The Core Structures of Roseophilin and the Prodigiosin Alkaloids Define a New Class of Protein Tyrosine Phosphatase Inhibitors

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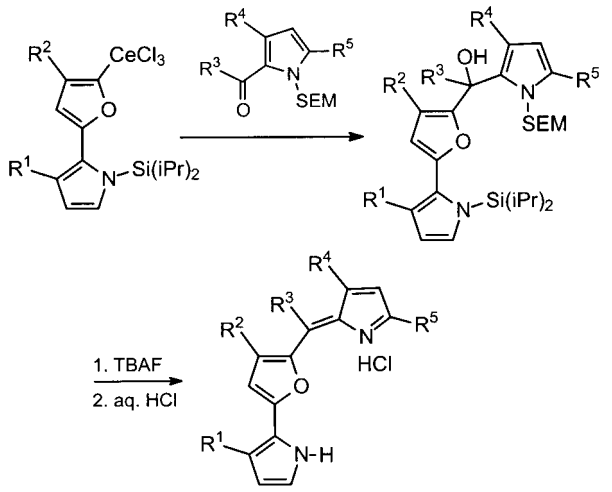
Protein phosphorylation and dephosphorylation reactions are at the heart of innumerable biological processes, and aberrant protein phosphorylation contributes to the development of many human diseases including cancer and diabetes.[1] Due to this biological importance, protein kinases that catalyse protein phosphorylation belong to the most intensively investigated targets of current medicinal chemistry and chemical biology research. Very recently, their natural antagonists, the protein phosphatases (PPs), have also moved into the focus of a rapidly growing number of research programs. However, despite the fact that naturally occurring PP inhibitors are widely used research tools in biology,[2] only a very limited number of different PP inhibitors is currently available.[3–5]

This is particularly the case for the protein tyrosine phosphatases[4] and the dual-specificity phosphatases,[5] which are the most promising drug targets. Therefore, the identification and development of novel PP-inhibitor classes that are amenable to rapid structure variation and optimisation of biological activity and selectivity is of major importance to research in chemical biology and medicinal chemistry.

In this paper we disclose that the pyrrole alkaloids roseophilin **1** and nonylprodigiosin **6** (Tables 1 and 2, below) as well as analogues thereof comprise such a new class of PP inhibitors. Roseophilin, a secondary metabolite isolated from a culture broth of *Streptomyces griseoviridis* displays potent cytotoxicity against various human cancer-cell lines but its biological mode of action remains elusive.[6] The prodigiosin antibiotics produced by a restricted group of actinomycetes are antibacterial, cytotoxic and antimalarial agents,[7–9] and exhibit significant immunosuppressive properties at noncytotoxic doses.[8–10] First insights into the structural requirements for the biological activity of these compounds have been gained and indicated that an intact azafulvene structure might be required for biological activity.[12,13] However, the molecular and cellular targets of these compounds have not been identified unambiguously.

Having access to roseophilin and the prodigiosins through total synthesis,[8,11–15] and in the course of a program directed at the identification and development of novel classes of phosphatase inhibitors[16–18] we investigated whether these alkaloids are able to modulate the activity of selected but representative members of this protein-dephosphorylating enzyme family. Since the blueprint of the chosen synthesis routes (Schemes 1 and 2) is inherently flexible, various analogues and congeners of the natural products have also been obtained. Specifically, the heterocyclic perimeter of roseophilin was formed by addition of the metallated pyrrolylfuran segment to the macrotricy-



Scheme 1. *General route for the synthesis of roseophilin and analogues thereof.*[15]

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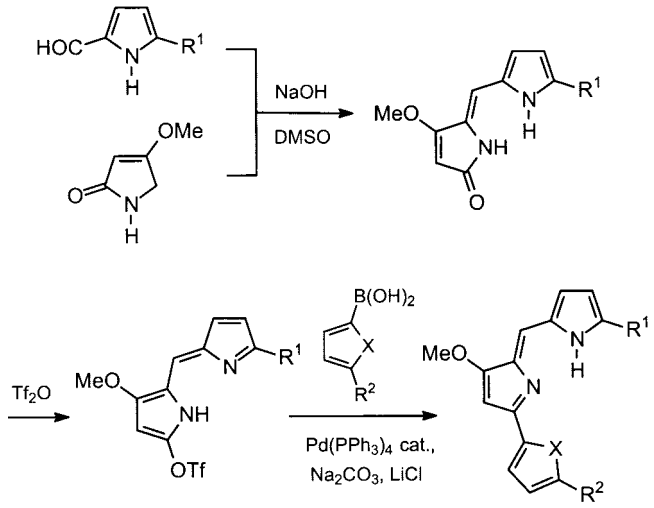
clic ketopyrrole unit followed by deprotection and dehydration of the resulting alcohol with formation of the azafulvene motif. Variations of the substituents R^1–R^5 as indicated in Scheme 1 opened access to the functional analogues depicted in Table 1.[15] Compounds belonging to the prodigiosin series were formed by condensation of a suitable pyrrole-2-carbaldehyde with commercial 4-methoxypyrrolin-2-one followed by conversion of the resulting enelactam into the corresponding aryltriflate (Scheme 2). Subsequent palladium-catalysed cross coupling with various (hetero)arylboronic acids completed the pyrrolopyrromethene chromophore and allowed for systematic variations of the substituents R^1 and R^2 as well as the heteroelement X in the A-ring.[11–13] This modularity sets the basis for a preliminary mapping of structure–activity relationships in this series.

The screen included Cdc25A and VHR as two typical dual-specificity phosphatases and PTP1B as a prototypical tyrosine phosphatase. The vaccinia VH1-related phosphatase VHR is a physiological regulator of extracellular regulated kinases belonging to the MAP kinase family and influences signalling through the MAP kinase pathway.[19] The family of the Cdc25 dual-specificity protein phosphatases is critically involved in cell-cycle control.[20]

PTP1B is a key negative regulator of insulin-receptor activity, and PTP1B-inhibitors are expected to enhance insulin sensitivity and act as effective therapeutics for the treatment of type II diabetes, insulin resistance and obesity.[4]

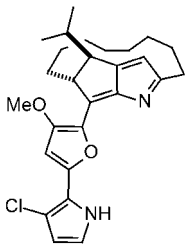
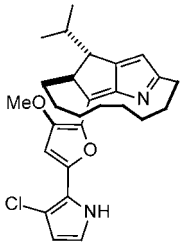
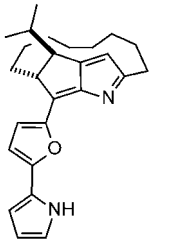
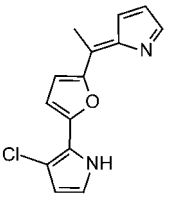
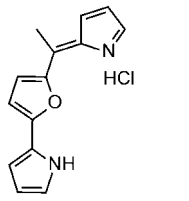
As compounds to be investigated in the phosphatase inhibition assay, roseophilin **1**, its enantiomer **2**, as well as analogues **3**–**5** were initially chosen (Table 1). Compound **3** lacks the two substituents on the aromatic rings attached to the tricyclic core of roseophilin that incorporates the electrophilic azafulvene unit. Compounds **4** and **5** no longer have an aliphatic macrocyclic ring.

Roseophilin **1** is a weak inhibitor of the three tyrosine and dual-specificity phosphatases investigated. However, its enantiomer **2** significantly inhibits PTP1B as well as VHR. This result is reminiscent of a previous report showing that roseophilin is one of the extremely rare examples in which the non-natural



Scheme 2. *General route for the synthesis of prodigiosin alkaloids.*[11–14]

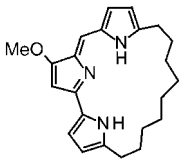
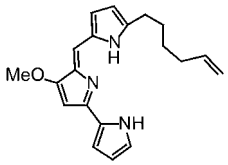
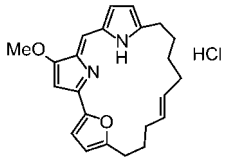
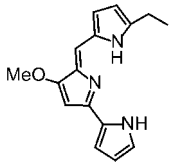
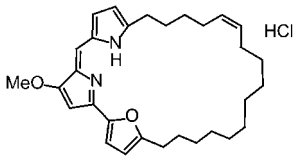
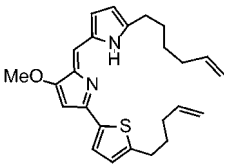
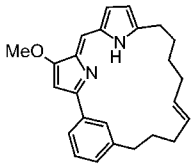
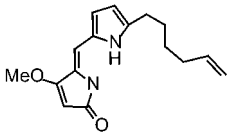
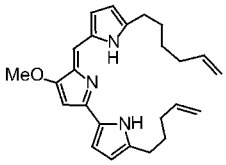
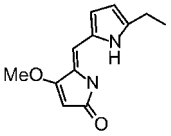
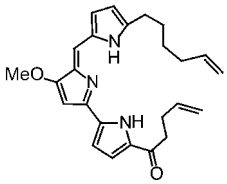
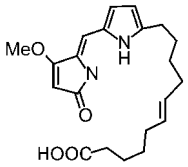
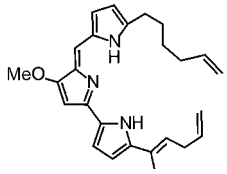
Table 1. *IC_{50} values [μM] of the phosphatase inhibition by roseophilin and selected analogues.*[a]

Compound	Structure	IC_{50} Cdc25A	IC_{50} PTP1B	IC_{50} VHR
1		28 ± 3 (3)	38 ± 14 (5)	32 ± 11 (4)
2		21 ± 6.6 (4)	6 ± 1 (3)	6 ± 2 (3)
3		> 50	10 ± 2 (3)	15 ± 3.4 (3)
4		34 ± 21 (3)	6 ± 2 (6)	9 ± 7 (3)
5		40 ± 16 (3)	3.3 ± 1 (6)	4.9 ± 3 (3)

[a] The dephosphorylation of 4-nitrophenyl phosphate (pNPP) was measured by its absorption change at 405 nm. The concentration [μM] of inhibitors for which the enzyme activity is reduced to 50% is shown for the phosphatases Cdc25A, PTP1B and VHR as average, standard deviation and number of independent experiments, respectively.

enantiomer exhibits higher cytotoxicity (factor 2–10) against several cancer cell lines than the naturally occurring antipode.[21] It is therefore tempting to speculate that phosphatase inhibition is intimately linked to the still unknown mechanism of action that is responsible for the cytotoxicity of this structurally rather unique agent. Analogue **3**, lacking the substituents on the periphery, and compounds **4** and **5**, devoid of the aliphatic ring, show similar activity. In general, the dual-specificity phosphatase Cdc25A was less prone to inhibition by compounds **1**–**5** than PTB1B and VHR.

Table 2. *IC_{50} values [μM] of the phosphatase inhibition by nonylprodigiosin and selected analogues.*[a]

Compound	Structure	Cdc25A	IC_{50} PTP1B	IC_{50} VHR	Compound	Structure	Cdc25A	IC_{50} PTP1B	IC_{50} VHR
6		>50	10±3 (6)	21±8 (8)	**13**		>50	11±5 (5)	11±3 (3)
7		>50	23±11 (5)	28±13 (4)	**14**		>50	10±3 (5)	21±4 (3)
8		>50	24±8 (6)	41±8 (3)	**15**		>50	28±18 (5)	39±12 (4)
9		>50	>50	30±9 (6)	**16**		>50	46±6 (5)	8±5 (4)
10		>50	>50	>50	**17**		>50	>50	>50
11		>50	32±4 (5)	>50	**18**		>50	16±9 (3)	21±12 (4)
12		>50	12±2 (5)	9±7 (5)					

[a] The dephosphorylation of 4-nitrophenyl phosphate (pNPP) was measured by its absorption change at 405 nm. The concentration [μM] of inhibitors for which the enzyme activity is reduced to 50% is shown for the phosphatases Cdc25A, PTP1B and VHR as average, standard deviation and number of independent experiments, respectively.

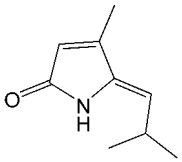
Based on the hypothesis that the electrophilic azafulvene incorporated into compounds **1**–**5** is the key structural element responsible for the observed activity, the related natural product nonylprodigiosin **6** and several cyclic and acyclic analogues thereof were investigated as possible inhibitors of the representative phosphatases chosen. All prodigiosins embody an azafulvene as characteristic motif, but differ from roseophilin in its exact positioning within the heterocyclic perimeter as well as in the type of aromatic rings forming their backbone.

The results of the inhibition studies are displayed in Table 2. In fact, nonylprodigiosin **6** inhibited the dual-specificity phosphatase VHR and the tyrosine phosphatase PTP1B. The cyclic analogues **7** and **8** displayed similar potency and selectivity. Investigation of the open-chain prodigiosin analogues **10**–**15** revealed an activity and selectivity pattern in which relatively small structural changes result in significant variation of inhibitory activity. Thus, compounds **10** and **11** either do not or only weakly inhibit any of the phosphatases, whereas the close ana-

logue **12** targeted PTP1B and VHR. Compounds **13** and **14**, which have only one side chain, showed a similar activity pattern. The thienyl analogue **15** was less potent.

Finally, congeners **16**–**18** were investigated as possible phosphatase inhibitors. Although these compounds no longer contain an azafulvene, two of them inhibited the chosen phosphatases. Specifically, the vinylogous urethane **16** displayed pronounced activity for VHR.

This result is particularly noteworthy since the natural product pulchellalactam **19**[22] was recently discovered to be an inhibitor of the tyrosine phosphatase CD45. This natural product, isolated from the marine fungus *Corollospora pulchella*, contains a 3-pyrrol-2-one moiety similar to that occurring in **16**–**18**. Together these findings indicate that this small heterocyclic system may constitute an interesting pharmacophore for further development of phosphatase inhibitors.



Pulchellalactam
19

Interestingly, none of the prodigiosin analogues inhibited the dual-specificity phosphatase Cdc25A; this indicates that the positioning of the electrophilic azafulvene in the heterocyclic framework may be the decisive factor for inhibition of this enzyme by roseophilin and its analogues.

From the data detailed above, however, the mechanism of inhibition is not clearly obvious and may differ between enzymes and inhibitors. Tyrosine and dual-specificity phosphatases employ a nucleophilic cysteine to cleave the P–O bond. Therefore, it appears possible that the electrophilic groups embodied in the natural products and their analogues may target active site nucleophiles.

In conclusion we have discovered that roseophilin- and prodigiosin-type compounds define a new class of potent inhibitors for tyrosine and dual-specificity phosphatases. Given the fact that efficient routes to these heterocyclic systems are available,[8, 11–15] the development of more potent and selective phosphatase inhibitors based on the structure of these alkaloids appears possible.

Experimental Section

Protein phosphatase inhibition assay: All assays were performed in 96-well microplates and a final volume of 100 μL. Buffer conditions were NaCl (50 mM), EDTA (1 mM), Dithioerythritol (DTE; 1 mM), Tris (50 mM, pH 8.0) for Cdc25; EDTA (5 mM), DTE (1 mM), MOPS (25 mM, pH 6.5) for VHR and NaCl (50 mM), EDTA 2.5 mM), DTE (2 mM), HEPES (25 mM, pH 7.2) for PTP1B. Enzyme concentrations were chosen to yield absorption changes of 0.2 OD at 405 nm within 80 min for 4-nitrophenyl phosphate (pNPP) in the absence of inhibitors. Inhibitors were added from 5 mM solutions in DMSO to 50 μM for the highest concentrations. A dilution series by a factor of 2 was generated for the inhibitors with the enzyme solutions. After an incubation time of 30 min at room temperature, pNPP (10 μL) was added to 50 mM for each well, and the plate was transferred into a Multiscan Ascent plate reader (Thermo Labsystems, www.thermo.com). The absorption at 405 nm was recorded at 37 °C for 80 min. Relative enzyme activities (slopes) were calculated from the absorption at 30 and 60 min. The IC_{50} values were obtained from a secondary plot of relative activity versus log inhibitor concentration as the inhibitor concentration for which the activity was reduced to 50 %. No assumptions concerning the inhibition mechanism (shape of the curve) were made.

Protein tyrosine phosphatase 1B (PTP1B) and the dual-specificity protein phosphatase VHR were obtained from Biomol GmbH, Hamburg. The dual-specificity phosphatase Cdc25A was expressed in *E. coli* from a human clone[23] as a cdc25a-His_6-Tag construct in pET9d, kindly donated by I. Hoffmann (DKFZ, Heidelberg). It was localised in inclusion bodies of *E. coli* and could be purified and stored in 8 M urea. Enzymatic activity was recovered by tenfold dilution in EDTA (1 mM), DTE (1 mM), Tris (50 mM, pH 8.0).

We note that the IC_{50} values presented in this work were obtained with the very simple non-natural model substrate *p*-nitrophenyl phosphate. This assay is widely established in screens aiming at the identification of phosphatase inhibitors (see refs. [16–20] and references therein). pNPP is not a physiological substrate of phosphatases. In fact, it is a relatively small substrate that has to be applied at high (50 mM) concentrations in order to monitor dephosphorylation. The physiological substrates are large proteins that generally are bound with much higher affinity (at much lower concentrations) before they are dephosphorylated specifically.

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Keywords: alkaloids · biological activity · enzymes · inhibitors · phosphatases · prodigiosin

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