N-Leu-EK, DiPEA, 89%; (d) i) TFA, DCM; ii) 17, DiPEA, DMF, 63%.

Fmoc-Ile-Thr(tBu)-OMe (26)
Fmoc-Ile-OH (1.2 eq., 13.3 mmol, 4.70 g) was dissolved in DCM (60 mL) and to this were added HCTU (1.2 eq., 13.3 mmol, 5.50 g), DiPEA (3.3 eq., 36.0 mmol, 6.0 mL) and HCl·H-Thr(tBu)-OMe (1 eq., 11.0 mmol, 2.50 g) successively. The mixture was stirred for 2 h after which TLC analysis indicated a completed reaction. DCM was evaporated under reduced pressure and the residue was dissolved in EtOAc, extracted with 1 M aq. HCl (2×), sat. aq. NaHCO₃ (2×) and brine, dried over MgSO₄ and concentrated under reduced pressure. The title compound was obtained after purification by column chromatography (10% → 50% EtOAc/PE) as a colourless solid (yield: 5.16 g, 9.83 mmol, 89%). ¹H NMR (400 MHz, CDCl₃): δ = 7.76 (d, J = 7.48 Hz, 2H), 7.60 (d, J = 7.41 Hz, 2H), 7.39 (t, J = 7.46 Hz, 2H), 7.31 (dt, J = 7.43, 0.98 Hz, 2H), 6.48 (d, J = 8.84 Hz, 1H), 5.58 (d, J = 8.70 Hz, 1H), 4.49 (dd, J = 9.00, 1.68 Hz, 1H), 4.44-4.33 (m, 2H), 4.28-4.21 (m, 2H), 4.18 (dd, J = 8.53, 6.39 Hz, 1H), 3.71 (s, 3H), 1.94-1.83 (m, 1H), 1.65-1.53 (m, 1H), 1.33-1.21 (m, 1H), 1.17 (d, J = 6.27 Hz, 2H), 7.39 (t, J = 7.46 Hz, 2H), 7.31 (dt, J = 7.43, 0.98 Hz, 2H), 6.48 (d, J = 8.84 Hz, 1H), 5.58 (d, J = 8.70 Hz, 1H), 4.49 (dd, J = 9.00, 1.68 Hz, 1H), 4.44-4.33 (m, 2H), 4.28-4.21 (m, 2H), 4.18 (dd, J = 8.53, 6.39 Hz, 1H), 3.71 (s, 3H), 1.94-1.83 (m, 1H), 1.65-1.53 (m, 1H), 1.33-1.21 (m, 1H), 1.17 (d, J = 6.27 Hz, 2H) ppm. ¹³C NMR (100 MHz, CDCl₃): δ = 171.42, 170.86, 156.07, 143.78, 141.24, 127.63, 127.01, 125.07, 119.90, 74.21, 67.19, 66.96, 59.30, 57.83, 52.13, 47.17, 38.18, 28.27, 24.82, 21.04, 15.08, 11.52 ppm. LC-MS (System B): R (min): 10.87 (ESI-MS (m/z): 525.0 (M + H⁺)).

Boc-Ile-Ile-Thr(tBu)-NHNH₂ (27)
DBU (1.05 eq., 10.3 mmol, 1.57 mL) was added to a solution of Fmoc-Ile-Thr(tBu)-OMe (26, 5.16 g, 9.83 mmol) in DMF (50 mL) and the mixture was stirred for 5 min. after which HOBr (1.5 eq., 14.7 mmol, 1.98 g) was added. After stirring the mixture for another 30 min. Boc-Ile-OH (1.2 eq., 11.8 mmol, 2.73 g), HCTU (1.2 eq., 11.8 mmol, 4.88 g) and DiPEA (3 eq., 29.5 mmol, 4.87 mL) were added. TLC analysis indicated sufficient product formation after 14 h and the mixture was concentrated under reduced pressure. The residue was redissolved in DCM and extracted with 1 M aq. HCl (2×), sat. aq. NaHCO₃ (2×) and brine, dried over MgSO₄ and concentrated in vacuo. The intermediate was purified by column chromatography (10% → 50% EtOAc/PE) and the obtained product (3.69 g, 7.15 mmol) was dissolved in MeOH (50 mL). Hydrazine monohydrate (30 eq., 215 mmol, 10.4 mL) was added and the mixture was stirred for 14 h, after which TLC analysis indicated complete consumption of starting material. Toluene was added and the mixture was concentrated under reduced pressure. The title compound was obtained after coevaporation with toluene (3×) as a colourless solid (yield: 3.66 g, 7.10 mmol, 72%). ¹H NMR (400 MHz, CD₃OD): δ = 4.34 (d, J = 3.53 Hz, 1H), 4.29 (d, J = 8.12 Hz, 1H), 4.05-3.99 (m, 1H), 3.92 (d, J = 7.90 Hz, 1H), 1.90-1.70 (m, 2H), 1.61-1.47 (m, 2H), 1.42 (s, 3H), 1.22-1.10 (m, 1H), 1.17 (s, 9H), 1.08 (d, J = 6.32 Hz, 3H), 0.92-0.85 (m, 12H) ppm. ¹³C NMR (100 MHz, CD₃OD): δ = 174.83, 173.39, 171.30, 157.91, 80.56, 75.84,
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68.52, 60.62, 59.22, 58.56, 37.94, 37.85, 28.77, 28.66, 25.94, 19.78, 16.23, 15.95, 11.39, 11.32 ppm. LC-MS (System A): R\( t \) (min): 6.08 (ESI-MS (m/z): 516.4 (M + H\(^+\)). HRMS: calcd. for \( \text{C}_{25}\text{H}_{49}\text{N}_{5}\text{O}_{6} \) 516.37556 [M + H\(^+\)]; found 516.37530.

Boc-Ile-Ile-Thr(tBu)-Leu-EK (28)

Boc-Ile-Ile-Thr(tBu)-NHNH\(_2\) (27, 1 eq., 3.87 mmol, 2.0 g) was dissolved in a 9:1 v/v mixture of DCM/DMF (40 mL) and cooled to –30 °C. To this were added \( t\text{BuONO} \) (1.1 eq., 4.25 mmol, 0.57 mL) and HCl (2.8 eq., 10.8 mmol, 2.70 mL; 4 M in 1, 4-dioxane). This mixture was stirred at –30 °C for 3 h, after which (S)-2-amino-4-methyl-1-((R)-2-methyloxiran-2-yl)pentan-1-one TFA salt\(^{23,24} \) (1.1 eq., 4.25 mmol, 1.16 g) in DMF (5 mL) and DiPEA (5 eq., 20.0 mmol, 3.31 mL) were added. The reaction was allowed to warm to RT and stirred for 14 h. Next, DCM (15 mL) was added and the mixture was extracted with 0.1 M aq., HCl (2×) and H\(_2\)O, dried over MgSO\(_4\) and concentrated under reduced pressure. The title compound was obtained after purification by column chromatography (20% → 50% EtOAc/PE) as a colourless solid (yield: 2.25 g, 3.43 mmol, 89%). 1H NMR (400 MHz, CDCl\(_3\)): \( \delta \) = 7.64 (d, \( J = 7.47 \) Hz, 1H), 6.99 (d, \( J = 5.63 \) Hz, 1H), 6.45 (d, \( J = 8.22 \) Hz, 1H), 5.22 (d, \( J = 7.84 \) Hz, 1H), 4.46 (ddd, \( J = 10.45, 7.55, 2.94 \) Hz, 1H), 4.41-4.32 (m, 2H), 4.14-4.07 (m, 1H), 3.94 (t, \( J = 7.34 \) Hz, 1H), 3.38 (d, \( J = 5.07 \) Hz, 1H), 2.89 (d, \( J = 5.06 \) Hz, 1H), 1.92-1.77 (m, 2H), 1.74-1.63 (m, 1H), 1.60-1.55 (m, 1H), 1.54-1.48 (m, 2H), 1.52 (s, 3H), 1.44 (s, 9H), 1.33-1.24 (m, 1H), 1.28 (s, 9H), 1.19-1.08 (m, 2H), 1.06 (d, \( J = 6.44 \) Hz, 3H), 0.96 (d, \( J = 6.54 \) Hz, 6H), 0.92-0.86 (m, 12H) ppm. 13C NMR (100 MHz, CDCl\(_3\)): \( \delta \) = 208.06, 171.59, 170.73, 169.51, 155.80, 79.76, 75.49, 66.14, 59.24, 57.68, 56.95, 52.39, 50.74, 39.80, 37.30, 36.97, 28.28, 28.08, 25.42, 24.88, 24.69, 23.35, 21.35, 16.75, 16.33, 15.53, 15.40, 11.28 ppm. LC-MS (System B): R\( t \) (min): 11.33 (ESI-MS (m/z): 655.27 (M + H\(^+\)). HRMS: calcd. for \( \text{C}_{34}\text{H}_{62}\text{N}_{4}\text{O}_{8} \) 655.46404 [M + H\(^+\)]; found 655.46451.

Boc-Ile-Ile-Thr(tBu)-Leu-EK (28)

N\(_3\)-Lev-phenol-epoxomicin (19)

Compound 24 (165 mg, 0.25 mmol) was treated with a 1:1 v/v mixture of DCM/TFA (2 mL) for 1 h and subsequently coevaporated with toluene (3×). The resulting intermediate was dissolved in DMF (2 mL) and to this were added compound 17 (1.1 eq., 0.258 mmol, 144 mg) and DiPEA (2 eq., 0.50 mmol, 83 \( \mu \)L). The reaction was stirred for 14 h before being concentrated under reduced pressure. The residue was dissolved in DCM (10 mL) and extracted with 1 M aq. HCl (2×), sat. aq. NaHCO\(_3\) (2×) and brine, dried over MgSO\(_4\) and concentrated under reduced pressure. The title compound was obtained after purification by column chromatography (25% → 100% EtOAc/PE) as a colourless solid (yield: 141 mg, 0.16 mmol, 63%). 1H NMR (400 MHz, CDCl\(_3\)): \( \delta \) = 8.75 (s, 1H), 8.28 (s, 1H), 8.24 (s, 1H), 7.46 (s, 1H), 6.97 (s, 2H), 4.94-4.76 (m, 3H), 4.63-4.55 (m, 1H), 4.34 (s, 1H), 4.09-4.01 (m, 1H), 3.31 (t, \( J = 6.64 \) Hz, 2H), 3.22 (s, 1H), 3.00-2.77 (m, 9H), 2.59 (t, \( J = 7.01 \) Hz, 4H), 1.88 (p, \( J = 6.89 \) Hz, 2H), 1.83-1.73 (m, 1H), 1.70-1.35 (m, 8H), 1.49 (s, 3H), 1.20-1.06 (m, 15H), 0.91-0.71 (m, 18H) ppm. 13C NMR (100 MHz, CDCl\(_3\)): \( \delta \) = 208.14, 207.22, 172.12, 171.40, 170.57, 143.75, 140.05, 138.75, 123.71, 67.38, 58.99, 57.44, 57.16, 52.39, 50.74, 39.80, 37.30, 36.97, 28.28, 28.08, 25.42, 24.88, 24.69, 23.35, 21.35, 16.75, 16.33, 15.53, 15.40, 11.28 ppm. LC-MS (System B): R\( t \) (min): 11.33 (ESI-MS (m/z): 898.40 (M + H\(^+\)). HRMS: calcd. for \( \text{C}_{47}\text{H}_{75}\text{N}_{7}\text{O}_{10} \) 898.56482 [M + H\(^+\)]; found 898.56539.

Biotin-Ahx-propargylamide (20)

Boc-Ahx-propargylamide\(^{25} \) (275 mg, 1.03 mmol) was treated with a 1:1 v/v mixture of DCM/TFA (6 mL) for 1 h and subsequently coevaporated with toluene (3×). The resulting intermediate was dissolved in DMF (5 mL) and to this were added biotin-OSu (1.1 eq., 1.1 mmol, 375 mg) and DiPEA (1.5 eq., 1.50 mmol, 248 \( \mu \)L). The reaction was stirred for 14 h before being concentrated under reduced pressure. The title compound was obtained after crystallisation from MeOH/Et\(_2\)O as a colourless solid. The compound was sufficiently pure based on LC-MS analysis and subjected to
the next step without further purification. LC-MS (System B): R_t (min): 4.27 (ESI-MS (m/z): 395.13 (M + H^+)).

**Biotin-Ahx-triazole-Lev-phenol-epoxomicin (1)**

To a solution of compound 19 (45.0 mg, 50.1 μmol) and biotin-Ahx-propargylamide (20, 1.5 eq., 70.0 μmol, 30.0 mg) in DMF (1.5 mL) were added CuSO_4 (0.2 eq., 10.0 μmol, 10.0 μL; 1 M in H_2O) and sodium ascorbate (0.3 eq., 15.0 μmol, 15.0 μL; 1 M in H_2O) and the mixture was stirred for 14 h at RT after which LC-MS analysis indicated a complete conversion of the azide. The mixture was concentrated under reduced pressure and the title compound was obtained after RP-HPLC purification (40% → 70% ACN/0.1% aq. TFA) as a colourless solid (yield: 26.3 mg, 20.4 μmol, 41%).

^1H NMR (400 MHz, CD_3OD): δ = 7.76 (s, 1H), 6.92 (s, 2H), 4.45 (dd, J = 10.68, 3.02 Hz, 1H), 4.38 (dd, J = 7.43, 5.04 Hz, 1H), 4.34-4.26 (m, 4H), 4.22 (d, J = 5.04 Hz, 1H), 4.19 (dd, J = 7.87, 4.49 Hz, 1H), 4.15 (d, J = 7.99 Hz, 1H), 4.10 (d, J = 8.14 Hz, 1H), 3.93 (dd, J = 6.19, 5.28 Hz, 1H), 3.15 (d, J = 5.07 Hz, 1H), 3.12-3.08 (m, 1H), 3.05 (t, J = 7.07 Hz, 2H), 2.87-2.70 (m, 10H), 2.59 (d, J = 12.70 Hz, 1H), 2.51-2.39 (m, 4H), 2.15-2.01 (m, 6H), 1.81-1.70 (m, 1H), 1.69-1.37 (m, 12H), 1.36 (s, 3H), 1.35-1.17 (m, 6H), 1.10-1.01 (m, 16H), 1.00-0.88 (m, 1H), 0.86-0.77 (m, 12H), 0.76-0.69 (m, 6H) ppm. 13C NMR (100 MHz, CD_3OD): δ = 209.52, 209.43, 176.00, 175.27, 174.10, 173.71, 173.61, 172.25, 145.32, 141.72, 140.24, 124.93, 68.57, 63.45, 61.69, 60.14, 59.84, 59.41, 59.32, 57.06, 53.10, 51.85, 50.58, 41.09, 40.37, 40.23, 39.53, 38.73, 38.04, 37.88, 37.79, 36.85, 35.64, 32.80, 30.16, 29.83, 29.54, 28.68, 28.61, 27.58, 26.98, 26.54, 26.26, 26.05, 25.46, 23.82, 21.53, 20.06, 17.06, 16.01, 15.97, 11.49, 11.37 ppm. [α]D23 = −1.28 (c = 1 in MeOH). LC-MS (System B): R_t (min): 8.42 (ESI-MS (m/z): 1292.53 (M + H^+)); HRMS: calcd. for C_66H_105N_11O_13S 1292.76868 [M + H]+; found 1292.76980.

**Stability/cleavage of the test-substrate**

The test-substrate N_3-CL-Lys-Gly-OH was made from compound 17 and dipeptide Fmoc-Lys(Boc)-Gly-OtBu via standard peptide chemistry. The test-substrate was dissolved (10 mM) in either 100 mM Tris pH 7.5, 100 mM HEPES pH 5.8, PBS (10 mM Na_2HPO_4, 1.8 mM KH_2PO_3, 0.14 M NaCl, 2.7 mM KCl) or McIlvaine’s buffers (combinations of 0.1 M citric acid and 0.2 M Na_2HPO_4, pH 3, 4, 5, 6). Hydrazine or hydroxylamine (100 mM final concentration) was added where appropriate. The samples were incubated for either 1 h or 15 h (o/n) at 23 °C or 37 °C. LC-MS samples were prepared by mixing 250 μL H_2O with 25 μL of the reaction mixture and 1 drop of TFA. The samples were analyzed by LC-MS by injection of 20 μL of the LC-MS sample and the applied gradient was 10% → 90% ACN/0.1% aq. TFA. LC-MS results: test-substrate: R_t (min): 5.60 (ESI-MS (m/z): 603.7 (M + H^+)); cleavage product azide: R_t (min): 2.71 (ESI-MS (m/z): 182.1 (M + H^+)); cleavage product phenol: R_t (min): 4.24 (ESI-MS (m/z): 436.4 (M + H^+)). No signals for hydrolyzed substrate was detected in either sample.

**Search for optimal cleavage conditions**

HEK-293T cell lysate (13.5 μg protein) was incubated with compound 1 (2.5 μM final concentration) for 1 h at 37 °C, after which the samples were denatured by addition of 4 μL 4× SB and boiling for 5 min. The appropriate amount of hydrazine (0, 1, 10, 50, 100 mM final concentration) was added and the samples were incubated at 23 °C for 15 h (o/n). After resolving of the protein content by 12.5% SDS-PAGE all biotinylated proteins were visualized by Western blotting. The blots were blocked with 1% BSA in TBS-Tween 20 (0.1 % Tween 20) for 30 min. at RT, hybridized for 1 h with Streptavidin-HRP (1:10,000) in blocking buffer, washed and visualized using an ECL+ kit (Amersham Biosciences).

**Pull-down experiments**

HEK-293T cell lysate (containing some 1.3 mg of protein) was incubated with 20 μM ABPs 1 or 21 for 1 h at 37 °C, denatured by boiling for 5 min. with 1% SDS and precipitated with
chloroform/methanol (C/M). The protein pellet was rehydrated in 180 µL 8 M urea/100 mM NH₄HCO₃, reduced with 10 µL 90 mM DTT for 30 min. at 37 °C, alkylated with 15 µL 200 mM iodoacetamide at RT in the dark, cleared by centrifugation at 13,000 g and desalted by C/M. The pellet was dispersed in 25 µL pull down (PD) buffer (50 mM Tris·HCl pH 7.5, 150 mM NaCl) with 2% SDS in a heated (37 °C) sonic bath. Stepwise (3 × 25 µL, 4 × 100 µL, 1 × 500 µL) addition of PD buffer afforded a clear solution that was incubated with 50 µL MyOne T1 Streptavidin grafted magnetic beads (Invitrogen) at RT with vigorous shaking for 1 h. The beads were stringently washed with 2 × 300 µL PD buffer with 0.1% SDS, 2 × 300 µL PD buffer, 2 × 300 µL wash buffer I (4 M urea/50 mM NH₄HCO₃), 2 × 300 µL wash buffer II (50 mM Tris·HCl pH 7.5, 10 mM NaCl) and 2 × 300 µL water. All 5 samples from ABP 1 were mixed and divided over 8 equal portions. The same was done for the 2 samples from ABP 21, which were divided over 3 equal portions. To the samples was added 65 µL of the appropriate cleavage cocktail (see Figure 3) and the samples were shaken for 15 h at RT or at 37 °C. Next, the supernatant was removed, diluted with 20 µL 4× sample buffer and boiled at 100 °C for 5 min. One sample from ABP 21 was treated directly with 85 µL 1× sample buffer containing 10 µM biotin, boiled at 100 °C for 5 min. and stored o/n at 4 °C. The samples were resolved by 12.5% SDS-PAGE and visualized by silverstain to determine the cleavage efficiency (see Figure 4).

**LC-MS/MS analysis**

Trytic peptides were analyzed on a Surveyor nanoLC system (Thermo) hyphenated to a LTQ-Orbitrap mass spectrometer (Thermo). Gold and carbon coated emitters (OD/ID = 360/25 µm tip ID = 5 µm), trap column (OD/ID = 360/100 µm packed with 25 mm robust Poros®10R2/15 mm BioSphere C18 5 µm 120 Å) and analytical columns (OD/ID = 360/75 µm packed with 20 cm BioSphere C18 5 µm 120 Å) were from Nanoseparations (Nieuwkoop, The Netherlands). The mobile phases (A: 0.1% formic acid/H₂O, B: 0.1% formic acid/ACN) were made with ULC/MS grade solvents (Biosolve). The emitter tip was coupled end-to-end with the analytical column via a 15 mm long TFE teflon tubing sleeve (OD/ID 0.3 x 1.58 mm, Supelco, USA) and installed in a stainless steel holder mounted in a nanosource base (Upchurch scientific, Idex, USA). General mass spectrometric conditions were: an electrospray voltage of 1.8 kV was applied to the emitter, no sheath and auxiliary gas flow, ion transfer tube temperature 150 °C, capillary voltage 41 V, tube lens voltage 150 V. Internal mass calibration was performed with air-borne protonated polydimethylcyclosiloxane (m/z = 445.12002) and the plasticizer protonated dioctyl phthalate ions (m/z = 391.28429) as lock mass. For shotgun proteomics analysis, 10 µL of the samples was pressure loaded on the trap column with a 10 µL/min flow for 5 min. followed by peptide separation with a gradient of 35 min. 5 → 30% B, 15 min. 30 → 60% B, 5 min. A, at a flow of 300 µL/min. split to 250 nL/min. by the LTQ divert valve. For each data dependent cycle, one full MS scan (300-2000 m/z) acquired at high mass resolution (60,000 at 400 m/z, AGC target 1 × 10⁶, maximum injection time 1,000 ms) in the Orbitrap was followed by 3 MS/MS fragmentations in the LTQ linear ion trap (AGC target 5 × 10³, max injection time 120 ms) from the three most abundant ions. MS/MS settings were: collision gas pressure 1.3 mT, normalized collision energy 35%, ion selection threshold of 500 counts, activation q = 0.25 and activation time of 30 ms. Fragmented precursor ions that were measured twice within 10 s were dynamically excluded for 60 s and ions with z < 2 or unassigned were not analyzed. Data from MS/MS was validated manually.

**Stability of the cleavable linker in blood serum and plasma**

Plasma and serum were collected from blood voluntarily donated by a healthy person. For plasma collection: blood was extracted in an EDTA tube and centrifuged for 15 min. at 4,000 g. For serum
collection no EDTA was used. All experiments were executed in duplo. A volume of 100 μL plasma or serum was incubated with compound 1 (10 μM final concentration) for 15 h at 37 °C. Next, 900 μL of cooled (–20 °C) acetone was added and the samples were kept at –20 °C for 2 h and an additional 1 h at –80 °C. This was followed by centrifuging of the samples for 10 min. at 14,000 g after which all acetone was evaporated under reduced pressure. The resulting yellowish pellet was extracted with 100 μL ACN/H2O/tBuOH (1:1:1) for 5 min. and the samples were analyzed by LC-MS (injection of 20 μL of the extract, system B, 10% → 90% ACN/0.1% aq. TFA).

References


