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Two-step bioorthogonal activity-based proteasome profiling using copper-free click reagents: A comparative study ☆

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ABSTRACT

The development and application of bioorthogonal two-step labeling techniques receives much attention. Employing bifunctional proteasome probe **2** the efficiency of two-step labeling of recently published biotinylated cyclooctynes **3–5** is compared to Staudinger–Bertozzi ligation in cell extracts and living cells. While cyclooctynes **3–5** react faster and at a much lower concentration then the Staudinger–Bertozzi benchmark, background labeling is considerable with these reagents.

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1. Introduction

Activity-based protein profiling (ABPP) has emerged in recent years as a powerful strategy to map enzyme activity in complex biological samples. Generally, an activity-based probe (ABP) consists of an electrophilic trap that reacts covalently and irreversibly with an enzyme active site residue, an enzyme (family) recognition element and a reporter group (biotin, epitope tag and/or fluorophore) to assist in visualization and/or identification of the modified enzyme(s).[1,2] ABPP has met with considerable success in the profiling of esterases[3,4] and proteases[5–7] and the numerous literature reports that have appeared over the years include examples in which photo-activable groups are used to target enzymes (for instance, matrix metalloproteases)[8,9] that do not employ an amino acid side chain functionality as the nucleophile in the hydrolysis of their substrates. In recent years other enzyme families (glycosidases,[10–12] kinases)[13–15] were successfully modified using ABPP protocols, pointing towards the general applicability of this strategy. Next to visualization and identification of enzyme activities, ABPP has proven its merit in the assessment of potency and specificity of enzyme inhibitors in cell extracts and living cells.[16] In these studies, cells or cell extract has first been treated with a prospective inhibitor, for instance designed to act against a specific member of an enzyme family, after which a broad-spectrum (ABP) aimed at the enzyme family at large has been added to the sample to visualize remaining enzyme activities.[17]

A specific class of ABPs and subject of this paper are those that employ bioorthogonal chemistry as a means to introduce the visualization/identification tag. Inspired by the cell-surface glycoprotein engineering work of Bertozzi and co-workers, who employed a Staudinger ligation handle to tag azide-modified sialic acid residues,[18] we developed a protocol in which the three proteasome catalytic activities have first been modified with a pan-reactive, cell permeable inhibitor. Next, the cells were lysed, the azides modified with a Staudinger–Bertozzi reagent and the proteins resolved by SDS-PAGE. In this fashion we could modify the proteasome active sites with high efficiency and with relatively little background labeling.[19] In a later study we revealed that Staudinger–Bertozzi ligation could be achieved with complete conversion of all azides, but that a large excess of the Staudinger–Bertozzi reagent is needed.[20] Coinciding with our first studies, Cravatt and co-workers in a study on esterase ABPP demonstrated that essentially the same objective could be reached by making use of copper(I)-catalyzed azide-alkyne cycloaddition 'click' ligation.[21] Two-step ABPP is now recognized as an attractive alternative for direct ABPP especially in cases where the tag interferes with enzyme recognition (such as where the enzyme/enzyme family at hand is rather particular with respect to the nature of its substrate/substrate analogue) or hampers bioavailability of the probe. In part for this reason several research groups worldwide are

☆ Electronic Supplementary data (ESI) available: ^{1}H and ^{13}C spectroscopic data of new compound, Western blot readout and optimization attempt.

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involved in the development and application of new, more efficient bioorthogonal ligation strategies. Two main developments in the field are the evaluation of the Diels–Alder ligation,[22,23] with major advances especially in the use of inverse-electron-demand Diels–Alder reactions,[24–26] and in the application of cyclooctynes in copper-free, strain-promoted click ligations.[27–29] In this paper we set out to compare the efficiency and selectivity of three recently reported cyclooctyne derivatives in the two-step bioorthogonal ABPP of proteasome activities in cell extracts and in living cells, set against Staudinger–Bertozzi ligation as the benchmark.

2. Results and discussion

The panel of reagents employed in our studies is depicted in Figure 1. The strategy is based on previous work[20] from our group in which we employed an azide-modified BODIPY containing proteasome probe, effectively a direct ABPP and two-step ABPP in one. After proteasome labeling, modification of the azide with a bioorthogonal tag can be monitored in two ways, either by fluorescence read-out and looking for a mobility-shift of the modified proteasome active sites or by biotin-streptavidin blotting if the bioorthogonal tag is equipped with a biotin. We demonstrated the validity of both protocols in our work detailing that Staudinger–Bertozzi phosphane **6** can be used to quantitatively tag all azide-modified proteasome active sites.[20] As a positive control in these studies we employed a biotin-BODIPY modified proteasome probe. For the purpose of this study we synthesized or acquired azido-BODIPY-epoxomicin **2**, biotin-BODIPY-epoxomicin **1**,[30] biotin-cyclooctyne derivatives **3** (DIBO),[31] **4** (BCN)[32] and **5** (MFCO).[33] Cyclooctyne derivatives **3–5** were selected for the dual reason that (a) they have proven their merit in bioorthogonal ligation and (b) their synthesis has been well described.

As the first research objective (Fig. 2) we set out to establish the labeling efficiency of the three strain-induced click reagents to label azide modified proteasome active sites in cell extracts. To this end, lysates derived from human embryonic kidney cells (HEK293T) that express the constitutive proteasome as the single proteasome particle, were treated with azido-BODIPY-epoxomicin **2** at 5 μM final concentration. As can be seen (for instance, Fig. 2A lane 1) three bands are apparent after SDS-PAGE separation of the proteome content followed by fluorescence read-out obtained by scanning the wet gel slabs using a TYPHOON fluorescence scanner. Based on previous work we assigned the three bands to the constitutive proteasome active sites, β1, β2 and β5, as indicated in Figure 2A.[20,34] Biotin-cyclooctynes **3** (Fig. 2A), **4** (Fig. 2B) and **5** (Fig. 2C) were added to the cell extracts to final concentrations ranging from 1 to 100 μM and incubated at 37 °C for 1 h.

As can be seen from gel mobility shift all azides are quantitatively modified within this concentration range for all three cyclooctyne derivatives. The fully and bioorthogonally modified proteasome active sites now appear at a position approximating that of proteasome active sites labeled with the dual ABPP **1** (for instance, compare Fig. 2A lane 1 versus lane 2). In contrast, labeling with Staudinger–Bertozzi reagent **6** (Fig. 2D) is nearing completion only at 200 μM final concentration (a value corresponding to what we observed in our previous work)[20] and from this experiment it is apparent that strain-induced click ligation is the more efficient approach at least where it concerns amounts of reagent used. Closer perusal of the experiments involving the three click reagents reveals a marked difference in efficiency, with ligation to dibenzylcyclooctyne derivative **3** complete at 5 μM final concentration and ligation to the two other reagents **4** and **5** complete between 50 and 100 μM final concentrations. However, streptavidin readout of the Western blot indicated that the expected proteasome signals were overshadowed by tremendous background (Supplementary

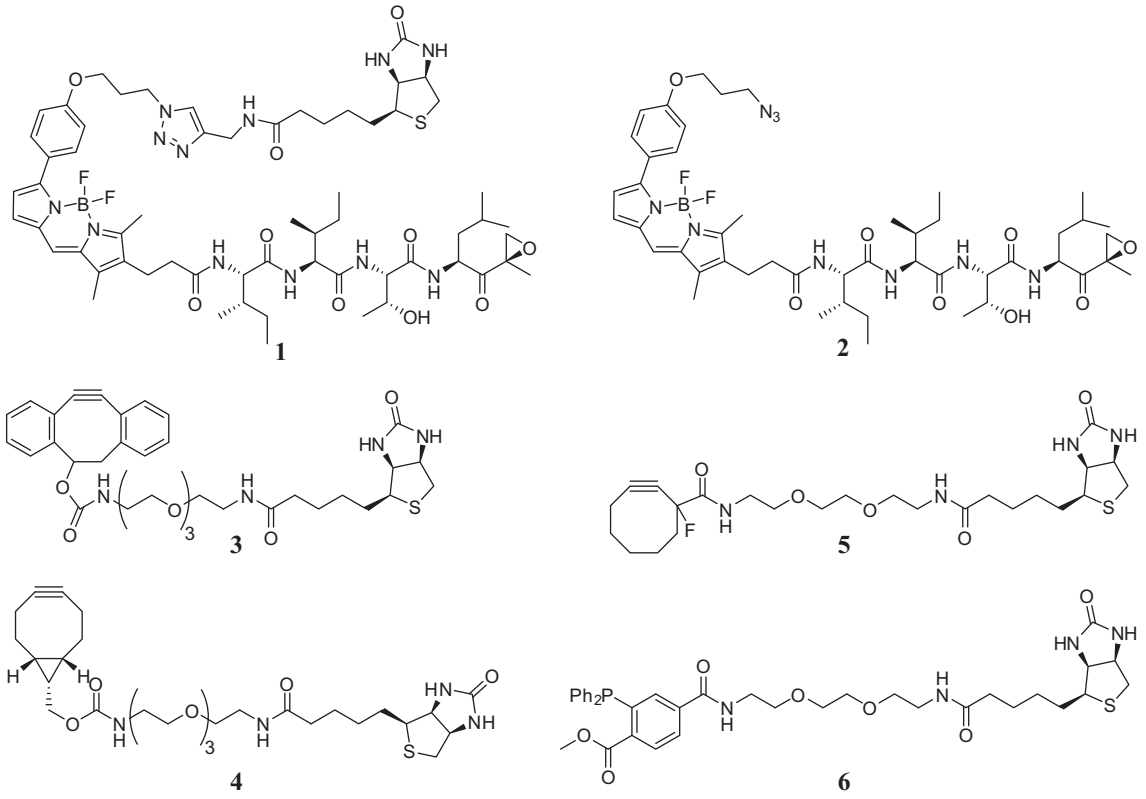

Figure 1. Structures of the compounds used in this study.

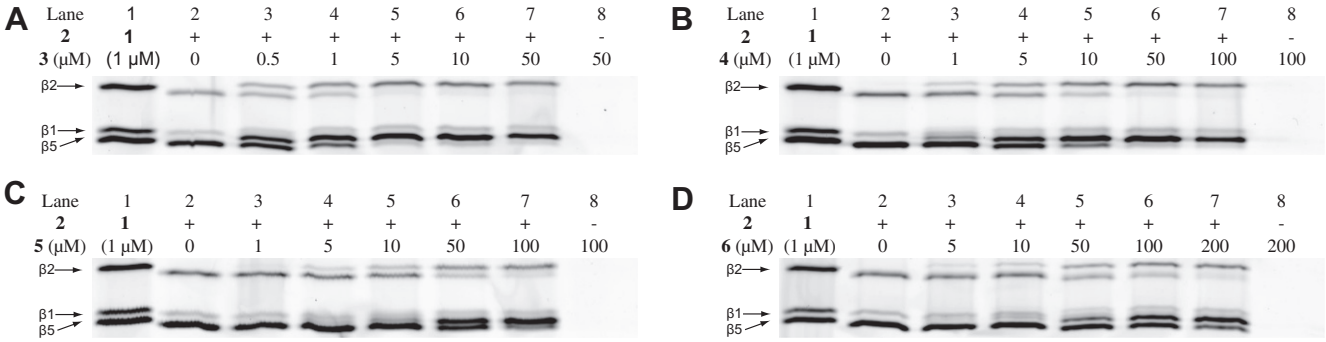

Figure 2. Determination of two-step label concentration for full conversion of labeled proteasome in cell lysate. HEK293T lysates labeled with fluorescent proteasome probe **2** were incubated with indicated concentrations of two-step labeling reagents (**A**. DIBO (**3**), **B**. BCN (**4**), **C**. MFCO (**5**) and **D** Biotin-phosphane (**6**)) for 1 h followed by SDS–PAGE separations of proteins and fluorescence readout of the wet gel slabs.

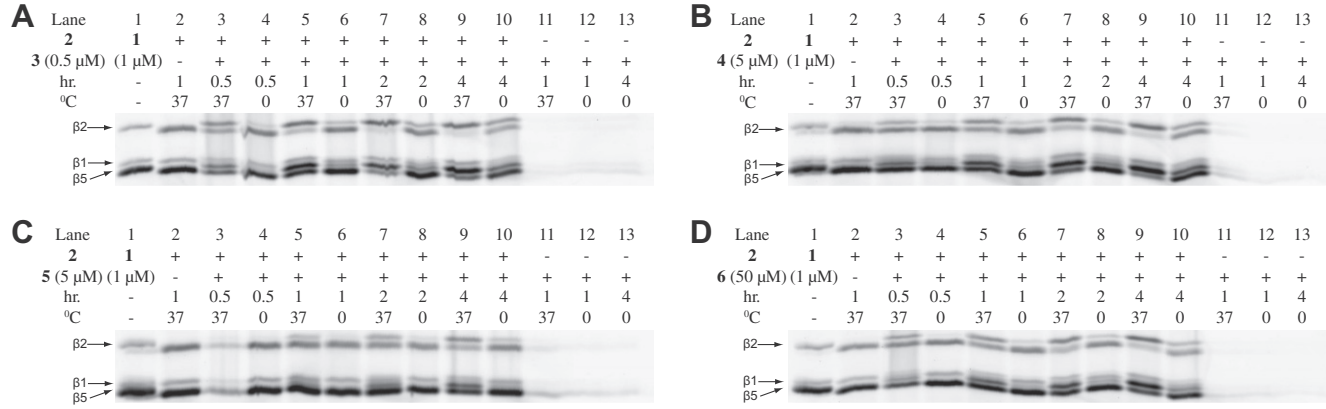

Figure 3. Time and temperature dependence of two-step labeling reaction in **2** labeled HEK293T cell lysates. Lysates (25 μg) were incubated with two-step reagents (**A**. DIBO (**3**), **B**. BCN (**4**), **C**. MFCO (**5**) and **D** Biotin-phosphane (**6**)) for indicated time and temperature followed by C/M precipitation. Proteins were resolved by SDS–PAGE and fluorescence detected in the wet gel slabs.

data Fig. S1), persisting through some preliminary optimization attempts (Supplementary data Fig. S2).

In the next experiment we investigated the time and temperature dependent labeling efficiency of the panel of bioorthogonal ligation reagents **3** (0.5 μM final concentration), **4** (5 μM), **5** (5 μM) and **6** (50 μM). As expected, labeling at 0 °C proceeded much slower than labeling at 37 °C for all reagents (Fig. 3). In these experiments we applied final concentrations for all compounds that are below those identified in the first experiment as needed for full conversion. From the experiments at 37 °C it is apparent that DIBO **3** is the most reactive of the series, with significant labeling after 30 min. Maximal labeling with compounds **4** was achieved after two hours, while compounds **5** and **6** show increase in labeling up to four hours of reaction time. These results roughly correspond to the reported reaction rates of the cyclooctynes in literature in which DIBO has the highest rate[31], followed by BCN[32] and MFCO[33] with the lowest reaction rate in this series. Here, it should be noted that one has to be careful in comparing the literature reaction rates, since experimental conditions in which these were obtained might differ.

In a final experiment we established whether both activity-based probe and bioorthogonal tag could be applied in living cells (Fig. 4). To our surprise, all three the click reagents, and indeed also the Staudinger–Bertozzi reagent, proved cell-permeable and in all four experiments significant bioorthogonal proteasome tagging was observed. This is apparent from the multiple bands that appear on gel as visualized by in-gel fluorescence detection, and in most cases also after biotin-streptavidin blotting. With respect to this latter analysis some important differences were observed. As expected based on previous work, Staudinger ligation gives clean bands corresponding to the proteasome active sites after Western blotting.

Compound **4** and to a lesser extend also compound **5** delivered proteasome bands based on probing for a biotin moiety. The background labeling, albeit much lower than in experiments in lysates, is much more prominent for **4** and **5** compared to Bertozzi-Staudinger **6**. Rather surprisingly, and as yet not understood, is the almost complete absence (vague bands are visible) in the experiment employing biotin-cyclooctyne **3**. This result is at odds with the clear positive bioorthogonal labeling of proteasome subunits as visualized by fluorescence read-out.

3. Conclusions

In conclusion, our results underscore previous reports on the versatility of strain-promoted cyclooctynes in bioorthogonal chemistry, in particular with respect to two-step activity-based protein profiling. The three reagents tested, and in particular cyclooctyne **3**, outperform Staudinger–Bertozzi ligation reagent **6** where it comes to reactivity and stoichiometry. At the same time, it is equally clear that neither of the reagents is truly bioorthogonal in the sense that they modify the desired target, and nothing else. Background labeling as observed with the cyclooctynes in general is extensive, rather more so than observed by Staudinger–Bertozzi ligation, even though lower final concentrations of the cyclooctynes are needed. Apparently reactions between these highly strained systems and functional groups inherent to biological samples are more prominent, whereas the intrinsic sensitivity of the

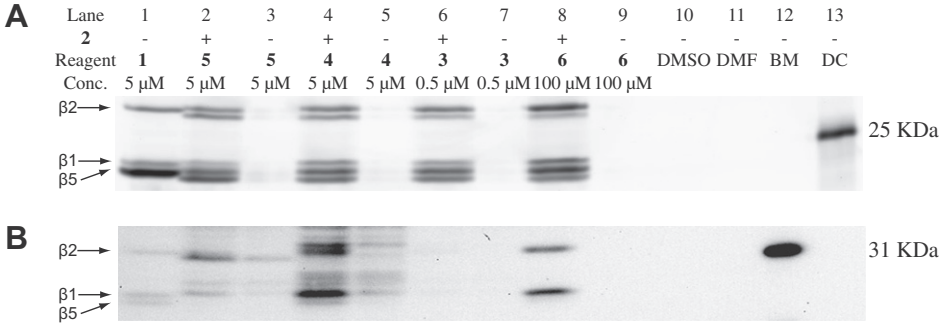

Figure 4. Cell permeability of two-step labeling reagents **3–6**. HeLa cells are incubated with **2** (5 μM) for 2 h followed, after washing, by **3–6** for 4 h. After washing, cells were lysed and proteins resolved by SDS–PAGE followed by western blotting. A. fluorescence readout and B. streptavidin blot of labeled cell lysates. BM = biotinylated protein marker. DC = dual color marker.

biotinylated phosphane towards oxidation[27,35] is the main causative for the need of large excesses of this reagent. Interestingly, all ligation reagents proved cell permeable and a general trend observed is that bioorthogonal ligation in living cells is much cleaner with respect to background labeling than performing this step in cell extracts. Future research involving pull-down followed by proteomics analysis is needed to unearth the nature of the proteins modified by the cyclooctyne reagents at functionalities other than an azide, and possibly also the nature of these functionalities. In addition, the stability of the cyclooctyne reagents under physiological conditions should be studied. In a more general sense, our results also underscore the need for the development of fast reacting, more selective bioorthogonal ligation reagents that can be used at low concentrations, especially for application in cell extracts.

4. Experimental

All reagents were commercial grade and were used as received unless indicated otherwise. Dichloromethane (DCM) and dimethyl formamide (DMF, Biosolve) were stored on 4 Å molecular sieves. Reactions were monitored by TLC-analysis using DC-alufolien (Merck, Kieselgel 60, F254) with detection by UV-absorption (254 nm), spraying with 20% H_2SO_4 in ethanol followed by charring at ~150 °C, by spraying with a solution of $(NH_4)_6Mo_7O_{24}$ $4H_2O$ (25 g/L) and $(NH_4)_4Ce(SO_4)_4{\cdot}2H_2O$ (10 g/L) in 10% sulfuric acid followed by charring at ~150 °C or spraying with an aqueous solution of $KMnO_4$ (7%) and KOH (2%). Column chromatography was performed on Screening Devices (0.040–0.063 nm). HRMS were recorded on a LTQ Orbitrap (Thermo Finnigan). ^{1}H- and ^{13}C-APT-NMR spectra were recorded on a Bruker AV-400 (400/100 MHz) equipped with a pulsed field gradient accessory. Chemical shifts are given in ppm (δ) relative to tetramethylsilane as internal standard. Coupling constants are given in Hz. All presented ^{13}C-APT spectra are proton decoupled.

DIBO **3**,[31] BCN **4**,[32] Staudinger–Bertozzi reagent **6** and compound **1** and **2**[30] were synthesized as described in literature. MFCO **5** was synthesized by literature procedure[33] from 1-fluorocyclooct-2-ynecarboxylic acid (21.2 mg, 126 μmol) and biotinylated diaminotriethyleneglycol[20] (60 mg, 160 μmol) to yield MFCO **5** (27 mg, 51 μmol, 41% over 2 steps). ^{1}H NMR (400 MHz, MeOD, mixture of diastereomers) δ ppm 4.52 (dd, J = 7.7, 4.9 Hz, 1H), 4.33 (dd, J = 7.8, 4.4 Hz, 1H), 3.73–3.54 (m, 8H), 3.47–3.35 (m, 4H), 3.29–3.15 (m, 1H), 2.95 (dd, J = 12.7, 4.9 Hz, 1H), 2.73 (d, J = 12.7 Hz, 1H), 2.40–2.21 (m, 6H), 2.18–1.56 (m, 9H), 1.54–1.41 (m, 3H). ^{13}C NMR (101 MHz, MeOD) δ ppm 176.22, 176.13, 171.03 (J = 24.9 Hz), 166.07, 110.19, 110.09, 95.2 (J = 185.4 Hz), 88.28 (J = 31.5 Hz), 71.27, 70.61, 70.20, 63.35, 61.60, 57.02, 47.69 (J = 24.8 Hz), 41.06, 40.42, 40.30, 36.73, 35.00, 30.12, 29.78, 29.50, 26.86, 26.76, 21.07, 21.05. HRMS Calcd for $[C_{25}H_{40}F_1N_4O_5S_1]^+$ 527.26980, found 527.26986.

5. Two-step labeling of fluorescently labeled proteasomes in lysate

HEK293T cells were cultured on DMEM supplemented with fetal calf serum (FCS, 10%), penicillin (10 units mL^{-1}), and streptomycin (10 mg mL^{-1}) in a CO_2 (5%) humidified incubator at 37 °C. Some 15×10^6 HEK293T cells were seeded on a 15 cm petri dish and cultured overnight. **2** (5 μM final concentration, 100x DMSO stock) or DMSO was added in 10 mL fresh medium and incubated for 2 h at 37 °C. The medium was removed and the cells were harvested in cold PBS and washed with cold PBS (3x) before the pellet was flash frozen (N_2 (l)). The pellet was resuspended in 400 μl digitonin lysis buffer [Tris pH 7.5 (50 mM), sucrose (250 mM), $MgCl_2$ (5 mM), dithiothreitol (DTT; 1 mM), digitonin (0.025%)] and incubated on ice for 10 min before spinning down at 16100 relative centrifugal force (rcf) at 0 °C. The supernatant was collected and the protein concentration was determined by the Bradford assay. 25 μg protein in 9 μl lysis buffer was treated with 10x stock of two-step labeling reagent (in DMSO for **3**, **4**, **5**, in DMF for **6**; no solubility issues were observed when dissolving the compounds in DMSO (20 μM) or adding the DMSO stocks to lysate) or **1** and incubated for 1 h at 37 °C before the reaction was stopped by chloroform/methanol precipitation.[36] The pellet was redissolved in 10 μl 2x Laemli's sample buffer. The proteins were resolved on 12.5% SDS-PAGE gel and the fluorescently labeled proteasome subunits visualized by scanning the wet gel slab on a Typhoon variable mode imager (Amersham Biosciences, using the Cy3/TAMRA setting (λ_{ex} 532 nm, λ_{em} 560 nm)). Proteins were electrotransferred to polyvinylidene (PVDF) membranes. The blots were blocked with BSA (1%) in TBS-Tween 20 (0.1% Tween 20) for 30 min at RT, hybridized for 30 min with streptavidin/HRP (1:10,000) in blocking buffer, washed, and visualized with the aid of an ECL+ kit (Amersham Biosciences).

6. Two-step labeling of fluorescently labeled proteasomes in living cells

HeLa cells (75. 10^4) were seeded and grown overnight on 2 mL medium mentioned above. The medium was removed and replaced with 1 mL fresh medium containing a 100x DMSO stock of **1** or **2** (5 μM end concentration) followed by 2 h incubation. The medium was removed and the cells were washed with PBS. 1 mL fresh medium containing a 100x stock solution of the click reagent (in DMSO for **3**, **4**, **5**, in DMF for **6**) was added and incubated for 4 h, after which the cells were scrutinized under visual light microscope. The cells were washed with PBS, harvested and washed with PBS (3x) before being lysed as described above.

25 μg protein was resolved on SDS-PAGE gel and western blotted as described above.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.06.037.

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