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Synthesis and evaluation of locostatin-based chemical probes towards PEBP-proteins

Jorin Hoogenboom [a], Martijn Fiers [b], Richard Immink [b], Han Zuilhof [a,c], Tom Wennekes [a,d,*]

[a] Laboratory of Organic Chemistry, Wageningen University, Dreijenplein 8, Wageningen 6703 HB, The Netherlands
[b] Plant Research International, Bioscience, Droevendaalsesteeg 1, Wageningen 6708 PB, The Netherlands
[c] Department of Chemical and Materials Engineering, King Abdulaziz University, Jeddah, Saudi Arabia
[d] Department of Chemical Biology and Drug Discovery, Utrecht Institute for Pharmaceutical Sciences and Bijvoet Center for Biomolecular Research, Utrecht University, The Netherlands

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ABSTRACT

Phosphatidyl ethanolamine-binding proteins (PEBPs) are implicated in various critical physiological processes in all eukaryotes. Among them is Flowering Locus T (FT), the protein recently discovered as the vital flowering hormone in plants. Small molecule inhibitors and activators of FT could provide control over plant flowering and are therefore an interesting target for industrial agriculture. No small molecule inhibitors or activators are known for FT, but for a structurally similar PEBP, RKIP, an inhibitor called locostatin has been reported to covalently bind in the RKIP ligand binding pocket. Herein, we report the synthesis of novel locostatin-based chemical PEBP probes and evaluate their ability and selectivity towards the binding of FT and RKIP.

The phosphatidyl ethanolamine-binding protein (PEBP) family compromises over 400 members from a variety of organisms, including plants, bacteria, yeast and mammals.[1] The PEBP family is thus a highly conserved group of proteins, that fulfil a plethora of different functions. Recently, a new functional addition to the PEBP family was made due to the search towards 'florigen'. Florigen is a crucial, long sought substance that relays a signal from the leaves to the growing tip of a plant, known as the shoot apical meristem, to induce flowering. The extensive hunt for florigen has led to the discovery of Flowering Locus T (FT) as the flowering inducing agent belonging to the PEBP family of proteins in the model plant *Arabidopsis thaliana*.[2,3] As a PEBP, FT also possesses the two notable features common to all PEBPs, namely (1) a compact globular structure, which provides ample surface area for interaction with other proteins and (2) a ligand binding pocket.[1] The crystal structures of PEBPs from rat, human, bovine and plant material reveal the evolutionary highly conserved nature of the PEBP ligand binding pocket.[4] For example, the very similar spatial arrangement of the amino acid residues in the ligand binding pocket of the FT-like protein Centrodialis (CEN) from Antirrhinum and bovine PEBP (bPEBP) is depicted in Figure 1.

The ligand binding pocket in FT is interesting as a potential target for the development of small molecules that can activate or inhibit the functioning of FT. In view of the importance of FT as the key flowering-inducing protein, the applications for such compounds in plant science and industry are obvious.

Currently, no small molecule inhibitor is known that can selectively bind FT. In contrast, several small molecule inhibitors have been identified for the Raf Kinase Inhibitory Protein (RKIP), a structurally similar mammalian PEBP. RKIP has been well studied as a potential therapeutic target involved in pathophysiological processes including diabetic nephropathy,[6] Alzheimer's disease[7–9] and immunotherapy against human cancer.[10–13] The extensive study of RKIP has also led to the discovery of locostatin (Fig. 1), named after its ability to inhibit cellular locomotion in multiple systems.[10] Besides claims about its potential therapeutic value,[10–12,14–16] locostatin's mode of action has been reported to involve covalent and irreversible binding of the RKIP ligand binding pocket.[10,14,17] The pocket contains a highly conserved histidine residue (His86) that was reported to act as a nucleophile on the Michael acceptor in locostatin[17] and which is also present in bPEBP, CEN and FT (Fig. 1). Because of this conservation and the overall structural similarity of the binding pocket, we hypothesized that FT might also be covalently bound by locostatin in a similar fashion and block the mode of action of FT, and hence influence flowering. We therefore set out to investigate if locostatin affects

* Corresponding author. Tel.: +31 (0)302534262.
E-mail address: t.wennekes@uu.nl (T. Wennekes).

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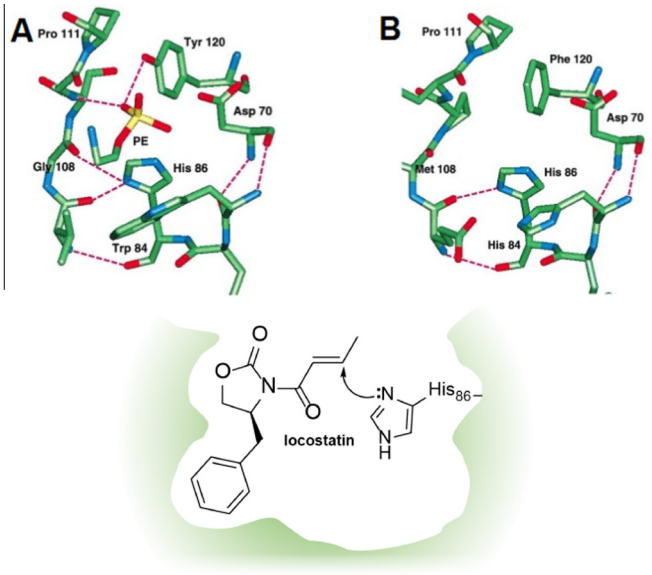


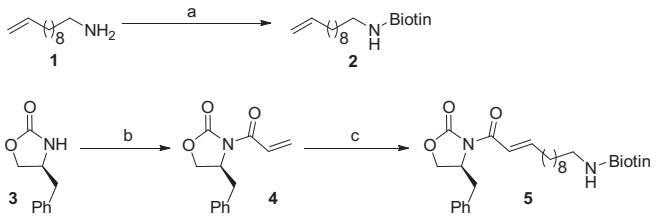
Figure 1. Top: conserved residues of the PEBP ligand binding pocket in the crystal structure of bPEBP (A) with bound phosphoryl group (PE) and FT-like protein CEN (B).[5] Bottom: Putative binding mechanism of locostatin in the ligand binding pocket of the PEBP, RKIP.[10,17]

the flowering-inducing properties of FT and by developing a set of novel locostatin-based chemical probes, if it selectively binds FT in its ligand binding pocket.

We started by studying the effect of locostatin on the flowering time of *Arabidopsis thaliana* Col0. We initially tested different concentrations of locostatin and noticed that this compound is toxic for Arabidopsis at higher concentrations (ESI, Fig. S1). Based on this pilot experiment a 25 μM locostatin solution in H_2O was used in a flowering time experiment. Although here we found some very early flowering plants, no significant flowering stimulating or repressing activity was observed at population level (ESI, Fig. S2). The only significant effect was that locostatin negatively affected root development of the plants (ESI, Fig. S1). It is improbable that locostatin is affecting the roots due to interaction with FT, since the FT gene is not expressed in roots.[18] A saturated version of locostatin did not have a significant effect on the root development (ESI, Fig. S3), indicating the crucial role of the reactive Michael acceptor of locostatin for its activity.

Despite the fact that no FT-related phenotypes could be observed in the plants treated with locostatin, we decided to investigate whether FT can be targeted by locostatin, based on the conservation of the PEBP ligand binding pocket and previous binding studies for locostatin and RKIP.[10,14,17] In order to visualize the covalent modification of FT by locostatin, novel chemical probe analogues of locostatin were designed. We aimed to modify the Michael acceptor tail of locostatin, since it has been previously reported for RKIP that this part of locostatin remains covalently bound to the PEBP protein, while the oxazolidinone-moiety may dissociate over time through hydrolysis.[17] In addition, a structure–activity relationship study of locostatin with RKIP has shown that structural modification of this part is tolerated.[10]

We aimed to synthesize target locostatin-based chemical probe **5** by starting with commercially available 1-amino-10-undecene **1**, which was coupled to biotin in good yield (Scheme 1). The resulting alkene **2** was then reacted with known compound **4** via a cross-metathesis reaction. Initial attempts to carry out this reaction with the 2nd generation Grubbs catalyst were unsuccessful, but we were able to obtain compound **5** using the more stable 2nd generation Hoveyda–Grubbs catalyst in AcOH, which exclusively gave



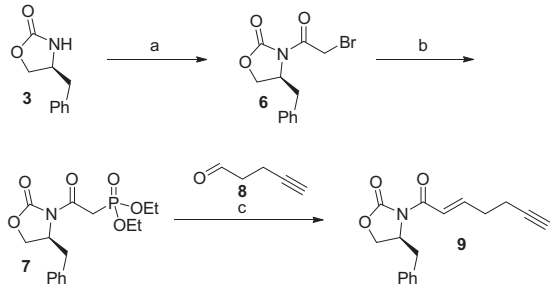
Scheme 1. Synthesis of locostatin-probe **5**. Reagents and conditions: (a) EDC, HOBt, D-Biotin, DMF, rt, 1 d, 85%; (b) *n*-BuLi, acryloyl chloride, THF, −78 °C, 1 h, 17%; (c) **2**, HG-II, AcOH, 40 °C, 1 d, 26%.

the *E*-isomer. Not surprisingly, this compound turned out to possess only limited water-solubility so we also set out to develop more water-soluble versions. To this end we aimed to synthesize locostatin analogue **9** (Scheme 2) and PEGylated compound **13** (Scheme 3), which could then be coupled to a PEGylated Biotin group through a Cu(I)-catalyzed alkyne–azide click reaction.[19]

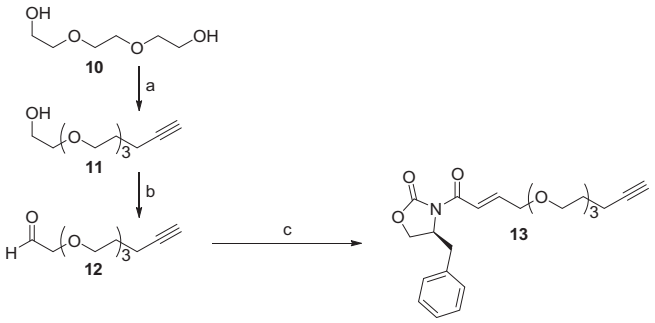
Compound **9** could be obtained from (*S*)-4-benzyloxazolidin-2-one **3**, which was acrylated in good yield with 2-bromoacetyl bromide and then converted to phosphonate **7** through an Arbuzov reaction in almost quantitative yield. The resulting intermediate was reacted through a Horner–Wadsworth–Emmons reaction with aldehyde **8**, providing the *E*-isomer as the major isomer that could be purified to provide compound **9** in 74% yield.

The other required building block, compound **13**, could be obtained from triethylene glycol, which was first alkylated to provide intermediate **11** (Scheme 3). Intermediate **11** was then oxidized via a Swern protocol to aldehyde **12**. This aldehyde was reacted with intermediate **7** through another Horner–Wadsworth–Emmons reaction to give compound **13**.

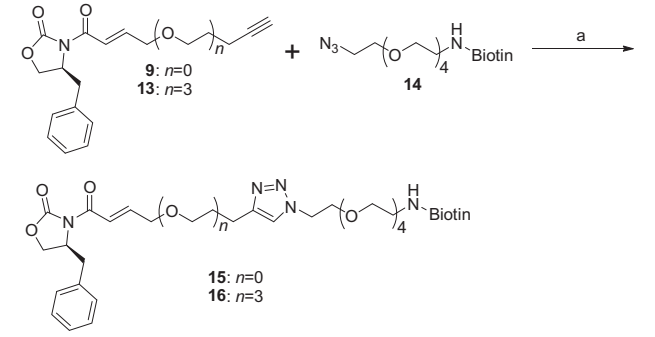
With both compound **13** and compound **9** in hand we set out to couple them to a PEGylated biotin **14** through a CuAAC click reaction, which was achieved in reasonable yield (Scheme 4). With probes **5**, **15** and **16** in hand, we set out to validate if these locostatin-based chemical probes could covalently bind FT. For this purpose FT was expressed in vitro and the protein mixture containing this FT was incubated with locostatin-probe **5** for 24 h at 37 °C (ESI, Fig. S4), similar to the conditions employed earlier by Beshir and co-workers[17] for locostatin and RKIP. However, instead of selective labelling of FT we observed that the probe seemed to label the majority of the proteins in the cell lysate. Similarly, when we used locostatin-probes **15** and **16** under milder conditions: 2 h at 4 °C or room temperature, we again did not see any selectivity for FT even at lower concentrations (Figs. 2A and ESI, S4). Nevertheless, it was unclear if this was due to poor selectivity or due to low expression levels of FT in our system. To that end, we expressed FT, fused to Glutathione S-Transferase (GST), in



Scheme 2. Synthesis of compound **9**. Reagents and conditions: (a) bromoacetyl bromide, *n*-BuLi, THF, −78 °C, 1 h, 70%; (b) $P(OEt)_3$, neat, 50 °C, 16 h, 100%; (c) **8**, NaH, THF, rt, 1 h, 74%.



Scheme 3. Synthesis of compound **13**. Reagents and conditions: (a) NaH, 5-chloro-1-pentyne, THF, 60 °C, 16 h, 60%; (b) oxalyl chloride, DMSO, DCM, −78 °C, Et_3N, 1 h, 47%; (c) NaH, **7**, THF, DCM, rt, 1 h, 26%.



Scheme 4. Synthesis of locostatin-probes **15** and **16**. Reagents and conditions: (a) $CuSO_4$, sodium ascorbate, DMF, H_2O, 64% (for **15**) or 68% (for **16**).

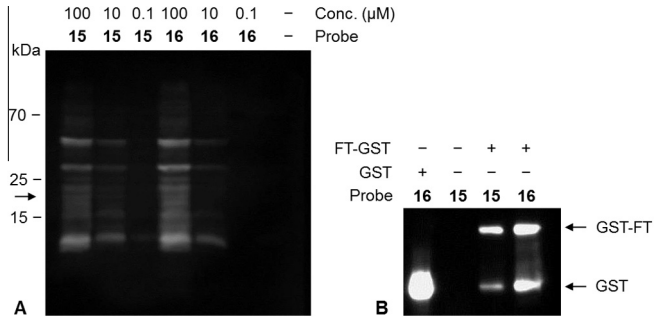


Figure 2. (A) Binding of probe **15** and **16** at room temperature for 2 h with in vitro expressed FT; (B) binding of 40 μM probe **15** and **16** with purified GST-FT and residual GST; binding of 200 μM probe **15** with GST. Arrow indicates proximate position where FT is expected.

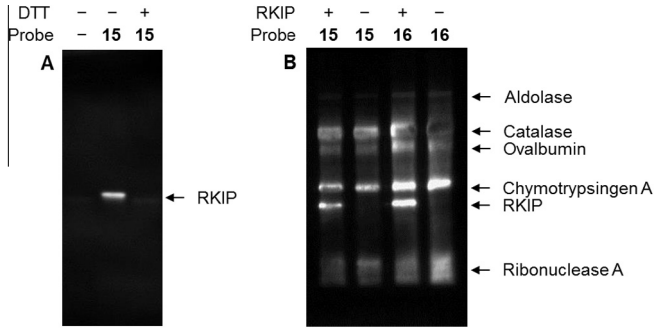


Figure 3. (A) Evaluation of binding of 2.38 μM RKIP by 200 μM locostatin probe **15** for 6 h at 37 °C with or without 2 mM DTT; (B) evaluation of selectivity of locostatin probes **15** and **16** towards 5 reference proteins (2.38 μM) with or without 2.38 μM RKIP for 1 h at rt.

Escherichia coli and purified this fusion protein using affinity chromatography.

The N-terminal GST fusion was used as an affinity tag. We then incubated GST-FT with locostatin probes **15** and **16**. Upon incubating GST-FT with probes **15** and **16** we found that both probes were able to successfully bind GST-FT (Fig. 2B). However, we observed that the probes also bound residual cleaved GST to a similar extent. This proved that locostatin-based probes **15** and **16** were not selectively binding FT. It also seemed to indicate that the Michael acceptor motif in locostatin is capable of reacting with the various nucleophiles present on any given protein.

In the past it has been reported that a tritium-labelled locostatin analogue was able to selectively label RKIP in MDCK cell extracts.[10] We therefore next investigated if (1) our locostatin-based chemical probes could also bind RKIP, and (2) if they display selectivity towards RKIP. As such, we incubated recombinant RKIP with locostatin probe **15** with or without 2 mM DTT. Although DTT has been used by others in incubation steps of locostatin with RKIP, we expected that the nucleophilic thiol of DTT might deactivate our locostatin-based chemical probes.[10,17] As expected we indeed observed that binding of our locostatin-based probe **15** proceeded much better in the absence of DTT (Fig. 3A). Similarly, locostatin probe **5** was also able to bind RKIP, but not quantitatively, probably due to a lower effective concentration of the probe as a result of its lower water solubility (ESI, Fig. S6).

Finally, we tried to visualize the selectivity of our locostatin probes towards RKIP by introducing equimolar amounts of commonly used, non-PEBP, reference proteins to a buffered solution with or without RKIP. We observed that locostatin-based chemical probes **15** and **16** seem to bind RKIP and the other negative control proteins more or less to the same extent (Figs. 3B and ESI, S7). In addition, the selectivity did not improve by performing the incubation for a shorter time at reduced temperatures. These results are in line with a recent study of locostatin towards a bacterial PEBP present in *Mycobacterium tuberculosis*, where the authors speculate that locostatin probably inhibits additional targets.[20]

Conclusions

We successfully synthesized novel locostatin-based chemical probes. These probes enabled us to study the locostatin binding selectivity in cell lysates and protein mixtures, which provided surprising and important new insights into the selectivity of locostatin. While our locostatin-based probes can covalently bind both FT and RKIP, we have shown that these probes are not selective and can also bind a multitude of non-PEBP proteins. In addition, the apparent toxicity of locostatin towards plants and lack of selectivity of locostatin-based chemical probes towards FT indicate that locostatin is not a suitable lead compound towards novel small molecule binders of FT and flowering time-modulating compounds.

Locostatin is currently sold commercially by many vendors as a small molecule inhibitor of cell migration through its supposed selective inhibition of RKIP. In addition, potential pharmacological applications[10–12,14–16] have been attributed to locostatin. However, our findings here highlight that future research into locostatin and its analogues and their use as inhibitors of RKIP and other PEBP targets should take into account that locostatin has limited selectivity towards these proteins.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tetlet.2016.04.071.

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