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Affinity labeling of the proteasome by a belactosin A derived inhibitor

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ABSTRACT

Belactosin A is a potent proteasome inhibitor isolated from *Streptomyces* metabolites. Here we show that a hydrophobic belactosin A derivative, dansyl-KF33955, can covalently, and specifically, affinity label the catalytic subunits of the 26S proteasome, which consists of the 20S protein degrading core particle and the 19S regulatory particles. The labeling of catalytic subunits proceeds faster in intact proteasomes in vivo than in isolated 20S core particles. These data suggest that the 19S regulatory particle may facilitate entry of the inhibitor into the 20S core particle. This cell-permeable chemical probe is an excellent tool with which to study the interactions of this proteasome inhibitor with proteasomes in intact cells.
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The 26S proteasome is a large protein complex containing enzymes that degrade proteins conjugated to ubiquitin and plays a central role in selective proteolysis in eukaryotes. The 26S proteasome is composed of the 20S core particle (CP) bearing several catalytic sites for protein degradation and two 19S regulatory particles (RP) involved in the recognition and unfolding of ubiquitinated proteins.[1] Structurally, the 20S CP is a 700-kDa complex with a hollow cylindrical-shape composed of four stacked heptameric rings. The two inner rings each consist of seven β subunits and the two outer rings each consist of seven α subunits, providing an enclosed cavity in which target proteins are degraded. Openings at the two ends of the CP enable proteins to enter the cavity. Each end of the CP associates with an RP that contains ubiquitin binding sites and ATPase active sites. Thus, the RP recognizes, unfolds and translocates target proteins into the catalytic cavity for degradation. Within the catalytic cavity, three β subunits (β1, β2 and β5) contain peptidase active sites for caspase-like peptidylglutamyl protein hydrolyzing (PGPH), trypsin-like, and chymotrypsin-like activities, respectively. Protein degradation by the proteasome plays key regulatory roles in metabolic adaptation, cell differentiation, cell cycling, stress response, regulation of transcriptional factors, as well as generation of epitopes presented by the major histocompatibility complex class I receptors and quality control through removal of abnormal proteins.

Small molecule inhibitors of the proteasome are important tools in developing new molecular-targeting drug for cancer chemotherapy, as well as providing a tool for understanding the ubiquitin-proteasome system.[2] We previously identified the novel proteasome inhibitor belactosin A (Fig. 1, Structure **1**) from *Streptomyces* metabolites and showed that this compound inhibits human cancer cell proliferation.[3] Interestingly belactosin A is a strong inhibitor of the chymotrypsin-like activity in the purified 20S proteasome CP in vitro.[3] Structure-function studies indicate that the β-lactone ring of belactosin A is necessary for full inhibition of proteasome activity.[3] More recently, an X-ray crystallographic analysis revealed that a belactosin analog, homobelactosin C, covalently binds to the active sites of the β5 subunit through esterification of the β-lactone ring to a threonine residue that is present in each catalytic center.[4] Thus, the fundamental mechanism by which peptidase activities in CP are inhibited by belactosins has been clarified. However, binding of the inhibitors to the 26S proteasome, the intracellular complex of CP and RP, is not well understood. RP may regulate inhibitor entry into the 20S CP cavity. To analyze intracellular interactions between inhibitors and the intact 26S proteasome, a cell-permeable affinity-labeling probe derived from a potent proteasome inhibitor is needed.

A hydrophobic belactosin A derivative, KF33955 (Fig. 1, Structure **2**), in which a benzyl group has been introduced at the carboxyl group of belactosin A, exhibits a 100-fold greater growth inhibition of HeLa cells, relative to belactosin A, which is presumably due to the improved cell-membrane permeability of KF33955.[3] To detect covalent complexes of the belactosin A derivative and proteasomal components with high sensitivity, we prepared dansyl-KF33955 (Fig. 1, Structure **3**) as an affinity probe. In this report, we demonstrate the successful labeling of β subunits

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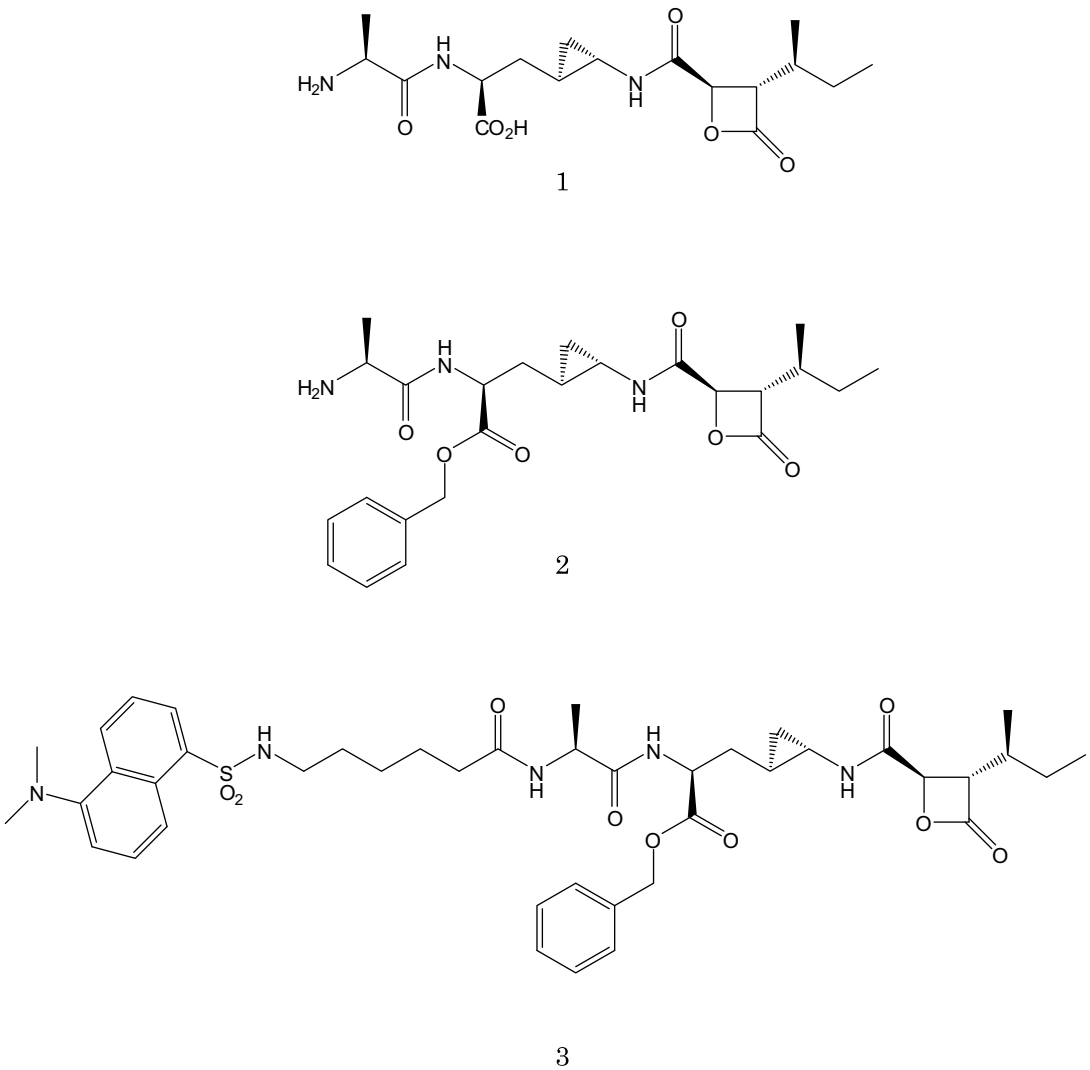

Figure 1. Structures of belactosin A (**1**), KF33955 (**2**) and dansyl-KF33955 (**3**).

responsible for peptidase activities and compare the temporal pattern of labeling between purified CP in vitro and the 26S proteasome in vivo.

Belactosin A was isolated from the broth of *Streptomyces* KY11780 cultures, and KF33955 was prepared from belactosin A, as described previously.[3] Dansyl-KF33955 was prepared by coupling a succinimidyl ester of dansylaminohexanoic acid to KF33955.[5] Figure 2 shows the MSMS spectrum of dansyl-KF33955 (precursor ion *m*/*z* 806.37). The mass signals *m*/*z* 347.14, 389.20, 418.17, and 698.32 were consistent with the predicted sizes of its cleavage products (Fig. 2 inset). Most of other signals in the low mass range (indicated by the asterisks in Fig. 2) were the same values as those observed in MSMS measurements of KF33955 (data not shown). These data are in full agreement with the structure proposed.

To examine inhibition by belactosin A derivatives and other well-known proteasome inhibitors, such as *clasto*-lactacystin β-lactone and MG132, different concentrations of each compound were added to reaction mixtures containing purified human erythrocyte 20S CP and a synthetic substrate specific for each peptidase.[6,7] The calculated IC_{50} values are shown in Table 1. Dansylation of the hydrophobic belactosin A (KF33955) resulted in a marked enhancement of the inhibition of the chymotrypsin-like activity and PGPH activity. Dansylation reduced inhibition of the trypsin-like activity, probably due to loss of the positive charge in the amino terminus of belactosin A. However, it is of interest that introduction of the dansyl moiety to KF33955 markedly increased the inhibitory effect on the chymotrypsin-like site, which is thought to be the major site of protein degradation by proteasomes.[6]

Since belactosin A represses proliferation of cancer cells[3], we compared the ability of the belactosin A derivatives to inhibit HeLa cell growth (Table 1).[8] The hydrophobic derivative KF33955 exhibited a more potent inhibition of cell proliferation compared to the parent compound, belactosin A. This high potency may be the result of the increased membrane permeability of KF33955. The inhibitory effect of dansyl-KF33955 on cell proliferation is greater than belactosin A, but 20-fold less potent than KF33955, suggesting that the dansyl moiety reduced the membrane permeability concomitantly with the enhanced potency of the proteasome inhibitory activity. Given that addition of a biotin moiety to belactosin A severely decreased its inhibitory potency in HeLa cells (IC_{50} value of ~350 μM, data not shown), dansylation appears to provide a more advantageous affinity label for tracing labeled components of the proteasome, as reported previously.[9]

To examine the binding characteristics of dansyl-KF33955 to the proteasome in vivo, we first determined an inhibitor concentration appropriate for clear detection of labeled catalytic β subunits by Western blotting using anti-dansyl antibody. When HeLa cells were cultured in the presence of 1 μM inhibitor, labeled

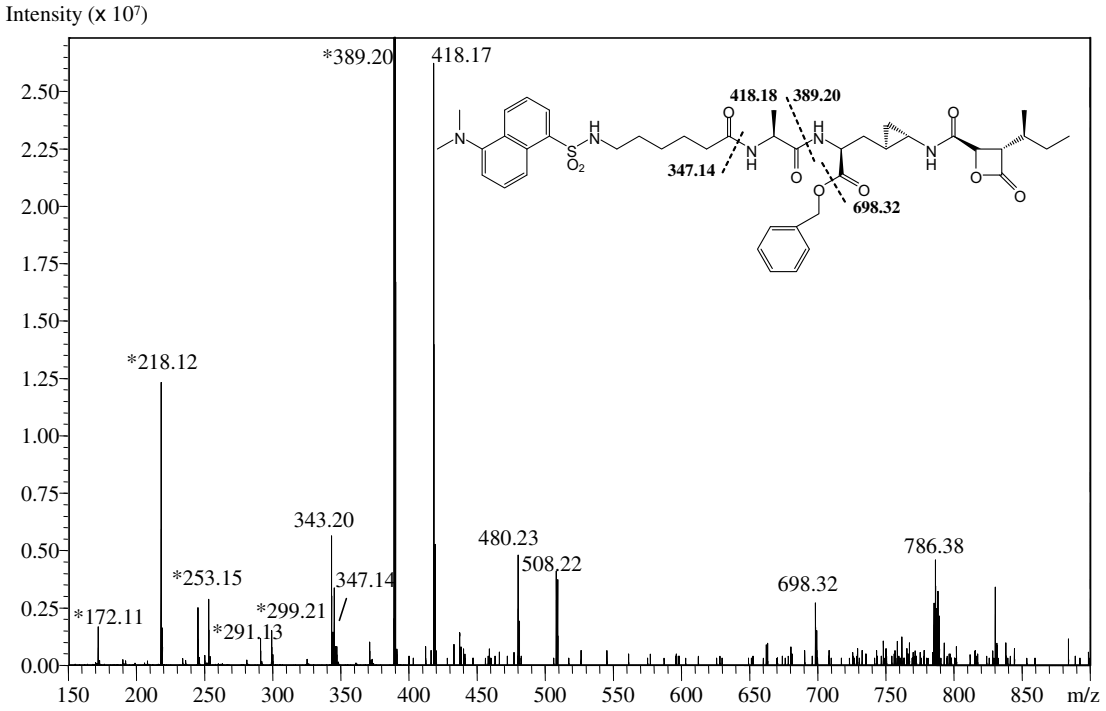

Figure 2. The MSMS spectrum of dansyl-KF33955. The asterisks indicate common peaks of KF33955.

Table 1
IC_{50} values of human erythrocyte 20S proteasome enzymatic activities and growth inhibition of HeLa cells by inhibitors

Compound	Proteasome inhibition[a] (μM)			Growth inhibition[b] (μM)
	Chymotrypsin-like activity	Trypsin-like activity	PGPH activity	
Belactosin A	0.82	4.9	14	37
KF33955	0.34	0.45	3.7	0.50
Dansyl-KF33955	0.029	2.1	0.15	9.8
clasto-lactacystin β-Lactone	0.029	0.69	8.3	ND
MG132	0.011	2.1	0.12	ND

ND: not determined.
[a] IC_{50} values of human 20S proteasome activity.
[b] IC_{50} values of growth in HeLa cell cultures.

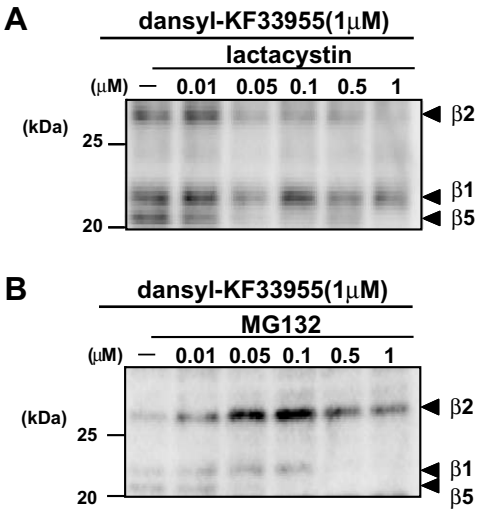

Figure 3. Western blotting of dansyl-crosslinked subunits of the 26S proteasome treated with 1 μM dansyl-KF33955 in intact HeLa cells in the presence of vatious concentrations of *clasto*-lactacystin β-lactone (A) and MG132 (B).

subunits could be detected without notable cell death (data not shown). Figure 3 shows dansyl-KF33955 labeling of the catalytic subunits in proteasomes in HeLa cells (in vivo) and its competitive inhibition by various concentrations of *clasto*-lactacystin β-lactone and MG132.[10] The bands detected by the anti-dansyl antibody were confirmed to be the β1, β2, and β5 subunits by performing Western blotting using antibodies specific for each subunit (data not shown). After incubation of HeLa cells with *clasto*-lactacystin β-lactone, dansyl-KF33955 only labeled the β1 subunit suggesting that dansyl-KF33955 and *clasto*-lactacystin β-lactone compete with another for binding to the same sites in the β2 and β5 subunits (Fig. 3A). After incubation of HeLa cells with MG132, dansyl-KF33955 only labeled the β2 subunit, suggesting that dansyl-KF33955 and MG132 compete with one another for binding to the same sites in the β1 and β5 subunits (Fig. 3B). These results indicate that dansyl-KF33955 specifically binds to the β1, β2 and β5 subunits of 20S CP in vivo. Figure 4A shows the time-dependent labeling of catalytic subunits in proteasomes in HeLa cells (in vivo) and Figure 4B shows the labeling in the 26S proteasome extracted from HeLa cells (in vitro).[11,12] Figure 4C shows labeling of subunits in the similar condition except for using the purified human erythrocyte 20S CP.[12] Within as little as 0.5 h, dansyl-KF33955 was covalently bound to the β1, β2, and β5 subunits in the 26S proteasomes, both in vivo *and* in vitro. The labeled subunits appear to have reached maximal levels within 0.5 h, and these levels were maintained after 1 h of culture, but decreased significantly by 3 h in the presence of the inhibitor (Fig. 4A and B). When labeling of subunits in the erythrocyte CP, in which the 19S RP was excluded, was examined, the kinetics of labeling of all subunits was slower than that of the 26S proteasome labeling; the maximal labeling required at least 1 h or more of inhibitor treatment, and the maximum level of labeled subunits was sustained for 10 h (Fig. 4C).

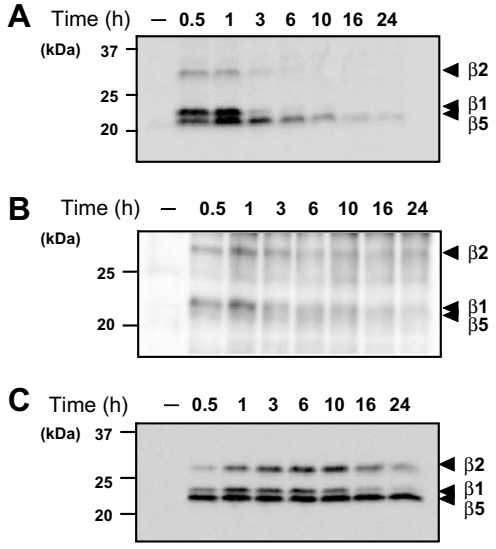

Figure 4. Western blotting of dansyl-crosslinked subunits of the 26S proteasome in intact HeLa cells (A) and HeLa cell extract (B) and the purified erythrocyte 20S CP (C) treated with 1 μM dansyl-KF33955 for the indicated times. The lane indicated by the bar contains untreated control samples. The proteasome subunit bands crosslinked to dansyl-KF33955 were detected using an anti-dansyl antibody.

Protein substrates to be degraded enter the CP cavity through a channel that is opened upon substrate association with the RP through a mechanism in which Rpt subunits with ATPase activity mediate the opening.[13] Without the 19S RP, the α ring of the 20S CP would have no obvious path for substrates to access the active center chamber of the β ring.[14] The in vivo and in vitro labeling of β subunits in the 26S proteasome, which consists of 20S CP and 19S RP, is faster than labeling of the isolated CP. This result suggests that entry of dansyl-KF33955 into the CP cavity may be facilitated through an RP-induced channel, although the interaction of the inhibitor with RP components responsible for the channel opening remains to be verified.

The time-dependent disappearance of labeled subunits in vivo may result from two events. First, the bound inhibitor may be gradually released through hydrolysis of the ester bond between the β lactone ring of the inhibitor and the threonine residue in the active site. Second, the inhibitor may be unstable under aqueous conditions so that the free inhibitor concentration can not be maintained. In concert with this, the inhibitor was gradually degraded, even in purified water, and it was completely lost overnight, observed in MALDI-TOF MS measurements (data not shown). The labeled subunits in the 26S proteasome disappeared more rapidly (Fig. 4A and B) than those in isolated erythrocyte CP (Fig. 4C). Although further studies are needed to understand the mechanism underlying this difference, an intriguing hypothesis is that the RP may stimulate hydrolysis of the ester bond formed between the inhibitor and threonine residue, suggesting a novel function of RP in the proteasomal protein degradation pathway. Alternatively, proteasomes containing subunits whose active sites have been esterified may be subject to protein degradation.

In this study, we report that a belactosin A derivative labeled with an immuno-detectable tag, dansyl-KF33955, covalently binds to the catalytic β-subunits of the proteasome both in vitro and in vivo. This chemical probe is an excellent tool to study proteasome function. Moreover, fluorescence detection of the dansyl moiety may allow in vivo identification of proteasomes with labeled subunits and the intracellular fate of the proteasome could thereby be traced. Optimization of conditions for fluorescence detection is underway.

Acknowledgments

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5. Five hundred nmol of KF33955 and an equivalent amount of the succinimidyl ester of dansylaminohexanoic acid (Invitrogen Co., Carlsbad, CA) were dissolved in 300 μl of dimethylformamide containing 0.01% triethylamine and stirred for 16 h at room temperature. Dansyl-KF33955 was purified by reversed-phase high-performance liquid chromatography. The structure was supported by ESI–MSMS analysis on an Shimadzu LCMS-IT-TOF. (M^++H): 806.370 (calcd 806.372).
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7. The inhibitors were mixed with 100–200 ng of the human 20S proteasome CP isolated from human erythrocytes (BIOMOL International L.P.) in enzyme assay buffer (25 mM HEPES pH 7.6, 0.5 mM EDTA, and 0.03% SDS) in 96-well plates. SDS was omitted in assays of the trypsin-like activity. The chymotrypsin-like, trypsin-like, and PGPH catalytic activities were assayed using the fluorogenic peptide substrates, Suc-LLVY-AMC, Boc-LRR-AMC, and Z-LLE-AMC, respectively (Peptide Institute Inc., Osaka, Japan). The assays were initiated by addition of the AMC-substrate. The linear increase in fluorescence was recorded for up to 1 h using a fluorescence plate reader (380 nm excitation, 460 nm emission). Enzyme activity was estimated using the slope of the fluorescence increase, and inhibition is represented by IC_{50} values.
8. HeLa cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum and penicillin/streptomycin with 5% CO_2. The viability of HeLa cells was tested using an MTT assay after incubation with inhibitors added at various concentrations for 48 h. Treatment with (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl) tetrazolium bromide (MTT) was performed according to the manufacturer's protocol. Absorbance at 570 nm was measured using a plate reader.
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10. HeLa cells were mixed with various concentrations of clasto-lactacystin β-lactone and MG132 and incubated for 1 h at 37 °C in serum-free medium. Next, they were mixed with 1.0 μM dansyl-KF33955 and incubated for another hour. To stop the assay, the cells were collected by centrifugation (15,000*g*, 5 min), treated with denaturing buffer (60 mM Tris–HCl pH 8.8, 5 M urea, 1 M thiourea, 5 mM EDTA, 1% CHAPS, 1% Triton X-100) at 4 °C, and lysed by sonication. The samples were separated by SDS–PAGE and protein bands on the gel were visualized by SYPRO-Red staining (Invitrogen Co.). Images were acquired using a FLA3000 laser scanner (Fuji Photo Film Co., Ltd., Tokyo, Japan). Following transfer onto a PVDF membrane, immunodetection using an anti-dansyl monoclonal antibody (Santa Cruz Biotechnology) was performed. HRP-conjugated anti-rabbit IgG (MBL) was used as the secondary antibody. Signals were visualized using an ECL Plus detection kit (Amersham Biosciences) and an LAS-1000 Plus image analyzer (Fuji Photo Film Co., Ltd.).
11. Extraction of 26S proteasome from HeLa cells was basically performed by the method reported by Kisselev, A.F; Callard, A; Goldberg, J. Biol. Chem. 2006, 281, 8582. Briefly, HeLa cells were grown up to 50–80% confluency. After harvested and washed three-times with ice-cold phosphate-buffered saline, the cells were resuspended in the homogenization buffer (50 mM Tris–HCl pH7.5, 250 mM sucrose, 5 mM $MgCl_2$, 0.5 mM EDTA, 1 mM dithiothreitol, and 1 mM ATP). The cells were permeabilized by the addition of 0.025% digitonin and incubation on ice for 5 min. The cytosol was "squeeze out" by centrifugation for 15 min at 15,000*g*. The supernatants were used for the next experiments.
12. HeLa cells were mixed with 1.0 μM dansyl-KF33955 and incubated for a given incubation time at 37 °C in serum-free medium. To stop the assay, the cells were collected by centrifugation (15,000*g*, 5 min), treated with the denaturing buffer. For assays using the extracts 26S proteasome and the purified 20S CP, solutions of them were mixed with 1.0 μM dansyl-KF33955 for various times. The cell extracts and the purified 20S CP solutions were then directly diluted with 2× denaturing buffer. The samples were separated by SDS–PAGE. Following transfer onto a PVDF membrane, immunodetection using the anti-dansyl monoclonal antibody was performed as described above.[10]
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