Bioorganic & Medicinal Chemistry 20 (2012) 655–661

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Protein enrichment by capture–release based on strain-promoted cycloaddition of azide with bicyclononyne (BCN)

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ARTICLE INFO

Article history:
Available online 3 August 2011

Keywords:
Protein enrichment
Cleavable linker
Strain-promoted cycloaddition
Bicyclononyne
Capture–release

ABSTRACT

An enrichment strategy was devised for azide derivatized macromolecules, based on strain-promoted alkyne–azide cycloaddition (SPAAC) and a cleavable linker. A ring-strained alkyne, bicyclo[6.1.0]non-4-yne (BCN), was covalently attached to agarose beads via a hydrazine-sensitive linker. Benchmark studies of the resulting 'azido-trap' beads were performed with a fluorogenic coumarin derivative, leading to efficient capture of the azidocoumarin with concomitant fluorescence staining of the beads via SPAAC. The versatility of the beads for specific protein enrichment was shown by an effective and highly specific capture–release strategy for enrichment of azido-containing *Candida antarctica* lipase *B* (CalB) from a mixture of proteins. This approach is suited for selective enrichment of (glyco)proteins after metabolic incorporation of azides for subsequent (glyco)proteomics studies.

1. Introduction

An essential aspect in proteomics, metabolomics, glycomics and other analytical life sciences is the identification, isolation, and structural characterization of a specific biomolecule of interest. Several strategies have been applied in the field of proteomics for the global analysis of protein expression and function. Examples include the shotgun analysis of protein expression and modification state by liquid chromatography–mass spectrometry (LC–MS), the large-scale mapping of protein–protein interactions by yeast two-hybrid assays, and the proteome-wide analysis of biochemical activities of proteins with protein microarrays. However, proteins and peptides containing post-translational modifications like glycans are often difficult to detect in a background of (unmodified) biomolecules, due to low ionization efficiencies and/or low abundance. An efficient and specific enrichment procedure is therefore required to study detailed structural aspects of a biomolecule of interest (peptide, protein, lipid or glycan) from complex mixtures like cell lysates. For example, studying proteome dynamics by mass spectrometry requires selective analysis of subsets of peptides that may be post-translationally modified, cross-linked or newly synthesized. Similarly, efficient separation of glycan-containing macromolecules from proteins and lipids in complex mixtures is required for their efficient analysis. Recent advances have shown the possibilities of selective labeling of glycans and proteins with azides, via metabolic labeling with azidosugars[1] like ManNAz, GalNAz and FucNAz or with azidohomoalanine.[2] Such procedures provide a selective handle to isolate subsets of a proteome. However, mild and effective methods for specific enrichment are still required.[3]

Several methodologies for protein enrichment have been developed over the years, such as activity-based protein profiling (ABPP)[4] and photoaffinity labeling.[5] For example, ABPP is an efficient method for detection of the activity of proteins, rather than their mere expression level. Similarly, photoaffinity labeling is a powerful strategy to study the interactions between biologically active compounds (ligands) and their target molecules. In both strategies, the reactive probe requires the presence of an analytical handle, typically a fluorophore for visualization or an immune epitope tag for binding and characterization. With respect to the molecular tag, biotin is a popular choice because a biotinylated molecule can be easily detected by immunological methods.[6] Moreover, the presence of biotin offers the possibility of isolation through binding to avidin beads, although harsh conditions are subsequently required to liberate the biotinylated molecule by disruption of the strong interaction between biotin and avidin; typically boiling of the beads with detergents. Clearly, such conditions may result in degradation of the target molecules as well as in contamination of other proteins that bind to the beads in a non-specific manner.

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doi:10.1016/j.bmc.2011.07.049

To overcome the drawbacks associated with biotin, a variety of linkers have been developed that can be cleaved under mild conditions. Examples of such cleavable linkers are constructs based on disulfides (reductive cleavage),[7] on peptide substrates (enzymatically cleavable),[8] on acetals (cleavable with acid),[9] on diazobenzene (cleavable with $Na_2S_2O_4$),[10] hydrazone-based linkers[11] and light-sensitive linkers.[12] An ideal cleavable linker is stable towards the various conditions to which the biological sample may be exposed (acidic, basic, reductive, including generally applied buffer systems), and can 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, compatible with functional groups inherent to the biological sample.

2. Design and synthesis of resin-bound BCN

Recently, Nessen et al. disclosed a general method to sequester peptides from complex mixtures by means of capture with an azido-reactive cleavable cyclooctyne resin.[13] Thus, metabolic incorporation of azidohomoalanine in the *Escherichia coli* proteome allowed the characterization of numerous newly synthesized peptides via trapping by strain-promoted alkyne–azide cycloaddition (SPAAC),[14] followed by reductive cleavage of the disulfide linker employed. Despite the elegancy of the procedure, broad application is unlikely because synthesis of the cyclooctyne probe is rather lengthy and its reactivity in strain-promoted cycloadditions is modest. Furthermore, the disulfide linker necessitates the strict avoidance of reductive conditions during the enrichment process, in order to avoid premature cleavage, while native protein disulfides are inevitably also reduced during cleavage.

We have recently reported on two cyclooctyne variants, that is, dibenzoazacyclooctyne (DIBAC)[15] and bicyclononyne (BCN),[16] which display high reaction rates in cycloadditions with a range of dipoles, including azides, nitrones,[17] and nitrile oxides.[18] Whereas both probes are synthetically readily accessible, BCN particularly stands out because it can be prepared from cheap starting materials in a matter of days. Furthermore, BCN is stable, fairly non-lipophilic and symmetrical in nature, which avoids the formation of regioisomers during cycloaddition. Based on these features, we considered BCN as a promising probe for the development of a novel enrichment tool. With respect to the choice of a linker, we were drawn towards a report by Geurink et al. on a cleavable linker inspired by the levulinoyl (Lev) protective group.[19] By careful fine-tuning of the phenolic leaving group, a construct was derived with significant advantages over earlier published strategies. The Lev-based linker was found to be robust enough to survive conditions commonly applied to cell extracts in biochemical experiments, including aqueous, acidic and basic media. Importantly, the linker was found resistant towards disulfide reducing conditions but was readily cleaved upon treatment with hydrazine. The versatility of the linker was nicely demonstrated in enrichment of proteasome active sites by activity-based profiling. Based on these arguments, we set out to prepare a novel, readily accessible and reactive probe for enrichment by a capture–release strategy based on a Lev-based linker and bicyclo[6.1.0]non-4-yne (BCN).

2.1. Attachment of BCN to cleavable linker

Our work started from the known levulinoyl-based 2,6-di-*iso*-propylphenolic ester **1**[19,20] (Scheme 1). We reasoned that the azido moiety of **1** could be readily reduced to an amine and therefore serve as a useful functionality for attachment to activated agarose beads. However, without taking appropriate precautions, an amino group at that position rapidly cyclizes onto the δ-keto function, leading to a non-nucleophilic dihydropyrrole. Based on this rationale, we opted to chain-extend **1** with a rigid aromatic moiety to preclude a potential ring-closure. Therefore, we performed a copper(I)-catalyzed alkyne–azide cycloaddition (CuAAC)[21] of **1** with Fmoc-protected propargylamine, leading to triazole adduct **2** in excellent yield. Next, in the reported two-step process, the *tert*-butyl ester was smoothly converted into the hydroxysuccinimide-activated ester **4**. The latter ester was efficiently coupled to BCN-amino derivative **5** via a short hydrophilic spacer. Finally, Fmoc-removal was accomplished by treatment of **6** with 20% piperidine in dichloromethane, leading to the effective and clean conversion of **6** into the desired amino derivative **7**.

2.2. Immobilization of BCN-Lev linker to agarose beads

Having obtained the desired amino derivative **7**, coupling to an insoluble bead was investigated. For this purpose, we selected cyanogenbromide-activated Sepharose™ 4B from GE Healthcare, a 4% cross-linked spherical agarose which is known to react rapidly to amines without the need for additional reagents. The resulting carbamate bonds are stable and resistant to a wide range of conditions, including base and high temperatures. Therefore, compound **7** was coupled at pH 8.3 to the Sepharose™ beads at 1, 2, and 10 mM concentration, thus affording the desired 'azido-trap' beads.

3. Protein enrichment by capture–release

As delineated above, our strategy for protein enrichment involved a selective pull-down from a mixture of proteins, or ideally whole cell lysate, followed by filtration and chemical release. However, before the suitability of the agarose-BCN conjugate for capture–release of azido-containing proteins could be performed, it was mandatory to validate the versatility of beads for such purpose.

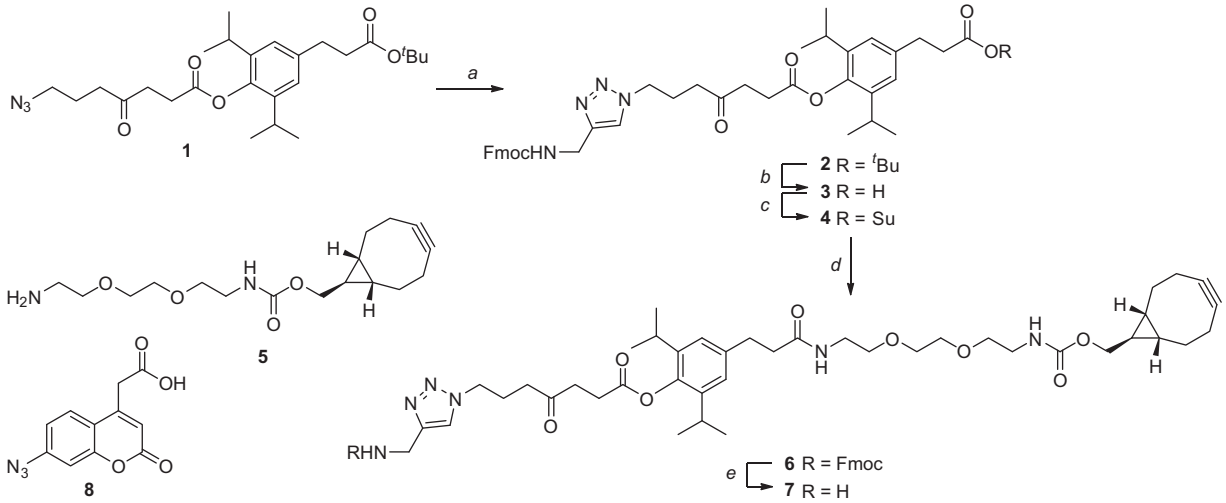
3.1. Evaluation of on-resin SPAAC and cleavage

The rapid and reagent-free reaction of ring-strained alkynes with azides allows a straightforward validation of the versatility of the BCN-modified beads with an azidocoumarin probe. The unique aspect of specific azidocoumarins is that they are fluorogenic, that is, they become fluorescent only upon conversion of the azide moiety into a triazole.[22] Therefore, the linker-coupled beads were incubated with 7-azidocoumarin derivative **8** (1 mM) and aliquots were taken at specific time intervals for analysis with fluorescence microscopy (Fig. 1A). The reaction was carried out with beads obtained by loading at three different linker concentrations (1, 2, and 10 mM).

A time-dependent increase of fluorescence staining of the beads became apparent, which strongly indicated that the SPAAC reaction between immobilized BCN and azidocoumarin had taken place (Fig. 1A). We also found that fluorescent intensities of the beads increased with higher loading concentrations of the linker and thus, the highest signal was established with beads charged at 10 mM (see Supplementary data). For this reason, the latter beads were selected for forthcoming experiments.

The fluorophore-attached beads also enable a straightforward evaluation of the release concept by cleavage of the levulinoyl linker. To this end, the fluorescent beads were incubated with 100 mM hydrazine, leading to a rapid and irreversible decrease in fluorescence (Fig. 1B).

In order to quantitate the loading of BCN on the Sepharose beads, the beads were treated, after fluorophore attachment, with 100 mM hydrazine, with the objective to directly correlate fluorescence in the resulting supernatant to beads loading. Unfortunately, it was



Scheme 1. Reagents and conditions: (a) *tert*-BuOH, $FmocNHCH_2C{\equiv}CH$, $Cu(OAc)_2$, Na-ascorbate, TBAT (quant), (b) CF_3CO_2H, CH_2Cl_2 (1:1), 1 h, then (c) CH_2Cl_2, EDC, HOSu, 40 h (88%, two steps), (d) **5**, DIPEA, DMF, 16 h (63%), (e) piperidine, CH_2Cl_2 (1:4), 10 min (95%).

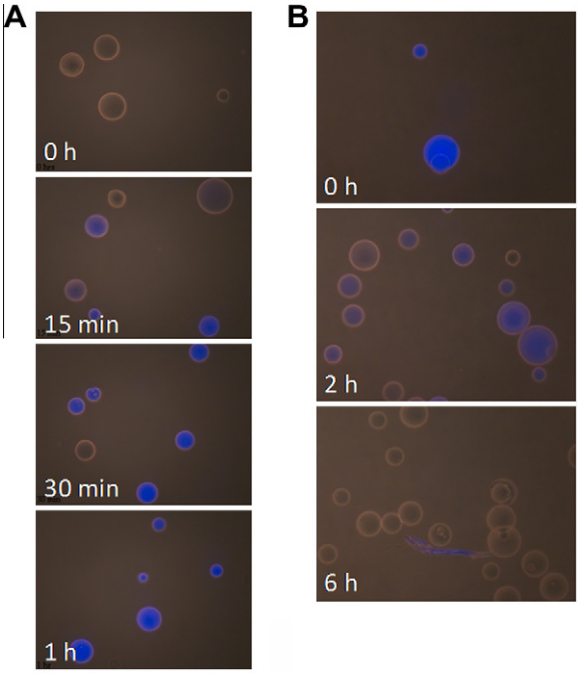


Figure 1. (A) Treatment of BCN-modified Sepharose™ beads with the fluorogenic substrate **8** leads to a time-dependent fluorescent staining of the beads. (B) Cleavage of the linker on the beads using 100 mM hydrazine releases the fluorophore from the beads.

found that the fluorophore underwent rapid degradation in 100 mM hydrazine, leading to complete disappearance of fluorescent signal. Therefore, a BCN derivative lacking the Lev-linker but including a base-sensitive ester (**S1**, see Supplementary data) was coupled to CNBr-Sepharose, followed by SPAAC with azido-ester-coumarin **S2** under the same conditions as described for the levulinoyl linker (10 mM). As expected, following the SPAAC reaction by fluorescence microscopy indicated comparable fluorescent staining of the beads. Next, loading of the beads was determined by measuring the fluorescent emission of the coumarin adduct (ex 325 nm, em 452 nm) from a beads sample (25 µL), relative to a standard curve of 7-hydroxycoumarin in 10 mM NaOH. Under these conditions, coumarin ester **S1** showed only minimal degradation (data not shown). After correction for background hydrolysis, it was determined that loading of the beads with BCN amounts to 0.39 µmol mL^{-1} gel (Fig. S1C), which correlates nicely to the loading capacity as indicated by the manufacturer (25–60 mg α-chymotrypsinogen mL^{-1}).

Taken together, these finding established the suitability of the BCN-modified 'azido-trap' beads for an enrichment protocol of azido-containing molecules.

3.2. Selective enrichment of an azido-modified protein from a protein mixture

To test whether the 'azido-trap' beads are useful for selective enrichment of a specific protein of interest from a mixture of proteins, we selected azido-modified *Candida antartica* lipase B (CalB) as a model protein. This modified CalB, obtained by expression in methionine auxotrophic *E. coli* in the presence of azidohomoalanine (AHA), contains five azides, of which typically only one to two are accessible for reaction with an alkyne probe.[15,23] Thus, AHA–CalB was mixed with proteins lacking an azido function, a mixture consisting of α-lactalbumin, trypsin inhibitor, carbonic anhydrase, ovalbumin, albumin, and phosphorylase B.

The mixture of proteins, including azido-CalB, was incubated with the BCN derivatized beads at three different temperatures and two different reaction times. Of each sample, the supernatant from the beads after the SPAAC reaction was resolved on SDS–PAGE (Fig. 2A). After SPAAC, the initial amount of CalB was clearly reduced in all experiments, best conditions involving a prolonged reaction time (18 h) at 37 °C. Under these conditions, a near quantitative removal of CalB from the mixture was observed, whereas no detectable change of the other proteins became apparent.

Next, cleavage of the linker with hydrazine was performed at 4 and 37 °C for 18 h. At 4 °C, a protein with a similar mass as AHA–CalB appeared in the gels, although at a slightly higher molecular weight. A logical explanation for the latter observation lies in the presence of the residual part of the linker, covalently attached to the protein. At 37 °C, the optimal temperature reported by Geurink et al., we observed some protein precipitation (Fig. S2A). This indicates that, in contrast to the capture conditions, a lower reaction temperature (4 °C) is advantageous for the isolation of CalB from the mixture. Nevertheless, some non-specific binding of phosphorylase B and albumin during SPAAC and subsequent release was observed. To reduce the non-specific binding to the beads, we compared washing

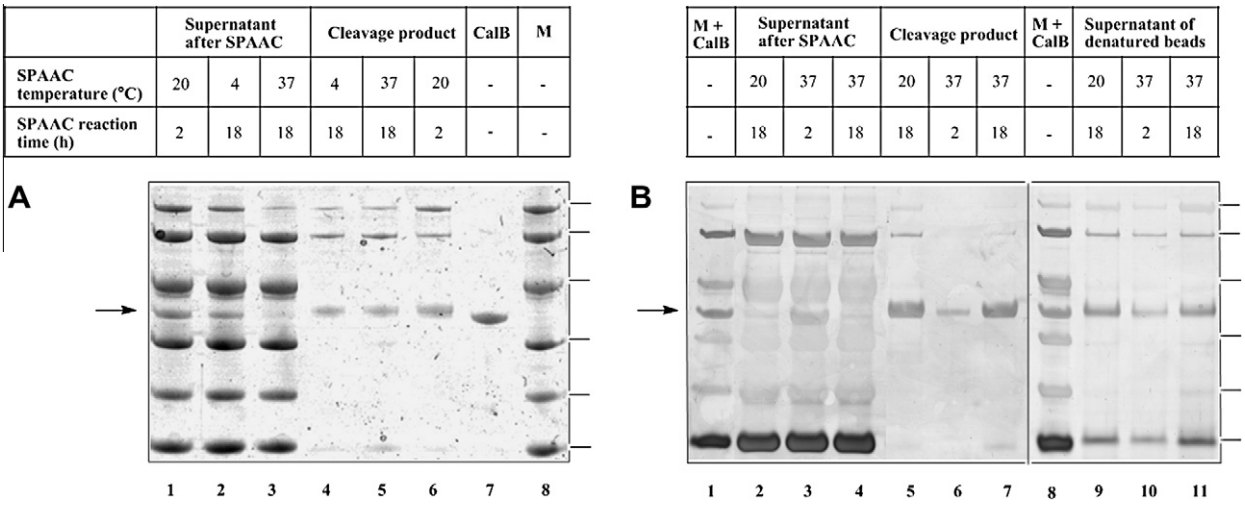


Figure 2. Enrichment of azido-modified CalB from a protein mixture. CalB (arrows) in a mixture of standard proteins M (from top to bottom phosphorylase B, albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor and α-lactalbumin) was used for SPAAC mediated enrichment. (A) Coomassie stained SDS–PAGE gel. Lanes 1–3 show the supernatants after SPAAC reaction. At 37 °C for 18 h (lane 3), almost all azido-modified protein was captured. Lanes 4–6 show the supernatant of the cleavage reaction. CalB is clearly enriched, although there is some non-specific binding as well. The cleavage product runs at a higher molecular weight than CalB, likely due to the part of the linker, that is, covalently bound to the protein. Lane 7 contains azido-modified CalB,[22] lane 8 contains protein mixture M. (B) Silver stained SDS–PAGE gel. Lanes 1 and 8 are CalB in protein mix M before capture. Lanes 2–4 are supernatants after the SPAAC reaction. Lanes 2 and 4 are comparable. From lane 3 it is clear that a reaction time of 2 h is not sufficient to capture all CalB. Lanes 5–7 show the supernatants after cleavage, indicating clean enrichment with only marginal non-specific binding in lane 7. Lanes 9–11 show the supernatants of the beads treated with denaturing buffer after specific release. It shows that non-specific binding is comparable for these samples.

the beads in PBS with washing in 0.5% SDS in PBS, and washing the beads an additional three times at 37 °C with larger volumes while shaking for 2 h instead of just washing three times with a larger volume. It turned out that the effect of addition of SDS to the washing buffer was negligible, while more extensive washing steps seemed to decrease the recovery of modified CalB (Fig. S2B and C). Varying the amount of beads per sample indicated that use of 50% of the beads as used in Figure 2A resulted in slightly less non-specific binding, while CalB recovery remained similar (Fig. S2A). Apparently, the lower amount of beads is sufficient to capture CalB specifically, while higher amounts lead to more non-specific binding of other proteins. The SDS–PAGE profile of hydrazine-released proteins under optimized conditions (Fig. 2B) showed a single band at the position of CalB without the presence of other proteins, indicating a highly specific enrichment of modified CalB from the protein mixture.

Finally, the specificity of the azido-trap capture was unequivocally established by mass spectrometry of bead-enriched CalB. Hydrazine-released CalB was analyzed by nanoLC–MS/MS after tryptic digestion and compared to unbound CalB. The N-terminal peptide, known to harbor an accessible azide moiety,[23] was shown to contain the expected triazole adduct of BCN and azide (Fig. S3B), which was absent in the unreacted CalB sample (Fig. S3A). Other tryptic fragments without (accessible) azidohomoalanine residues were comparable in both CalB samples. These results prove the mechanism for selective binding and hydrazine-mediated release of azido-modified proteins using BCN-beads.

4. Conclusions

We have synthesised an effective 'azido-trap' bead based on a conjugate of Sepharose™ beads, a hydrazine-sensitive cleavable linker, and a strained alkyne (BCN). We demonstrated the concept by fluorogenic staining of the beads with azidocoumarin, which displayed a clear time and concentration dependence. Next, we succeeded in the selective pull-down of an azido-modified protein (AHA–CalB) from a mixture of proteins by covalent attachment to the beads. Finally, both the coumarin derivative and the protein were effectively cleaved from the beads by treatment with hydrazine. By optimization of conditions, highly enriched CalB could be recovered from the protein mixture in two simple and straightforward steps.

Based on these findings, it can be concluded that a versatile construct has been prepared for enrichment of azido-containing molecules, including proteins. The strategy presented here has several distinct advantages over the earlier reported capture–release approach involving strain-promoted cycloadditions. First of all, the cyclic alkyne applied here (BCN) is more readily obtained by synthesis (and commercially available) and displays much higher reactivity to azides. Secondly, we have exploited a stable, levulinoyl-based cleavable linker that does not preclude reductive conditions during the capture step, yet the linker is readily cleaved upon treatment with hydrazine. Thirdly, it must be noted that the symmetrical nature of BCN (versus all other cyclooctynes employed for SPAAC published to date) will facilitate detailed structural analysis of the enriched products after cleavage, due to the absence of regioisomeric triazole products.

Taken together, the successful capture–release of CalB from the protein mixture constitutes a promising general strategy for enrichment of azido-containing (glyco)proteins from complex biological samples after metabolic incorporation.

5. Experimental section

5.1. General methods

^{1}H NMR spectra were recorded in $CDCl_3$ on Bruker DMX 300 or Varian Inova-400 spectrometers at 300 K. TMS (δ_H 0.00) was used as the internal reference. ^{13}C NMR spectra were recorded in $CDCl_3$ at 75 MHz on a Bruker DMX 300 spectrometer, using the central resonance of $CDCl_3$ (δ_C 77.0) as the internal reference. Mass spectra of synthesized compounds were obtained on a JEOL AccuToF. Chemicals were purchased from Aldrich and used without further purification. CH_2Cl_2, acetonitrile, THF, Et_2O and toluene were obtained dry from a MBRAUN SPS-800 solvent purification system; and CH_3OH was distilled from magnesium and iodine. Aqueous solutions are saturated unless otherwise specified. All reactions

were performed under anhydrous conditions under argon and monitored by TLC on Kieselgel 60 F254 (Merck). Detection was by examination under UV light (254 nm) and by charring with 10% sulfuric acid in methanol or with aqueous $KMnO_4$. Silica gel (Acros 0.035–0.070 mm) was used for chromatography. Bicyclo[6.1.0]non-4-yne was a kind gift from SynAffix B.V. 7-Azidocoumarin-4-acetic acid was prepared according to a literature procedure.[24] Fluorescence microscopy was done on a Zeiss Axioskop 20, with an HB050 mercury lamp.

5.2. Synthesis of the BCN-cleavable linker conjugate

5.2.1. 4-(3-(*tert*-Butoxy)-3-oxopropyl)-2,6-diisopropylphenyl 7-(4-(((((9*H*-fluoren-9-yl)methoxy)carbonyl)amino)methyl)-1*H*-1,2,3-triazol-1-yl)-4-oxoheptanoate (2)

To a solution of sodium ascorbate (3.5 mg, 17.8 μmole) in *tert*-BuOH (1 mL) was added successively tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA, 4.7 mg, 8.9 μmole), azide **1** (42 mg, 88.6 μmole) and Fmoc-*N*-propargylamine[25] (27 mg, 97.6 μmole) and the mixture was gently heated until clear. Then 250 μL from a stock solution of $CuSO_4$ (11 mg in 2.5 mL H_2O) was added to the mixture and the reaction was stirred at room temperature until TLC analysis (EtOAc/heptane, v/v, 1:1) after 16 h indicated the complete consumption of **1** and the formation of a more polar product (R_F 0.1). The reaction was diluted with EtOAc (20 mL) and washed successively with H_2O (4 mL) and brine (4 mL). The organic layer was dried ($MgSO_4$), filtered, concentrated and purified by silica gel column chromatography (EtOAc/heptane, v/v, 1/1 → 2/1 → 3/1) to give title compound **2** as a colorless oil. Yield 67 mg (quant). R_F 0.1 (EtOAc/heptane, v/v, 1:1). IR (neat) 2958, 1718, 1524, 1450, 1368, 1256, 1145, 741, 612 cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz): δ 7.75 (2H, d, J = 7.2 Hz), 7.57 (2H, d, J = 7.6 Hz), 7.48 (s, 1H), 7.39 (2H, t, J = 7.6 Hz), 7.29 (2H, t, J = 7.4 Hz), 6.95 (2H, s), 5.50 (1H, br s), 4.45 (2H, d, J = 6.0 Hz), 4.40 (2H, d, J = 6.8 Hz), 4.34 (2H, t, J = 6.8 Hz), 4.20 (1H, t, J = 7.0 Hz), 2.92–2.84 (6H, m), 2.84–2.80 (2H, m), 2.55–2.49 (4H, m), 2.21–2.14 (2H, m), 1.42 (9H, s), 1.16 (12H, d, J = 6.8 Hz). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 207.0, 172.3, 171.6, 156.4, 145.0, 143.8, 143.7, 141.3, 140.1, 138.8, 127.7, 127.0, 125.0, 123.8, 122.1, 119.9, 80.3, 66.8, 49.1, 47.2, 38.7, 37.0, 36.9, 36.4, 31.0, 29.7, 28.1, 27.7, 27.4, 24.1. HRMS (ESI+) m/z calcd for $C_{44}H_{55}N_4O_7$ $(M+H)^+$: 751.4071, found: 751.4096.

5.2.2. 4-(3-((2,5-Dioxopyrrolidin-1-yl)oxy)-3-oxopropyl)-2,6-diisopropylphenyl 7-(4-(((((9*H*-fluoren-9-yl)methoxy)carbonyl)amino)methyl)-1*H*-1,2,3-triazol-1-yl)-4-oxoheptanoate (4)

Trifluoroacetic acid (1 mL) was added to a solution of *tert*-butyl ester **2** (67 mg, 88.6 μmole) in CH_2Cl_2 (1 mL) and the mixture was stirred until TLC analysis (EtOAc/heptane, v/v, 3:1) showed complete consumption of starting material (30 min). Toluene (3 mL) was added and the mixture was concentrated under reduced pressure, which was repeated twice to give carboxylic acid **3** as an oil (crude). R_F 0.25 (EtOAc). Without further purification, compound **3** was dissolved in CH_2Cl_2 (2 mL) and to this solution was added *N*-hydroxysuccinimide (15 mg, 0.133 mmole) and EDC (25 mg, 0.133 mmole). The mixture was stirred overnight after which time TLC (EtOAc) indicated the incomplete conversion of **3** into a more lipophilic product (R_F 0.35, EtOAc). To the mixture was again added *N*-hydroxysuccinimide (7.5 mg, 66.5 μmole) and EDC (12.5 mg, 66.5 μmole) and the mixture was stirred overnight again, leading to the complete disappearance of starting material. The mixture was diluted with CH_2Cl_2 (10 mL), and washed with 1 N HCl-solution (2 × 1 mL) and H_2O (1 mL). The organic layer was dried ($MgSO_4$), filtered, concentrated and purified by silica gel column chromatography (CH_2Cl_2/acetone, v/v, 90/10 → 85/15) to give title compound **4** as a colorless oil. Yield 62 mg (88%, two steps). R_F 0.35 (EtOAc). IR (neat) 2958, 1735, 1524, 1442, 1364, 1208, 1161, 1143, 1070, 906, 733, 651, 616 cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz): δ 7.75 (2H, d, J = 7.6 Hz), 7.57 (2H, d, J = 7.2 Hz), 7.47 (1H, s), 7.40 (2H, t, J = 7.4 Hz), 7.30 (2H, t, J = 7.6 Hz), 6.98 (2H, s), 5.40 (1H, br s), 4.45 (2H, d, J = 6.0 Hz), 4.40 (2H, d, J = 7.2 Hz), 4.34 (2H, t, J = 6.6 Hz), 4.21 (1H, t, J = 7.0 Hz), 3.04–3.00 (2H, m), 2.93–2.77 (11H, m), 2.51 (2H, t, J = 6.8), 2.21–2.14 (3H, m), 1.17 (12H, d, J = 6.8). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 207.0, 171.6, 169.0, 167.9, 156.3, 145.0, 144.2, 143.8, 141.3, 140.6, 137.2, 127.7, 127.0, 125.0, 123.8, 122.1, 120.0, 66.8, 49.1, 47.2, 38.8, 37.0, 36.5, 32.7, 30.4, 27.7, 27.5, 25.6, 24.2. HRMS (ESI+) m/z calcd for $C_{44}H_{50}N_5O_9$ $(M+H)^+$: 792.3609, found: 792.3655.

5.2.3. *N*-Fmoc-protected levulinoyl-BCN conjugate (6)

Activated ester **4** (30 mg, 37.9 μmole) was dissolved in DMF (1 mL) before the addition of DIPEA (13 μL, 75.8 μmole) and BCN–$(POE)_3$–NH_2 (**5**, 15 mg, 46.2 μmole). The mixture was stirred overnight, after which time TLC analysis (EtOAc) indicated complete conversion of **6** into a more polar product (R_F 0.13). The mixture was concentrated, applied onto a column of silica gel and eluted with 4% MeOH in CH_2Cl_2. Title compound **6** was obtained as a colorless oil. Yield 24 mg (63%). R_F 0.13 (EtOAc). IR (neat) 3320, 2959, 2923, 1714, 1667, 1533, 1450, 1364, 1257, 1142, 1102, 1047, 800, 762, 741, 619 cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz): δ 7.76 (2H, d, J = 7.2 Hz), 7.57 (2H, d, J = 7.2 Hz), 7.47 (1H, s), 7.40 (2H, t, J = 7.4 Hz), 7.30 (2H, t, J = 7.2 Hz), 6.96 (2H, s), 6.02 (1H, br s), 5.49 (1H, br s), 5.20 (1H, br s), 4.45–4.32 (6H, m), 4.22–4.12 (3H, m), 3.63–3.49 (8H, m), 3.47–3.40 (2H, m), 3.39–3.31 (2H, m), 2.96–2.86 (6H, m) 2.80–2.77 (2H, m), 2.53–2.46 (4H, m), 2.27–2.17 (8H, m), 1.61–1.52 (2H, m), 1.36–1.31 (1H, m), 1.16 (12H, d, J = 6.8 Hz), 0.96–0.88 (2H, m). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 207.0, 172.2, 171.7, 162.5, 156.4, 145.0, 143.8, 141.3, 140.2, 139.0, 127.7, 127.0, 125.0, 123.8, 122.1, 120.0, 98.8, 70.2, 70.1, 69.9, 66.8, 62.8, 49.1, 47.1, 40.7, 39.1, 38.7, 38.6, 36.9, 36.5, 31.7, 31.4, 29.7, 29.0, 27.7, 27.4, 24.1, 21.4, 20.1, 17.7. HRMS (ESI+) m/z calcd for $C_{57}H_{72}N_6O_{10}Na_1$ $(M+Na)^+$: 1023.5208, found: 1023.5207.

5.2.4. Amino-levulinoyl-BCN conjugate (7)

To a solution of Fmoc-protected conjugate **6** (11 mg, 10.8 μmole) in CH_2Cl_2 (800 μL) was added piperidine (200 μL) and the solution was stirred for 10 min. The mixture was concentrated and purified by silica gel column chromatography (CH_2Cl_2/MeOH/Et_3N, v/v/v, 94:5:1 → 89:10:1) to give title compound **7** as a white solid. Yield 8 mg (95%). R_F 0.1 (CH_2Cl_2/MeOH, v/v, 9:1). IR (neat) 3320, 2959, 2923, 1714, 1667, 1533, 1450, 1364, 1257, 1142, 1102, 1047, 800, 762, 741, 619 cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz): δ 7.76 (2H, d, J = 7.2 Hz), 7.57 (2H, d, J = 7.2 Hz), 7.47 (1H, s), 7.40 (2H, t, J = 7.4 Hz), 7.30 (2H, t, J = 7.2 Hz), 6.96 (2H, s), 6.02 (1H, br s), 5.49 (1H, br s), 5.20 (1H, br s), 4.45–4.32 (6H, m), 4.22–4.12 (3H, m), 3.63–3.49 (8H, m), 3.47–3.40 (2H, m), 3.39–3.31 (2H, m), 2.96–2.86 (6H, m) 2.80–2.77 (2H, m), 2.53–2.46 (4H, m), 2.27–2.17 (8H, m), 1.61–1.52 (2H, m), 1.36–1.31 (1H, m), 1.16 (12H, d, J = 6.8 Hz), 0.96–0.88 (2H, m). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 207.0, 172.2, 171.7, 162.5, 156.4, 145.0, 143.8, 141.3, 140.2, 139.0, 127.7, 127.0, 125.0, 123.8, 122.1, 120.0, 98.8, 70.2, 70.1, 69.9, 66.8, 62.8, 49.1, 47.1, 40.7, 39.1, 38.7, 38.6, 36.9, 36.5, 31.7, 31.4, 29.7, 29.0, 27.7, 27.4, 24.1, 21.4, 20.1, 17.7. HRMS (ESI+) m/z calcd for $C_{42}H_{63}N_6O_8$ $(M+H)^+$: 779.47074, found: 779.47329.

5.3. Immobilization of BCN-Lev linker to agarose beads

Coupling linker-BCN conjugate **7** to agarose was performed with Sepharose™ beads from GE Healthcare (formerly Amersham, cat. no. 17-0430) according to the instructions of the manufacturer. The linker-BCN conjugate **7** was coupled to the beads at 1, 2, and

10 mM concentrations, for 16 h by end-over-end rotation. Beads were stored in 20% ethanol at 4 °C.

5.4. SPAAC of linker-coupled beads with 7-azidocoumarin

Bead solutions obtained above were individually incubated with 1 mM azidocoumarin in 50% methanol (1:1, v/v) at 20 °C in the dark, by shaking at 400 rpm. After 0.25, 0.5, 1, and 4 h, 1 μL of the mixture was used for fluorescence microscopy.

5.5. Cleavage of the linker

To follow the cleavage in time, samples of 15 μL beads of the 10 mM linker concentration and 15 μL 1 mM azidocoumarin were incubated for 2 h. Cleavage was carried out by addition of 30 μL 200 mM hydrazine and shaking at 400 rpm at either 4 or 37 °C in the dark. At each time point, a sample was centrifuged, and 50 μL of the supernatant was removed. The remaining 10 μL was used for fluorescence microscopy.

5.6. Determination of the loading

BCN–$(POE)_3$–NH_2 S1 (10 mM in DMSO/H_2O 1:9 v/v) was coupled to CNBr-activated Sepharose beads (0.1 g freeze-dried beads, GE Healthcare, cat. no. 17-0430-01) according to the instructions of the manufacturer. The beads were stored in EtOH/H_2O 1:4 (v/v) at 4 °C. Beads (100 μL) were incubated with azido-ester-coumarin S2 (100 μL of a 1 mM solution in DMSO/H_2O 1:99 v/v) for 4 h at rt, shaking in the dark at 600 rpm. Fluorescence of the beads was checked with fluorescence microscopy (Zeiss Axioskop 20, serial no. 451487, with an HB050 mercury lamp, using the UV filter).

Cleavage of the ester linkage was effected by incubation of 25 μL beads with 100 μL 10 mM NaOH at rt. The reaction was followed in time until fluorescence (ex 325 nm, em 452 nm) in solution showed saturation (about 3 h). A standard curve was prepared of 7-hydroxycoumarin in H_2O, which was followed in time to correct for coumarin degradation by NaOH.

5.7. Isolation of *Candida antarctica* lipase B from a protein mixture

For all experiments, a protein mixture of 10 μL 1 μg/μL AHA–CalB and 10 μL 1 μg/μL low molecular weight marker (LMW, Amersham, 17-0446-01) was used. To the mixture, 10 or 20 μL beads of the 10 mM linker concentration was added. This SPAAC mixture was shaken at 600 rpm for 2 or 18 h at 4, 20 or 37 °C, centrifuged, and the supernatant was transferred to a new tube and stored at 4 °C until loading it on SDS–PAGE. The beads were washed three or five times with 30 or 200 μL PBS with or without 0.5% SDS, possibly followed by three washing steps with 200 μL PBS, 2 h at 37 °C, shaking at 600 rpm. Cleavage was carried out for 18 h at 4 or 37 °C, shaking at 600 rpm. After cleavage, the samples were centrifuged and the supernatant was transferred to a new tube. 15 μL of both the SPAAC and cleavage supernatants, together with 5 μL 4× Laemmi sample buffer was used for SDS–PAGE (BioRad mini Protean 3). Markers were made by using 1 or 2 μL CalB and/or LMW, adding MQ up to 15 μL and 5 μL 4× sample buffer. To the remaining beads, 10 μL 4× sample buffer was added. All samples and markers were boiled at 95 °C for 5 min before loading them on the gel. Of the bead samples, 10 μL supernatant was loaded on gel, the other samples and markers (20 μL) were loaded completely.

Optimized conditions: to the protein mixture was added 10 μL beads of the 10 mM linker concentration. Subsequently, SPAAC was performed for 18 h at 37 °C. The beads were washed three times with 200 μL PBS and cleavage was performed for 18 h at 4 °C.

After running, the gels were stained with Coomassie brilliant blue or silver stain (PlusOne/PhastGel SDS, fixing solution and silver solution 30 min instead of 10 min.

5.8. NanoLC–MS/MS analyzes of tryptic protein digests

Proteins were in-solution digested using LysC and trypsin as described elsewhere.[26] Tryptic peptides were desalted and concentrated using stop and go elution (STAGE) tips according to Rappsilber et al.[27] NanoLC–MS/MS analyzes were performed using an Easy-nLC (Proxeon) liquid chromatograph coupled online to a LTQ FT Ultra (Thermo Fisher Scientific) mass spectrometer as described previously.[26] Chromatographic separations were performed using an in-house packed reversed-phase C18 column. Instrument settings are depicted in Supplementary Table A.

For peptide identification purposes, mass spectrometric data files were searched using the database search program Mascot and Mascot Distiller (Matrix Science Inc., USA, version 2.2 and version 2.3.2.0, respectively). A combined database was used for the searches containing the exact sequence of CalB, the *E. coli* Refseq database (release 29) and sequences of known contaminants such as human keratins, trypsin and Lys-C. Validation criteria are described in Supplementary Table A.

Acknowledgments

Prof. Hermen Overkleeft, Paul Geurink and Rian van den Nieuwendijk (Leiden Institute of Chemistry, Leiden University, the Netherlands) are kindly acknowledged for a generous gift of compound **1**. This research has been financially supported (in part) by the Council for Chemical Sciences of the Netherlands Organization for Scientific Research (NWO-CW, to F.L.D and NWO-Middelgroot, to D.J.L.).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.07.049.

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