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A trifluoromethylphenyl diazirine-based SecinH3 photoaffinity probe†

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The synthesis of a trifluoromethylphenyl diazirine photoaffinity probe of the cytohesin inhibitor SecinH3 is described. The probe exhibits improved labelling efficiency over a benzophenone-based probe and thus is more suitable for photoaffinity labelling in complex biological samples.

Photoaffinity probes are powerful tools for investigating interactions between small organic molecules and proteins. When combined with mass spectrometry, the identification of both the interacting proteins and their respective interaction sites becomes possible.[1–4] These probes contain three crucial elements: (i) an affinity unit, *i.e.* the ligand which interacts with the target protein, (ii) a photoreactive moiety, which upon irradiation covalently binds the probe to the protein, and (iii) a reporter group, for detection and enrichment of the labelled protein. An ideal photoactivatable group should be small, stable in the absence of light, and highly and indiscriminately reactive upon irradiation at wavelengths which do not damage the biological sample.[5] Commonly used photoactivatable groups include aryl azides, benzophenones, and 3-aryldiazirines.[6] Though the advantages of 3-trifluoromethyl-3-phenyl-diazirine (TPD)[7] over the other photoactivatable groups were recognised from their inception, its use in photoaffinity labelling was limited in the past by synthetic restrictions and limited availability of TPD derivatives with convenient reactive groups.[8,9] The recent establishment of various diazirine compatible reactions[8,10] and the increasing commercial availability of TPD derivatives now allow their more widespread use. The exceptional reactivity at relatively long wavelengths ($\approx$360 nm), the superior properties, and its structure make TPD an ideal photoactivatable group for the modification of our small organic cytohesin inhibitor SecinH3. In particular the lack of formation of slowly reacting isoforms, as observed for benzophenones and aryl azides,[5,11,12] is an important advantage of TPD for experiments aimed at binding site identification.[13]

Cytohesins are a class of small guanine nucleotide exchange factors (GEFs) for the Arf (adenosine diphosphate ribosylation factor) family of G-proteins. They are $\approx$47 kDa multidomain proteins with a coiled-coil amino terminal domain, a central Sec7 domain bearing the GEF function, and a pleckstrin homology (PH) domain. The 1,2,4-triazole derivative SecinH3 **1** (Fig. 1) binds the Sec7 domain of cytohesin-1, -2 (ARNO), and -3 and inhibits the GDP/GTP exchange.[14,15] Application of SecinH3 in human cells, flies and mice revealed the involvement of cytohesins in insulin signalling[14,16] and recently their role as cytoplasmic ErbB receptor activators.[17] Moreover, SecinH3 is increasingly being used as an indirect Arf6-inhibitor, owing to the requirement of cytohesins for Arf6 activation and the absence of any direct, specific Arf6 inhibitor.[18–24]

Using the benzophenone derivatised probe Bio-SecinPP **2**, we have previously demonstrated specific binding of SecinH3 to the Sec7 domain of the members of the cytohesin family.[25] However, the labelling efficiency was insufficient for allowing the determination of the binding site by mass spectrometry of LC-separated protein fragments or affinity based protein profiling in whole proteomes.

Here we report the design and synthesis of SecinH3-TPD **3** (Fig. 1), a SecinH3 probe for the determination of the SecinH3 binding site on cytohesins, the analysis of specificity in complex mixtures and as a lead structure for analyses in living cells, tissues, or organisms. Additionally, we compare the results of the SecinH3-TPD probe with those of an analogous benzophenone containing compound. For SecinH3-TPD we expected an improved labelling efficiency, which we confirmed by this work.

Structure–activity-relationship (SAR) studies on SecinH3 showed that removal of the thiophenol moiety in R_1 led to loss of inhibition of GEF activity. On the other hand, modification of this group was tolerated to some extent. Similarly, the replacement of the methoxy group for other ethoxy substituents affects the biological activity of the compound only to a minor extent.[25] These results indicate the suitability of the terminal thiophenyl group as a site for substitution with the TPD moiety by amine coupling reaction with commercially available 4-(1-azi-2,2,2-trifluoroethyl)-benzoic acid (TPD-COOH), and of the 3-methoxy residue as a site for reporter group attachment.[25]

To allow the enrichment of labelled protein in proteome-wide profiling experiments, we employed desthiobiotin coupled to the SecinH3-probe *via* an aminoethoxyethyl spacer (Fig. 1). Desthiobiotin, an analogue of biotin, binds less tightly to streptavidin. Desthiobiotin-containing probes can therefore be eluted from streptavidin by washing with biotin-containing buffers, allowing for straightforward recovery of proteins labelled with desthiobiotin after enrichment by streptavidin chromatography.[26]

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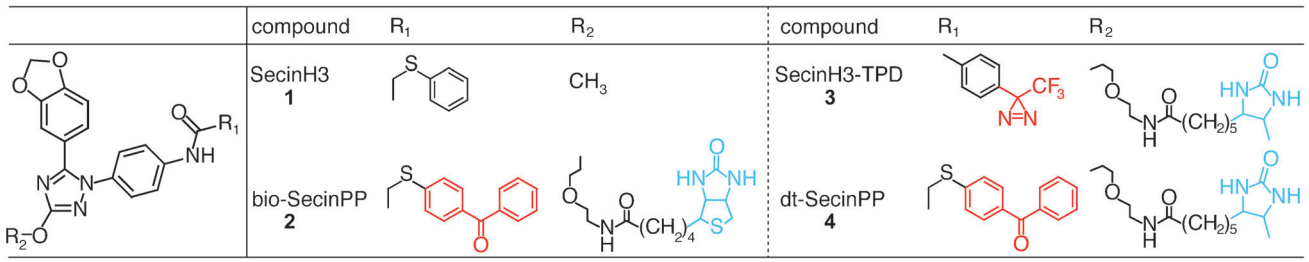


Fig. 1 Structures of the cytohesin inhibitor SecinH3 and the photoreactive probes SecinH3-TPD and dt-SecinPP. Blue: reporter group (desthiobiotin or biotin). Red: photoactive group (benzophenone or 3-trifluoromethyl-3-phenyl-diazirine, TPD).

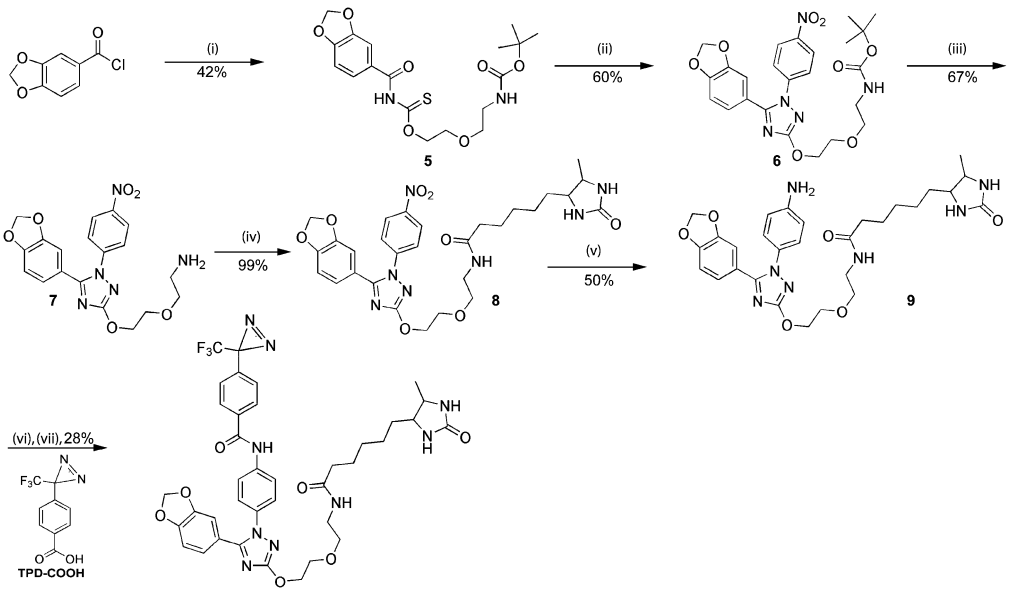
The synthesis of SecinH3-TPD **3** is shown in Scheme 1. Treatment of piperonoyl chloride with potassium thiocyanate to obtain the acylthioisocyanate *in situ*, followed by addition of the Boc-protected diglycol gave thiocarbamate **5**. The 1,2,4-substituted triazole ring **6** was obtained by cyclisation of **5** with 4-nitrophenylhydrazine.[24] Deprotection of **6** with formic acid led to amine **7**, which was then coupled to desthiobiotin by a standard peptide coupling reaction. The nitro-triazole **8** was subsequently reduced employing a standard palladium catalyzed hydrogenation to afford **9**. The benzoic acid TPD-COOH was converted to the corresponding benzoyl chloride before reaction with **9** giving the requisite final product SecinH3-TPD **3**. All steps led to the desired product in good purity and satisfactory yield.

The ability of SecinH3-TPD to label cytohesins in an irradiation-dependent way was tested on the purified Sec7 domain of cytohesin-2, called ARNO-Sec7. SecinH3-TPD in 5-fold excess was incubated on ice with a His_6-tagged ARNO-Sec7 construct (0.5 μM) for 10 min in the dark, before irradiation with UV light at 365 nm. The proteins were separated by 10% SDS-PAGE and transferred on nitrocellulose membranes by Western blotting. Labelled proteins were detected with a NeutrAvidin DyLight 800 fluorescent conjugate, while the total amount of protein was detected with a hexahistidine-specific antibody (His-tag in Fig. 2A). A strong biotin signal was observed only in the irradiated samples, demonstrating that SecinH3-TPD covalently reacts with ARNO-Sec7 upon UV activation (S in Fig. 2A).

As a control of specificity we used the purified PAZ domain of Argonaute 1 and measured the efficiency of its labelling by Secin-TPD (P in Fig. 2A). A slight degree of labelling could be detected, but when the same experiment was performed in the presence of ARNO as a specific competitor, no residual labelling of PAZ was obtained (S + P in Fig. 2A), indicating that SecinH3-TPD labels cytohesins in a specific fashion.

To verify that the SecinH3-TPD probe has the same binding site on ARNO as SecinH3 we performed a competition experiment by adding an excess of unmodified SecinH3 to the labelling reaction. Indeed, when ARNOΔPBR (an ARNO construct lacking the last 14 amino acids required for its autoinhibition)[27] was incubated with equimolar concentration of SecinH3-TPD (1 μM) and excess of SecinH3 (14 μM), the amount of labelled protein was strongly reduced (Fig. 2B), indicating that the binding sites of SecinH3-TPD and SecinH3 on cytohesins overlap.

Next, we directly compared the efficiencies of the TPD and benzophenone derivatives of SecinH3 in labelling ARNOΔPBR. To ensure optimal comparability, the desthiobiotinylated probe dt-SecinPP **4** (Fig. 1) was used instead of biotinylated **2**.[25]



Scheme 1 Synthesis of the photoreactive probe SecinH3-TPD: (i) potassium thiocyanate, acetone, 55 °C, then Boc-2-(2-aminoethoxy)ethanol, 55 °C; (ii) 4-nitrophenylhydrazine, EtOH, 80 °C, 8 h; (iii) formic acid, 2.5 h; (iv) D-desthiobiotin, HBTU, *N*,*N*-diisopropylethylamine, DMF, 1 h; (v) 10% Pd/C, EtOH/THF, 9 bar H_2, 2 h; (vi) TPD-COOH, oxalyl chloride, THF, DMF (catalytic), 2.5 h; (vii) *N*,*N*-diisopropylethylamine, CH_2Cl_2, 2 h.

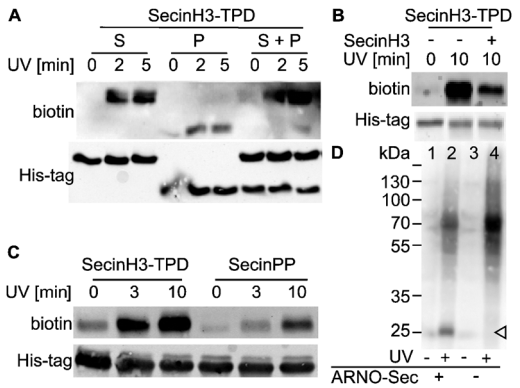


Fig. 2 SecinH3-TPD labels ARNO-Sec7. (A) Photoaffinity labelling of ARNO-Sec7 (S) and the unrelated PAZ domain (P). (B) Competition of labelling with SecinH3. (C) Comparison of labelling by SecinH3-TPD and the benzophenone probe dt-SecinPP. (D) ARNO-Sec7 (arrowhead) can be labelled with SecinH3-TPD in cell lysate.

ARNOΔPBR (1 μM) was incubated with 20 μM of either SecinH3-TPD or dt-SecinPP for 10 min on ice before irradiation for the indicated times at 365 nm. Detection of labelling was performed as above. As evident from Fig. 2C, the SecinH3-TPD treated samples display a considerably increased band intensity than the Secin-PP treated ones, directly proving the superior labelling efficiency of the TPD probe (Fig. 2C, left panels) as compared to the benzophenone-containing photoaffinity probe (Fig. 2C, right panels).

Finally, to test whether **3** can label ARNO-Sec7 in the presence of a whole proteome we performed a reaction in a cell lysate prepared from human embryonic kidney 293 (HEK293) cells in the presence and absence of purified ARNO-Sec7 (Fig. 2D). After UV irradiation, a band corresponding to ARNO-Sec7 was detected (lane 2) that was absent in the sample that did not contain ARNO-Sec7 (lane 4). A few additional bands of larger size were detected that might correspond to biotin-binding proteins (see ESI† for a discussion). As for the purified protein (Fig. 2B), the labelling of ARNO-ΔPBR by **3** was competed by SecinH3 also in cell lysate (Fig. S1, ESI†). These results show that ARNO-Sec7 can be labelled in cell lysates, indicating that SecinH3-TPD is, in principle, suitable for determining the binding site of SecinH3 on ARNO-Sec7.

A SecinH3-TPD photoaffinity probe with a desthiobiotin reporter group was prepared and examined in photo-cross-linking studies with ARNO-Sec7 *in vitro*. SecinH3-TPD showed improved labelling efficiency in direct comparison with the benzophenone-containing photoaffinity probe dt-SecinPP. Already after three minutes of UV-irradiation the amount of SecinH3-TPD labeled cytohesin significantly exceeded that of SecinPP-labeled cytohesin after ten minutes of UV irradiation, indicating that SecinH3-TPD not only increases the yield of labeled protein, but also can be employed under significantly milder conditions. The long wavelengths and short times needed for activation of TPD minimize the damage of biological samples. As ARNO-Sec7 is labelled in cell lysates SecinH3-TPD and its derivatives will thus favour binding site determination and specificity analyses in whole proteomes and should be well suited for labelling cytohesins in living cells, tissues or even whole organisms.[3,28–30]

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