Biological Activity

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Neuritogenic Militarinone-Inspired 4-Hydroxypyridones Target the Stress Pathway Kinase MAP4K4**

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Abstract: *Progressive loss and impaired restoration of neuronal activity are hallmarks of neurological diseases, and new small molecules with neurotrophic activity are in high demand. The militarinone alkaloids and structurally simplified analogues with 4-hydroxy-2-pyridone core structure induce pronounced neurite outgrowth, but their protein target has not been identified. Reported herein is the synthesis of a militarinone-inspired 4-hydroxy-2-pyridone collection, its investigation for enhancement of neurite outgrowth, and the discovery of the stress pathway kinase MAP4K4 as a target of the discovered neuritogenic pyridones. The most potent 4-hydroxy-2-pyridone is a selective ATP-competitive inhibitor of MAP4K4 but not of the other stress pathway related kinases, as proven by biochemical analysis and by a crystal structure of the inhibitor in complex with MAP4K4. The findings support the notion that MAP4K4 may be a new target for the treatment of neurodegenerative diseases.*

Severe neurological diseases like Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS) are characterized by progressive loss and impaired restoration of neuronal activity leading to reduced neuronal complexity and function.[1] These diseases impose a large and increasing burden on society, and it has been estimated that by 2050 AD will affect 1 in 85 persons.[1a] Despite the importance and the advances made in understanding the molecular basis of neurodegenerative disorders, current treatments mostly achieve symptomatic benefits but do not target the root cause of the diseases.[1–3] Therefore, the discovery of new small-molecule classes with neurotrophic or neuroprotective activity which may restore neuronal function and viability and prevent neuronal decline, as well as the identification of their target proteins are of major current interest.

Frequently, natural products command this kind of bioactivity.[4] Since their underlying scaffolds define biologically prevalidated structures in chemical space, compound collections inspired by them may be valuable sources for the discovery of novel bioactive compounds endowed with the same or similar activity.[5]

In this respect the militarinones **1** and **2** and related alkaloids,[6,7] like farinosone A (**3**; Figure 1) are of particular interest. These fungal metabolites induce pronounced neurite outgrowth from neuronal precursor PC-12 cells.[6] This kind of bioactivity is conserved in structurally simplified analogues,[7b] and the side-chain of the natural products has only a minor influence on neuritogenic activity.[7c] However, the protein target of these militarinone analogues which induce neurite outgrowth has not yet been identified.

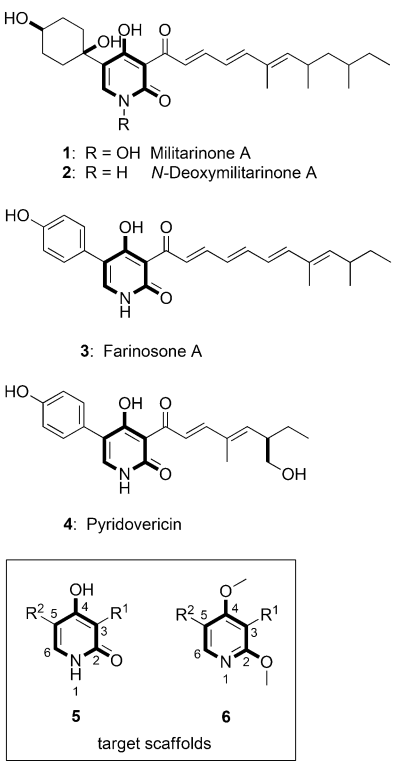

Figure 1. Selected alkaloids having 4-hydroxy-2-pyridone structures and the core scaffolds **5** and **6** used for library design and synthesis.

Herein we report on the synthesis of a militarinone-inspired 4-hydroxy-2-pyridone collection, its phenotypic

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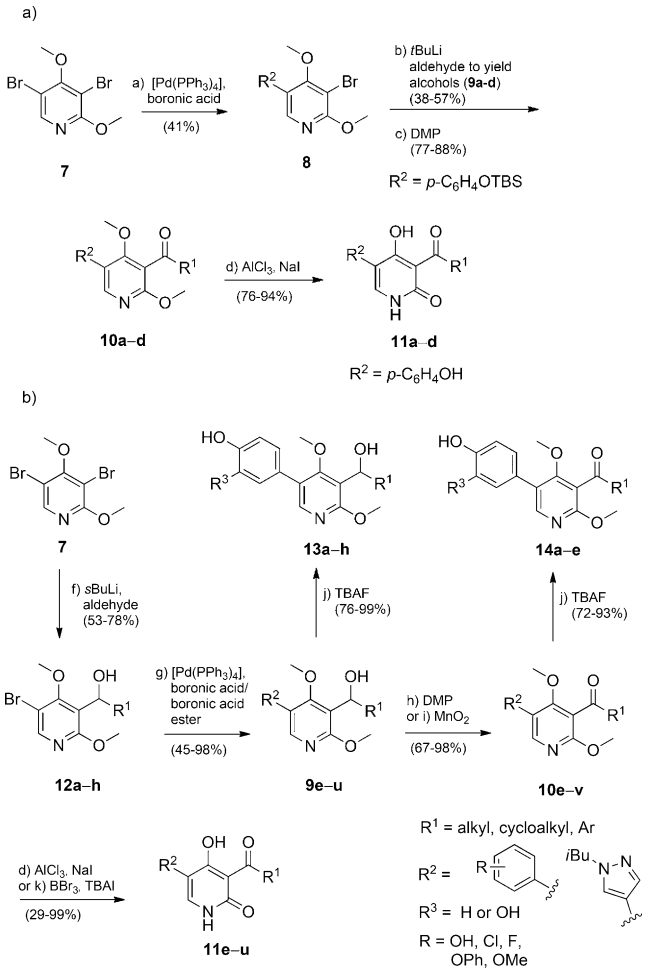
investigation for enhancement of neurite outgrowth, and the discovery of the stress pathway serine/threonine kinase MAP4K4 as a target of the newly discovered neuritogenic 4-hydroxy-2-pyridones.

Militarinone-A-related neuritogenic alkaloids frequently embody the 4-hydroxy-2-pyridone scaffold **5**, which is substituted at the 3- and 5-positions, as the characteristic structural core,[6,7] and was therefore chosen as a target for compound collection synthesis (Figure 1). To determine whether the 4-hydroxy-2-pyridone structure is required for bioactivity, we also envisaged synthesizing the corresponding 2,4-dimethoxypyridines **6**. Initially the desired compounds were synthesized by means of a regioselective Suzuki coupling employing the dibromopyridine **7**[8] and *p*-TBSO-substituted phenylboronic acid (Scheme 1 a). The resulting monobromopyridine **8** was lithiated at C3, and after nucleophilic addition to different aldehydes, the resulting secondary alcohols **9a–d** were oxidized to the corresponding ketones **10a–d**. Demethylation and simultaneous desilylation by means of treatment with $AlCl_3$ and NaI yielded the desired pyridones **11a–d**. Since the initial Suzuki coupling yielded **8** and other related coupling products with unsatisfying yields, a different strategy was developed and it employs regioselective lithiation of **7** at C3 as the first key step (Scheme 1 b). Treatment of **7** with *s*BuLi and subsequent reaction with different aromatic and (cyclo)aliphatic aldehydes gave the secondary alcohols **12a–h** in moderate to good yields. Suzuki couplings with boronic acids or boronic acid pinacol esters generated the pyridines **9e–u** and the corresponding ketones **10e–v** in viable yields. This strategy allowed successful introduction of a broader range of substituents compared with that of the initial synthesis sequence. For final demethylation either $AlCl_3$/NaI or BBr_3/TBAI was employed to yield the desired pyridones **11e–u**. In addition, the intermediate pyridines **9** and **10**, supporting a TBS-protected phenyl moiety, were desilylated to deliver the alcohols **13a–h** and ketones **14a–e** (see the Supporting Information for further details of the synthesis). In total 21 pyridones and 38 pyridines were synthesized.

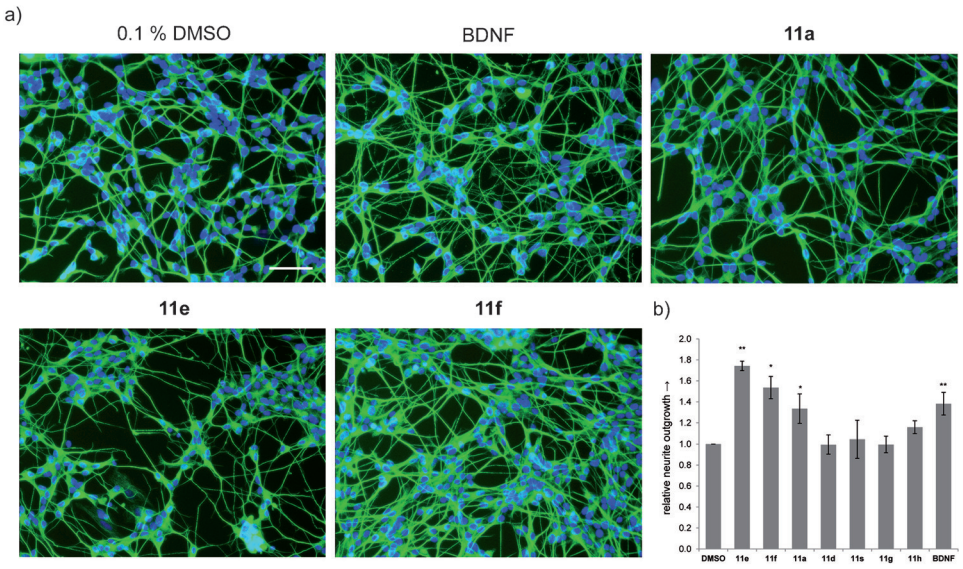


Scheme 1. Strategies for the synthesis of functionalized pyridones and pyridines. DMP = Dess–Martin periodinane, TBAF = tetra-*n*-butylammonium fluoride, TBAI = tetra-*n*-butylammonium iodide, TBS = *tert*-butyldimethylsilyl.

For evaluation of their neurotrophic potential the compounds were subjected to phenotypic screening in human neuroblastoma SH-SY5Y cells to monitor neurite outgrowth.[9] To this end, upon differentiation for 72 hours in a serum-free N2 medium supplemented with retinoic acid (RA), SH-SY5Y cells were treated with the respective compounds at concentrations of 1 μM and 10 μM for 96 hours. 0.1% DMSO was used as a vehicle control, while 1% DMSO served as a negative control because of its retracting properties (see Figure S1 in the Supporting Information).[10] Brain-derived neurotrophic factor (BDNF) was used as a positive control (Figure 2). Cells were stained for α-tubulin and DNA to visualize neurites and cell nuclei prior to high-content analysis.

Gratifyingly, three pyridones embodying a *p*-hydroxyphenol substituent significantly enhanced neurite outgrowth in SH-SY5Y cells at a concentration of 10 μM (Figures 2 and 3). The compound **11e** was the most active and increased the relative neurite outgrowth, defined as total neurites length per cell, by 74% compared to that of the 0.1% DMSO control. The pyridones **11f** and **11a** exhibited a relative neurite outgrowth enhancement of 54 and 33%, respectively. Apparently, the active small molecules display structural similarity to farinosone A (**3**) which also contains a 4-hydroxy-2-pyridone scaffold and bears a 4-hydroxyphenyl moiety at C5 (Figure 1).

Pyridones with residues, which are bulkier than cyclopentyl or longer than *n*-pentyl, at C3 were either inactive or cytotoxic. For instance, **11b** and **11i** (see Figure S2 a), having longer alkyl chains (*n*-nonyl and *n*-heptyl, respectively), were cytotoxic at 10 μM and were inactive at a concentration of 1 μM. Replacing the alkyl chains with aromatic rings yielded **11d** and **11s** and abolished the activity, thus demonstrating that only limited hydrophobic bulk is tolerated at this position for neuritogenic activity (Figure 2). Notably, replacing the phenol substituent by a *p*-fluorophenyl group (compare **11g** and **11h** with **11f** and **11e**) led to the loss of neuritogenic properties. The 2,4-dimethoxypyridines **14a**,**b** and **14d**, embodying the same C3- and C5-appendages as those in the active pyridones **11f**, **11e**, and **11a**, were either inactive or cytotoxic at 10 μM (see Figure S2), thereby clearly highlight-



Compound	11e	11f	11a	11d	11s	11g	11h
R^1	pentyl	propyl	*cyclo*-pentyl	*p*-$C_6H_4CH(CH_3)_2$	2-naphthyl	propyl	pentyl
R^2	OH	OH	OH	OH	OH	F	F

Figure 2. Influence of militarinone-inspired small molecules on neurite outgrowth in SH-SY5Y cells. Upon initiation of differentiation, SH-SY5Y cells were incubated with the compounds at a concentration of 10 μM or 0.1% DMSO and 200 ng mL^{-1} BDNF as controls for 96 h. Cells were fixed, permeabilized, and stained for DNA (blue) and microtubules (green) using DAPI and an anti-α-tubulin antibody and a secondary antibody coupled to Alexa 488. Scale bar: 50 μm. Representative images (a) and the calculated relative neurite outgrowth (b) are shown. Data are mean values ± s.d. ($n = 3$). Mean neurite outgrowth values were normalized to the mean neurite outgrowth of cells that were treated with 0.1% DMSO. Statistical significance was analyzed by one-sample t-test (**: $p < 0.01$, *: $p < 0.05$). c) Structures of active and inactive pyridones.

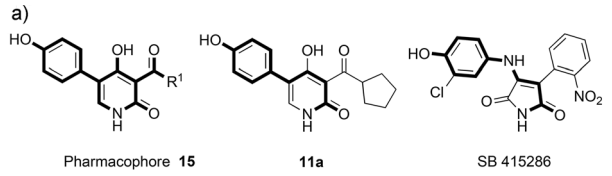
ing the importance of the core pyridone scaffold for neurotrophic activity. At 1 μM the pyridines were inactive. These data show that the 4-hydroxy-2-pyridone core in conjunction with a 4-hydroxyphenyl residue at C5 is mandatory for neurotrophic activity. Substitution at C3 is variable to a limited extent and strongly influences the neuritogenic properties. These observations led us to conclude that the pharmacophore-determining neuritogenic activity is defined by the 3-acyl-4-hydroxy-5-(4-hydroxyphenyl)pyridin-2(1*H*)-one **15** (Figure 3). The close structural similarities of the identified neuritogenic compounds (**11a** and **11e**,**f**) indicate a distinct mode of action.

Neuroprotection and neurite outgrowth have been linked to the modulation of different kinases.[1c,11–13] An RNAi-based genetic screen in SH-SY5Y cells identified kinases which are essential for growth-cone collapse, and either neurite retraction or neurite outgrowth.[12] Moreover, kinase inhibitors can promote neurite outgrowth from primary rat hippocampal cells.[13] Notably, the GSK3 inhibitor SB415286 possesses neuritogenic and neuroprotective properties, and clearly shares structural features with **11a**,**e** and **11f** (Figure 3).[14] In addition, the natural product pyridovericin (**4**), which is similar in structure to the neurite outgrowth inducing compounds identified by us, inhibits tyrosine kinases.[6d]

These findings and structural similarities suggested that the 4-hydroxy-2-pyridones might target kinases. To investigate this possibility we profiled **11a** and **11e** for the inhibition of 67 kinases involved in neurite outgrowth (see Table S1).[12] Both compounds almost completely inhibited the kinase MAP4K4 (mitogen-activated protein kinase kinase kinase kinase 4, also termed HPK/GCK-like kinase HGK or MEKKK4) at a concentration of 20 μM (see Table S1). The siRNA-mediated knock-down of MAP4K4 induces neurite outgrowth,[12] and therefore MAP4K4 inhibition also might bear a similar consequence. The dimethoxypyridines **14a**,**b** and **14d**, bearing the same substitution pattern as the active pyridones (**11a** and **11e**,**f**), neither promoted neurite outgrowth nor inhibited MAP4K4 (see Figure S2 and Table S2). Furthermore, **11d**,**s** and **11g**,**h**, which did not induce neurite outgrowth, were only very weak inhibitors of MAP4K4 (see Table S2). The most active pyridones, **11a**, **11e**, and **11f**, inhibited MAP4K4 with IC_{50} values in the range of 1.9–3.6 μM and the potency of MAP4K4 inhibition directly correlated with degree of neuritogenic activity (Figure 3b).

This observed parallel in structure–activity correlation for inhibition of MAP4K4 and neurite outgrowth induction strongly supports the notion that the biological activity of the 4-hydroxy-2-pyridones may be mediated by MAP4K4 inhibition. In comparison, biological evaluation of **1** revealed activation of MAP and Akt kinase pathways as well as a potentiation of NGF-mediated SAPK/JNK1 activation.[6e] This finding suggests a different mode of action for **11e**. Further enzymatic analysis revealed an increase in IC_{50} values for MAP4K4 inhibition by **11e** with increasing ATP concentrations (Figure 4a,b). These findings are indicative of an ATP-competitive mode of inhibition.

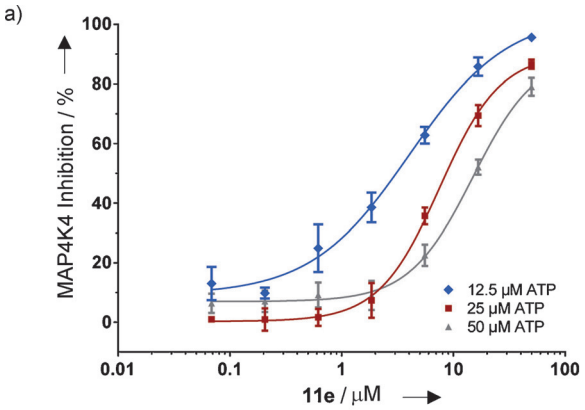
To characterize the binding of **11e** to the kinase in detail, the compound was co-crystallized with the kinase domain of human wild-type MAP4K4. The crystal structure clearly revealed that **11e** binds in a type I fashion (Figure 4c). The



b)

Comp.	R^1	Relative neurite outgrowth	IC_{50} for MAP4K4 inhibition [μM]
11e		1.7 ± 0.1	1.9 ± 0.1
11f		1.5 ± 0.1	3.2 ± 0.5
11a		1.3 ± 0.1	3.6 ± 0.5

Figure 3. Scaffold structures of 4-hydroxypyridone neurite outgrowth enhancers and correlation between neurite outgrowth and MAP4K4 inhibition. a) The potential pharmacophore **15**, **11a**, and GSK3 inhibitor SB 415286. b) Correlation between neurite outgrowth and inhibition of MAP4K4. Neurite outgrowth was determined in SH-SY5Y cells at a concentration of 10 μM after treatment for 96 h. Mean neurite outgrowth was normalized to the mean neurite outgrowth of cells that were treated with 0.1% DMSO. Data are mean values ($n = 3$) ± s.d.



b)

ATP / μM	IC_{50} / μM
12.5	4.0
25	7.5
50	14.9

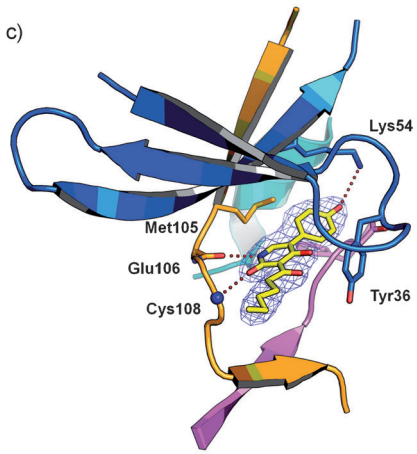


Figure 4. **11e** is an ATP-competitive inhibitor of MAP4K4. a,b) Dose-response data for MAP4K4 inhibition by **11e** in presence of different concentrations of ATP. MAP4K4 enzymatic activity was determined by means of Z′-Lyte Kinase Ser/Thr 7 Assay. a) Dose-response curves. Data are mean values ($n = 3$) ± s.d. Data were fitted using four-parameter Hill equation. b) Estimated IC_{50} values for MAP4K4 inhibition by **11e** at different ATP concentrations. c) The compound **11e** complexed with wild-type MAP4K4 (PDB code: 4RVT). Experimentally determined electron density of **11e** at 2.4 Å resolution is displayed ($2F_o - F_c$ map contoured at 1 σ). Hydrogen-bond interactions are displayed by red dotted lines. The kinase domain is in the active DFG-in conformation and compound **11e** is caged by the glycine-rich loop and flanked by Tyr36. Direct hydrogen-bond interactions are formed between **11e** and the hinge region by addressing the peptide backbone of Glu106 and Cys108 as well as the side chain of Lys54 adjacent to the glycine-rich loop. Color code: hinge region (orange), helix C (turquoise), DFG motif (pink), glycine-rich loop (blue), **11e** (yellow sticks).

pyridone core forms two hydrogen bonds to the kinase hinge region, thus addressing the peptide backbone of Glu106 and Cys108. The glycine-rich loop is folded over the inhibitor such that the side-chain of Tyr36 partly shields the 4-hydroxy-2-pyridone core of the ligand from the solvent, thereby stabilizing inhibitor binding. Similar binding characteristics, ligand-induced structural plasticity of the glycine-rich loop, and binding interactions with the conserved Tyr36 have also been reported for the Ser/Thr kinase p38α.[15] The 4-hydroxyphenyl group forms an additional hydrogen bond to Lys54, adjacent to the glycine-rich loop. This particular hydrogen bond seems to be mandatory for an effective inhibition of MAP4K4 and explains why the corresponding fluorinated compounds **11g** and **11h** fail to inhibit MAP4K4 and therefore do not induce neurite outgrowth. In addition, the *n*-pentyl chain of **11e** resides in the hydrophobic front pocket which is limited in space by the surrounding helices. Therefore propyl (**11f**) and cyclopentyl (**11a**) groups are accommodated, however, spatially more demanding residues such as aromatic moieties (**11d**,**s**) and longer alkyl chains are not. Our structural observations within the MAP4K4 binding cleft are consistent with findings of Crawford et al. on various quinazoline-based inhibitors in complex with MAP4K4.[16] Wang et al. reported on selective 4-amino-pyridopyrimidines as MAP4K4 inhibitors and disclosed complex structures revealing binding modes which translate into fairly different structural conformations as the ligands are not strictly caged by the glycine-rich loop.[17]

Rubin et al. recently demonstrated that the structurally unrelated kinase inhibitor kenpaullone inhibits the JNK-dependent apoptotic cascade by simultaneously blocking GSK3 and MAP4K4 activity and thereby increases survival of motor neurons.[3d] Prolonged over-activation of JNKs is a hallmark of many neurodegenerative diseases and can lead to activation of this apoptotic cascade,[18] and the use of selective JNK inhibitors may circumvent the activation of this pathway. JNK isoforms share high structural homology and selective inhibition of JNK3 over JNK1 and JNK2 is desirable because of the importance of JNK1 and JNK2 in neurogenesis and learning.[1c,18a] However, selective inhibition of JNK3 over

JNK1 and JNK2 using competitive inhibitors is challenging. Thus, the inhibition of an upstream kinase of the apoptotic cascade, for example, MAP4K4, TAK, and MKK4, could solve this fundamental issue.[19] Therefore, selective MAP4K4 inhibition could serve as a potentially new therapeutic approach for the treatment of neurodegenerative disorders.[3d]

Analysis of the inhibition of JNK1–JNK3 and other upstream kinases, which can lead to an activation of this apoptotic pathway by **11e** (see Table S3),[18a] revealed that, gratifyingly, **11e** does not inhibit either JNK1–JNK3 or the other selected kinases at a concentration of 20 μM. Thus, **11e** defines a very promising and novel small-molecule kinase inhibitor class which may serve as a starting point to develop novel approaches for the treatment of neurodegenerative diseases. In addition, in comparison to the structurally similar natural product **3**, **11e** is active at much lower concentration in the cellular neurite outgrowth assay and may exhibit better pharmacokinetic properties. The long polyene chain in the natural products **1**–**3** could in fact limit solubility and membrane penetration and could potentially lead to micelle formation.[6e] Therefore, **11e** appears to be more druglike than **3** and is readily accessible by a six-step synthesis.

In conclusion, we have developed a synthesis of a militarinone-inspired collection of 4-hydroxy-2-pyridones which yielded enhancers of neurite outgrowth in a human neuroblastoma cell line at low micromolar concentrations. The neuritogenic compounds selectively inhibit MAP4K4 kinase and inhibition correlates with biological activity. Our findings support the notion that MAP4K4 may be a new target for the treatment of neurodegenerative diseases and simultaneously provide a kinase inhibitor scaffold class that may inspire novel medicinal chemistry programs aimed at the treatment of neurodegenerative disorders. To this end, the crystal structure of the most potent inhibitor bound to MAP4K4 may be of particular relevance since it should enable structure-based design and synthesis for hit optimization.

MAP4K4 inhibitors have been described in only very few cases[3d,16,17,20] and in the context of different diseases, notably cancer and diabetes, and their structures are unrelated to the 4-hydroxy-2-pyridone inhibitor class described herein. Thus, our finding of a natural-product-based class of molecules may prove relevant to other areas of kinase inhibition, medicinal chemistry and the treatment of disease as well.

Keywords: alkaloids · biological activity · drug discovery · natural products · neurological agents

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