CHEMMEDCHEM

DOI: 10.1002/cmdc.200700175

Probing Novel 1-Aza-9-oxafluorenes as Selective GSK-3β Inhibitors

Burkhardt Voigt,[a] Martin Krug,[a] Christoph Schächtele,[b] Frank Totzke,[b] and Andreas Hilgeroth*[a]

Within the histopathology of Alzheimer's disease (AD) certain hallmarks are beeing observed. The occurance of protein deposits belong to such characteristic features. Such deposits can be found extracellular as β-amyloid (Aβ) plaques and intracellular as neurofibrillary tangles (NFTs). In the search for novel AD therapeutics it became of great interest to investigate the formation of NFTs and their contribution to the AD symptomatic. NFTs consist of hyperphosphorylated tau protein. Within the phosphorylation process of tau protein two kinases are of great importance: cyclin dependent kinase 5 (cdk5) and its truncated regulatory subunit p25 and glycogen synthase kinase 3β (GSK-3β). The role of both kinases within the NFT formation process is still under debate. To better understand the pathophysiological process highly selective inhibitors of both kinases are of value. Known inhibitors lack the necessary selectivity. We developed novel 1-aza-9-oxafluo-renes as selective GSK-3β inhibitors. Structure–activity relationships of a series of 4-phenyl substituted derivatives are discussed. Variation of the 3-side chain led to selective carbonyl amide derivatives with selectivity factors of more than 100 at the tested ATP competitor concentrations. Such selectivities permit specific investigation of the role of GSK-3β within the NFT formation processes.

Introduction

In the therapy of Alzheimer's disease several approaches have been made to improve the symptoms, such as the use of acetylcholine esterase inhibitors.[1] In the brain of Alzheimer patients extracellular and intracellular deposits of insoluble proteins are found. Extracellular protein deposits are β-amyloid (Aβ) plaques which mainly consist of Aβ-peptides of 39 to 43 amino acids.[2,3] With the most toxic species being Aβ-42, they result from the amyloid precursor protein (APP) by the activity of β- and γ-secretases which cleave the Aβ-peptides from the integral membrane protein APP.[4–6]

The second histopathologic hallmark in AD is the occurance of insoluble intracellular protein tangles, termed neurofibrillary tangles (NFTs). They consist of tau protein which is found hyperphosphorylated at the phosphorylation sites of more than 30 amino acids.[7,8] The hyperphosphorylation results mainly from the abnormal activity of two kinases.[9,10] One of them is the cyclin dependent kinase (cdk) 5 which is normally regulated in its physiological activity by the protein p35. The cleavage of p35 to p25 leads to a subunit that activates cdk5 constitutively and results in hyperphosphorylation of tau protein, its aggregation, and accumulation to form NFTs in transgenic mice models.[11,12]

However, the other kinase involved in NFT formation is glycogen synthase kinase (GSK) 3β. The discussion of a direct or indirect influence is still under debate. GSK-3β is known to phosphorylate several sites of tau protein.[10] It is also thought to activate cdk5 and so indirectly influence the cdk5-mediated tau phosphorylation.[13]

Therefore it would be of great value to have an inhibitor of GSK-3β which selectively inhibits only this kinase as a tool for the functional analysis of NFT formation. For such an inhibitor, the selectivity is of much more interest than the degree of activity itself.

Reported GSK-3β inhibitors belong to the family of the paullones which have been investigated as anticancer drugs with cdk-inhibitory activities.[14] They show activities in the submicromolar ranges but the selectivity factor for GSK-3β inhibition of the early compounds is only ten.[14,15] 1-Azakenpaullone, **1** (Figure 1) showed improved selectivity for GSK-3β compared to cdk1 and its regulatory subunit cyclin B, but information concerning inhibition of other related cdks or other kinases is very poor.[15,16]

So such a compound is not suitable as a tool for functionality investigations of NFT formation. Compounds belonging to the indirubin **2** family show better inhibitory activities towards the three investigated kinases including GSK-3β, but the selectivity factor was only two.[17] With uninvestigated inhibition profiles of related cdks these compounds are not suitable for analysing purposes.[16] The development of maleimide compounds **3** also led to insufficiently specific inhibitors.[15]

[a] *Dr. B. Voigt, M. Krug, Dr. A. Hilgeroth*
Institute of Pharmacy
Martin-Luther University
Wolfgang-Langenbeck Straße 4, 06120 Halle (Germany)
Fax: +49 345 5527026
E-mail: andreas.hilgeroth@pharmazie.uni-halle.de

[b] *Dr. C. Schächtele, Dr. F. Totzke*
ProQinase GmbH
Breisacher Strasse 117, 79106 Freiburg (Germany)

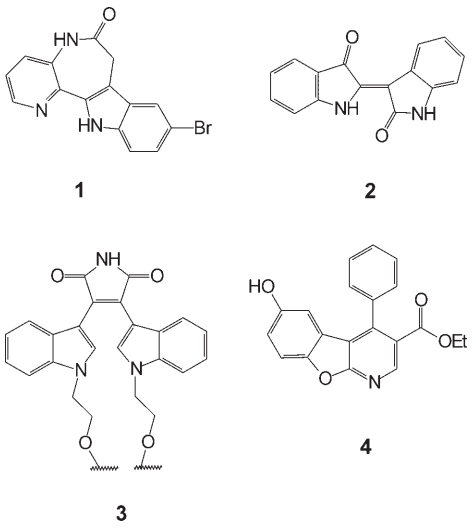


Figure 1. Structure of compounds **1**–**4**.

Design and synthesis

1-Aza-9-oxafluorenes have been discovered as a novel class of protein kinase inhibitors by a COMPARE analysis carried out with the 4-phenyl 1-aza-9-oxafluorene **4**.[18] However, screening against cyclin dependent kinase cdk1/cyclin B showed some activity as a cdk1 inhibitor wheras the activity against cdk5/p25, cdk2/cyclin E, and cdk4/cyclin D1 was poor.

One structurally essential element for activity has been the 4-phenyl substituent, because compounds with a 4-methyl group proved to be not active.

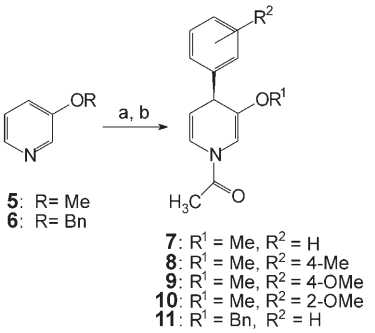
The 3-acyl function as a structural element in the active compounds was suggested to work as a hydrogen bond acceptor from the protein backbone of the enzyme. Moreover, the 6-hydroxy function also serves as an essential element for activity because the acylation of this hydroxy function leads to a loss of activity.

To further investigate the role of both the 3-substituent as a hydrogen bond acceptor and the role of the 4-phenyl substituent with respect to enzyme inhibiting activities, we structurally varied both substituents. The 3-substituent was varied by an alkoxy function and an amide function whereas the 4-phenyl substituent was substituted by varying lipophilic substitution patterns as discussed below.

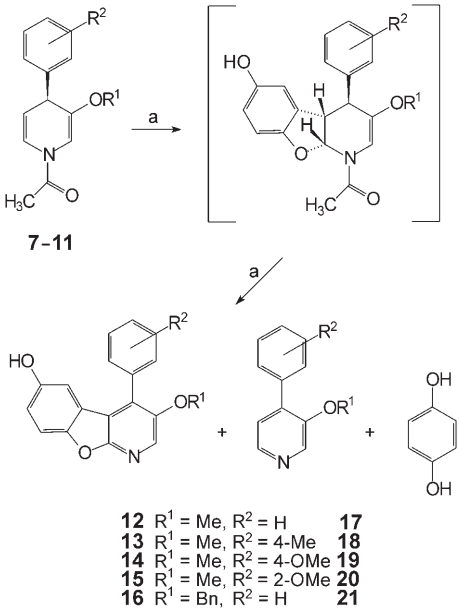
The synthesis of target compounds started from 3-methoxy pyridine **5** which was acetylated using equimolar amounts of acetyl chloride in THF at −20 °C. The resulting pyridinium intermediate was arylated using equimolar amounts of the corresponding grignard reagents and a combination of 10% of copper(I) iodide and 20% of lithium chloride.

Under these conditions the regioselective arylation of the 4-position of the pyridinium ring occured yielding the 1,4-dihydropyridines **7**–**10** as reaction products (see Scheme 1).

Then these compounds reacted with *p*-benzoquinone in dioxane under perchloric acid catalysis via a michael addition analogue product and a following cyclization reaction to primary tetrahydro 1-aza-9-oxafluorene intermediates which were detected by TLC.[18] Smooth oxidation of the unisolated intermediates with an excess of *p*-benzoquinone led to the formation of the 1-aza-9-oxafluorene target compounds **12**–**15** accompanied by the oxidative fragmentation of the tetrahydro intermediates to pyridine compounds **17**–**20** (see Scheme 2).



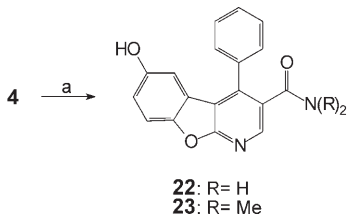
Scheme 1. Reagents and conditions for compounds **7**–**11**: a) 10% $Cu^{I}I$, 20% LiCl, acetyl chloride, THF, −20 °C; b) aryl magnesium chloride, THF, −20 °C.



Scheme 2. Reagents and conditions for compounds **12**–**16**: *p*-benzoquinone, dioxane, $HClO_4$ (2%), RT.

The formation of the 3-benzyloxy pyridine starting compound **6** resulted from a benzylation reaction of 3-hydroxy pyridine with benzyl chloride by phase transfer catalysis. An acetylation procedure and 1-aza-9-oxafluorene formation were carried out as described to yield compound **16**.

The formation of the 3-carbonyl amide compounds **22** and **23** (Scheme 3) started from the 1-aza-9-oxafluorene **4** which was treated with ammonia and dimethyl amine solutions, respectively.



Scheme 3. Reagents and conditions for compounds **22**, **23**: $NH(R)_2$, MeOH, $-8\,^{\circ}C$.

Results and Discussion

1-Aza-9-oxafluorene **4** with cdk1/(cyclin) B inhibiting properties had two structural features, namely the 4-phenyl and the 6-hydroxy function, which were found to be essential elements for kinase inhibition.

In a first series we substituted the 3-carbonyl ester which function as a potential hydrogen bond acceptor by methoxy functions and a benzyloxy function, respectively. The alkoxy oxygen atom would influence a potential hydrogen bond acceptor function of the pyridine nitrogen compared to the 3-carbonyl function in compound **4** because the carbonyl function has an electron withdrawing effect whereas the oxygen function also has electron pushing effects. The lipophilicity within the 3-side chain was changed to investigate potential effects on kinase binding possibilities.

The 3-methoxy compound **12** only showed activity as an cdk1/B inhibitor and some activity as a GSK-3β inhibitor determined at an ATP competitor concentration of 1 μM whereas at the higher concentration of 15 μM no activity was found (Table 1).[19] The change of the methoxy against the benzyloxy function in compound **16** changed the inhibitory properties because a better inhibition of both, cdk1/B and GSK-3β, was determined at the usual higher ATP competitor concentration of 15 μM. So the introduction of a large lipophilic substituent within the 1-aza-9-oxafluorene side chain is advantageous with respect to the binding affinity and comprises a hydrophobic interaction with a lipophilic enzyme binding site.

Next, we varied the structurally essential 4-phenyl substituent in compound **12** by the introduction of functional groups, namely a methyl function and a methoxy function. The methyl function in derivative **13** led to a decrease in the inhibition of cdk1/B and cdk2/E whereas GSK-3β inhibition improved. As the lipophilic 4-phenyl substituent may fill a hydrophobic binding pocket because of its space demand, the additional methyl group which affects the space demand of the substituent is a suggestion for a better accommodation of this functional group.

The introduction of the methoxy group at the same position led to a more significant decrease in activity towards cdk1/B in derivative **14** and to a loss of inhibition towards cdk2/E, cdk4/D, and cdk5/p25. The more lipophilic methoxy group strengthened the space demand properties of the related 4-methyl compound and thus led to selectivity of GSK-3β inhibition compared to the noninhibited kinases. However, compared to the cdk1/B inhibition selectivity with a factor of 2 is low.

The positioning of the methoxy group in the 2-position of the phenyl ring in compound **15** led to a decrease in activity towards the kinases which may be caused by a steric hindrance between the 2-methoxyphenyl function and the 3-methoxy substituent of the 1-aza-9-oxafluorene scaffold on the free rotation of the 4-aryl substituent which is obviously a disadvantage.

Finally, we varied the substitution patterns of the 3-carbonyl function side chain which has been our early functional cdk1/B protein kinase inhibitor template. The amide function of the synthesized compound **22** surprisingly led to a complete loss of cdk1/B inhibition and a GSK-3β inhibition constant in the micromolar range. This compound could serve as a tool for a functional analysis as a GSK-3β inhibitor on NFT formation and disaggregation. To further characterise its selectivity profile we determined exactly the binding affinity constants towards cdk5/p25 and cdk4/D1. The selectivity factor towards cdk4/D1 is about 100, whereas that towards cdk5/p25 is about 50. Therefore, compound **22** is the first selective GSK-3β inhibitor within the investigated series as desired for the purpose of functional neuronal analysis.

Improvements in activity and, furthermore, selectivity could be realised by the methylation of the amide function in the dimethyl amide derivative **23**. Whereas compound **23** did not inhibit cdk5/p25, the selectivity factor towards cdk4/D1 increased to 125.

We investigated structure–activity relationships of novel 1-aza-9-oxafluorenes. Increasing lipophilicity of the aromatic 4-substituent led to more selectivity towards GSK-3β. Moreover, a lipophilic character in the side chain of the 3-oxygen hydrogen bond acceptor function is also valuable with respect to more

Table 1. K_i values of 1-aza-9-oxafluorene target compounds **12**–**16**, **22**, and **23**.[a]

Compd.	K_i value [μM] cdk1/B	cdk2/E	cdk4/D1	cdk5/p25	GSK-3β
indirubine-3′-monoxime[b]	3.5	1.3	0.52	n.d.[c]	0.99
12[d]	5.3 ± 1.3	24.0 ± 5.1	92.6 ± 24	217 ± 30	14.8 ± 3.1
13[d]	27.4 ± 6.9	50.0 ± 6.7	783 ± 113	490 ± 46	9.9 ± 0.8
14[d]	31.3 ± 9.2	241 ± 67	n.a.[e]	n.a.[e]	16.3 ± 3.1
15[d]	n.a.[e]	267 ± 18	135 ± 12	n.a.[e]	n.a.[e]
16[f]	2.3 ± 0.3	6.4 ± 0.2	36.6 ± 8.3	n.a.[e]	5.8 ± 1.2
22[f]	n.a.[e]	n.a.[e]	462 ± 130	293 ± 17	4.1 ± 0.4
23[f]	n.a.[e]	n.a.[e]	194 ± 44	n.a.[e]	1.5 ± 0.2

[a] The selectivity towards the MAR-kinase (ERK2) which may attribute to the tau phosphorylation will be investigated in later studies. [b] Values taken from literature.[23] [c] not determined. [d] K_i values determined with an ATP competitor concentration of 1 μM. [e] $K_i > 1000$ μM. [f] K_i values determined with an ATP competitor concentration of 15 μM.

selective GSK-3β binding. Finally, the dimethyl amide function led to the highest selectivity, superior to known GSK-3β inhibitors.

Docking studies[20] carried out with compounds **16**, **22**, and **23** showed hydrogen bonding of the 6-hydroxy function of the molecular scaffold to the NH function of Cys 199 of the protein backbone of GSK-3β (Figure 2).

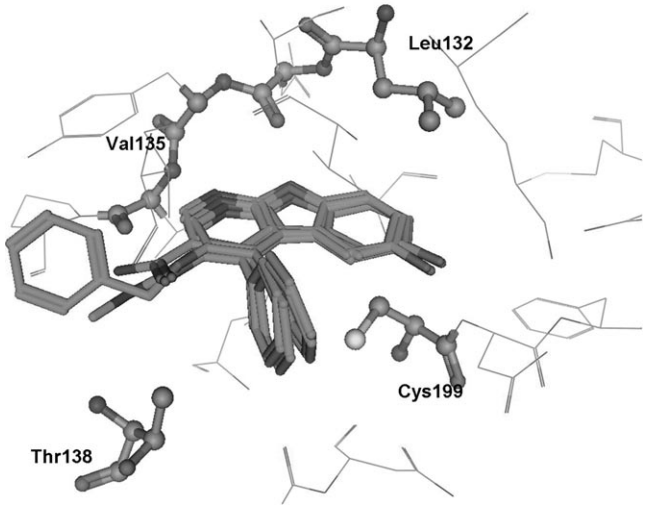


Figure 2. Docking results for target compounds **16**, **22**, **23**, and GSK-3β. Superimposition of the docked inhibitors.

The aromatic benzyloxy residue in compound **16** is positioned towards Arg 141 because the gatekeeper amino acid Leu 132 sterically limits the binding pocket. In the case of GSK-3β there is a hydrophobic binding area in the entrance location of the co-substrate pocket which favorably interacts with the aromatic residue of compound **16**. Whereas all the investigated compounds show hydrogen bonding of the scaffold nitrogen to the NH amide function of Val 135, only the amide derivatives **22** and **23** show an additional hydrogen bond to the amide carbonyl function of Thr 138 via a water molecule. This binding may contribute to the selectivity of compounds **22** and **23**.

Moreover, we investigated the inhibition constant of the most selective target compound **23** towards EGFR (epidermal growth factor receptor) kinase belonging to another class of tyrosine kinases. Paullone compounds were reported to additionally inhibit kinases which belong to this family and thus proved to be nonselective.[21] Interestingly, we found no activity against EGFR of **23** even at the lower ATP competitor concentration of 1 μM. So compound **23** is a perspective selective GSK-3β inhibitor.

Experimental Section

Chemistry: All the chemical agents used were either synthesized or are commercially available. Melting points were determined using a Boetius melting desk microscope and are uncorrected. Infrared spectra were recorded either on a Bruker or a Perkin–Elmer FITIR-spectrometer named IFS 28 and Spectrum BX, respectively. Mass spectra were recorded on an AMD 402 AMD INTEGRA or a Finnigan LCQ Classic mass spectrometer. Proton NMR spectra were recorded on a Varian Gemini 200 at 400 MHz or a Varian Inova Unity 500 at 500 MHz. Chemical shifts are reported in ppm units with tetramethylsilane as internal reference standard. Elemental analyses of target compounds were performed using a Leco CHNS-932 apparatus.

Procedure for the synthesis of 3-benzyloxy pyridine (6): 3-Hydroxy pyridine (5 g, 53 mmol), powdered KOH (5.9 g, 110 mmol), and nBu$_4$NBr (0.85 g, 2.6 mmol) were dissolved in dried toluene (150 mL). After addition of benzyl chloride (10.6 g, 84 mmol) the stirring mixture was heated under reflux for 12 h. Then distilled water (200 mL) was added and the solution was extracted twice with a 10% water solution of HCl (100 mL). The united water phases were then alkalined with 10 M of NaOH and extracted with $CHCl_3$ (75 mL) three times. After drying over Na_2SO_4 the organic layer was removed in vacuum. The remaining oil was purified by column chromatography using silica gel and a mixture of cyclohexane/EtOAc (1:1). After evaporation of the eluent the remaining oil of **6** was given in a yield of 59%: IR (ATR): $\tilde{\nu}$ = 1227, 1260 (C-O-C); MS (ESI): m/z 186 (100) [M+H$^+$]; ^{1}H NMR (400 MHz, [D$_6$]aceton): δ = 5.18 (s, 2H, OCH$_2$), 7.26 (ddd, ^3J (H/H) = 8.4, 4.6 Hz, ^4J (H/H) = 0.6 Hz, 5-H), 7.31–7.41 (m, 4H, aromat. H), 7.50–7.57 (m, 2H, aromat. H), 8.18 (dd, ^3J (H/H) = 4.6 Hz, ^4J (H/H) = 1.3 Hz, 1H, 6-H), 8.37 ppm (d, ^3J (H/H) = 2.9 Hz, 1H, 2-H).

General procedure A for the synthesis of 4-aryl-substituted *N*-acetyl 1,4-dihydropyridines 7–11: 3-Alkyloxy pyridine (10 mmol) was dissolved in dried THF (50 mL). After addition of Cu$^{\text{I}}$I (1 mmol) and LiCl (2 mmol) the stirred mixture was cooled to - 20 °C. After 20 min of additional stirring freshly distilled acetyl chloride (10 mmol) was added dropwise. After 15 min aryl grignard reagent (10 mmol) was added dropwise and then stirring continued for 15 min. The mixture was left standing without cooling at RT. Hydrolysis with an NH_4Cl solution in water (50 mL, 20%) followed. Then it was extracted with diethyl ether (50 mL) three times. The united organic layer was extracted twice with a solution of NH_4Cl/NH_3 in water (50 mL), once with water, twice with a solution of HCl in water (10%), once with water and, finally, with a saturated solution of NaCl. After drying over Na_2SO_4 and filtration the solvent was removed in vacuum and the resulting oil was purified by column chromatography over silica gel using a mixture of cyclohexane/EtOAc (60:40). After removal of the eluent the 1,4-dihydropyridines crystallised from MeOH.

(*EZ,RS*)-1-(3-Methoxy-4-phenyl-4*H*-pyridine-1-yl)-ethanone (7): Yield: 40%; white solid; mp: 50–70 °C; IR (ATR): $\tilde{\nu}$ = = 1674, 1633 (C=O); MS (ESI): m/z 252 (100) [M+Na$^+$]; ^{1}H NMR (400 MHz, CDCl$_3$): δ = 2.27 (spl s, 6H, NCOCH$_3$, *EZ*), 3.52 (s, 3H, 3-OCH$_3$, *Z*), 3.55 (s, 3H, 3-OCH$_3$, *E*), 4.23 (dd, ^3J (H/H) = 4.3 Hz, ^4J (H/H) = 1.3 Hz, 2H, 4-H, *EZ*), 5.03 (dd, ^3J (H/H) = 8.2, 4.3 Hz, 1H, 5-H, *E*), 5.12 (dd, ^3J (H/H) = 8.2, 4.3 Hz, 1H, 5-H, *Z*), 6.07 (d, ^4J (H/H) = 1.3 Hz, 1H, 2-H, *Z*), 6.64 (dt, ^3J = 8.2 Hz, ^4J (H/H) = 1.3 Hz, 1H, 6-H, *E*), 6.90 (d, ^4J (H/H) = 1.3 Hz, 1H, 2-H, *E*), 7.20–7.24 (m, 6H, aromat. H, *EZ*), 7.28–7.34 (m, 4H, aromat. H, *EZ*), 7.34 ppm (dt, ^3J (H/H) = 8.2 Hz, ^4J (H/H) = 1.3 Hz, 1H, 6-H, *Z*).

(*EZ,RS*)-1-(3-Methoxy-4-(4-methylphenyl)-4*H*-pyridine-1-yl)-ethanone (8): Yield: 55%; white solid; mp: 102–106 °C; IR (ATR): $\tilde{\nu}$ = 1670, 1634 (C=O); MS (EI): m/z 243 (20) [M^+], 152 (31) [$M^+ - C_7H_7$], 110 (100) [$M^+ - C_7H_7$, $-OC_2H_5$]; ^{1}H NMR (400 MHz, CDCl$_3$): δ = 2.26 (spl s, 6H, COCH$_3$, *EZ*), 2.31 (s, 6H, 4′-CH$_3$, *EZ*), 3.52 (s, 3H, 3-OCH$_3$, *Z*), 3.54 (s, 3H, 3-OCH$_3$, *E*), 4.19 (d, ^3J (H/H) = 4.4 Hz, 2H, 4-H, *EZ*), 5.01 (dd, ^3J (H/H) = 8.3, 4.4 Hz, 1H, 5-H, *E*), 5.10 (dd, ^3J = 8.3, 4.4 Hz, 1H, 5-H, *Z*), 6.06 (s, 1H, 2-H, *Z*), 6.62 (dt, ^3J (H/H) = 8.3, ^4J (H/H) =

1.3 Hz, 1H, 6-H, *E*), 6.88 (s, 1H, 2-H, *E*), 7.09–7.13 (m, 8H, aromat. H, *EZ*), 7.31 ppm (d, ^3J = 8.3 Hz, 1H, 6-H, *Z*).

(*EZ,RS*)-1-(3-Methoxy-4-(4-methoxyphenyl)-4*H*-pyridine-1-yl)-ethanone (9): Yield: 33%; white solid; mp: 78–83 °C; IR (ATR): $\tilde{\nu}$ = 1668, 1633 (C=O); MS (EI): *m/z* 259 (50) [M^+], 152 (34) [$M^+ - C_7H_7O$], 110 (100) [$M^+ - C_7H_7O$, $-OC_2H_5$]; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 2.26 (spl s, 6H, $COCH_3$, *EZ*), 3.52 (s, 3H, 3-OCH_3, *Z*), 3.54 (s, 3H, 3-OCH_3, *E*), 3.77 (s, 6H, 4′-OCH_3, *EZ*), 4.17 (d, ^3J (H/H) = 4.3 Hz, 2H, *EZ*), 5.00 (dd, ^3J = 8.2, 4.3 Hz, 1H, 5-H, *E*), 5.10 (dd, ^3J = 8.2, 4.3 Hz, 1H, 5-H, *Z*), 6.04 (s, 1H, 2-H, *Z*), 6.62 (dt, ^3J = 8.2 Hz, ^4J = 1.4 Hz, 1H, 6-H, *E*), 6.83–6.85 (m, 4H, aromat. H), 6.87 (d, ^4J = 1.4 Hz, 1H, 4-H, *E*), 7.11–7.14 (m, 4H, aromat. H, *EZ*), 7.31 ppm (d, ^3J = 8.2 Hz, 1H, 6-H, *Z*).

(*EZ,RS*)-1-(3-Methoxy-4-(2-methoxylphenyl)-4*H*-pyridine-1-yl)-ethanone (10): Yield: 60%; white solid; mp: 94–98 °C; IR (ATR): $\tilde{\nu}$ = 1668, 1634 (C=O); MS (EI): *m/z* 259 (44) [M^+], 152 (29) [$M^+ - C_7H_7O$], 110 (100) [$M^+ - C_7H_7O$, $-OC_2H_5$]; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 2.24 (s, 3H, $COCH_3$, *E*), 2.26 (s, 3H, $COCH_3$, *Z*), 3.54 (s, 3H, 3-OCH_3, *Z*), 3.58 (s, 3H, 3-OCH_3, *E*), 3.83 (spl s, 6H, 2′-OCH_3, *EZ*), 4.75 (dd, ^3J (H/H) = 4.4 Hz, ^4J (H/H) = 1.2 Hz, 1H, 4-H, *E*), 4.77 (dd, ^3J (H/H) = 4.4 Hz, ^4J (H/H) = 1.2 Hz, 1H, 4-H, *Z*), 5.06 (dd, ^3J (H/H) = 8.3, 4.4 Hz, 1H, 5-H, *E*), 5.12 (dd, ^3J (H/H) = 8.3, 4.4 Hz, 1H, 5-H, *Z*), 6.20 (d, ^4J (H/H) = 1.2 Hz, 1H, 2-H, *Z*), 6.53 (dt, ^3J (H/H) = 8.3 Hz, ^4J (H/H) = 1.2 Hz, 1H, 6-H, *E*), 6.86 (d, ^3J (H/H) = 8.3 Hz, 2H, 3′-H, *EZ*), 6.92 (dt, ^3J (H/H) = 7.4 Hz, ^4J (H/H) = 0.8 Hz, 2H, 5′-H, *EZ*), 7.00 (d, ^4J (H/H) = 1.2, 1H, 2-H, *E*), 7.09 (dd, ^3J (H/H) = 7.4 Hz, ^4J (H/H) = 1.6 Hz, 2H, 6′-H, *EZ*), 7.18 (ddd, ^3J (H/H) = 8.3, 7.4 Hz, ^4J (H/H) = 1.6 Hz, 2H, 4′-H, *EZ*), 7.22 ppm (dt, ^3J (H/H) = 8.3 Hz, ^4J (H/H) = 1.2 Hz, 1H, 6-H, *Z*).

(*EZ,RS*)-1-(3-Benzyloxy-4-phenyl-4*H*-pyridine-1-yl)-ethanone (11): Yield: 58%; white solid; mp: 78–82 °C; IR (ATR): $\tilde{\nu}$ = 1672, 1635 (C=O); MS (EI): *m/z* 305 (15) [M^+], 166 (44) [M^+–Ph], 124 (100) [M^+–Ph, $-O{=}C{=}CH_2$]; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 2.16 (s, 3H, $COCH_3$, *Z*), 2.26 (s, 3H, $COCH_3$, *E*), 4.31 (br d, ^3J (H/H) = 4.3 Hz, 2H, 4-H, *EZ*), 4.74 (AB, ^2J (H/H) = 11.8 Hz, 2H, 3-O$CH_2C_6H_5$, *Z*), 4.77 (AB, ^2J (H/H) = 11.8 Hz, 2H, 3-O$CH_2C_6H_5$, *E*), 5.07 (dd, ^3J (H/H) = 8.2, 4.3 Hz, 1H, 5-H, *E*), 5.15 (dd, ^3J (H/H) = 8.2, 4.3 Hz, 1H, 5-H, *Z*), 6.10 (d, ^4J (H/H) = 1.3 Hz, 1H, 2-H, *Z*), 6.67 (dt, ^3J = 8.2 Hz, ^4J (H/H) = 1.3 Hz, 1H, 6-H, *E*), 6.98 (d, ^4J (H/H) = 1.3 Hz, 1H, 2-H, *E*), 7.10–7.13 (m, 4H, aromat. H, *EZ*, 6-H, *Z*), 7.20–7.33 ppm (m, 17H, aromat. H, *EZ*).

General procedure B for the synthesis of 4-aryl-1-aza-9-oxafluorenes 12–16: *N*-Acetyl 1,4-dihydropyridine (5 mmol) was dissolved in a minimum volume of dried dioxane under stirring. Then *p*-benzoquinone (6.2 mmol) was added and the solution volume was increased by the addition of dioxane and $HClO_4$ (70%) up to 50 mL with finally 2% of $HClO_4$. After 24 h *p*-benzoquinone (2 mmol) was added to react with the still TLC detectable tetrahydro intermediate to form the 1-aza-9-oxafluorene target compounds beside pyridine oxidation product. Then 10 mL of distilled water were added and the pH-value was adjusted to pH 9 using NaOH (1 M). The solution was extracted with $CHCl_3$ (25 mL) three times. The united organic layer was dried over $NaSO_4$ and filtered. After removal of the organic layer in vacuum the resulting oily residue was purified by column chromatographie using silica gel and a mixture of cyclohexane/EtOAc (60:40). After evaporation of the corresponding fractions to dryness the remaining solids were recrystallised from MeOH to yield pure 1-aza-9-oxafluorenes as target compounds or crystalline pyridines by recrystallisation from diethyl ether.

3-Methoxy-4-phenylbenzo[4,5]furo[2,3-*b*]pyridine-6-ol (12): Yield: 10%; brownish powder; mp: 240–245 °C; IR (KBr): $\tilde{\nu}$ = 3434 (OH), 1275 (C-O-C), 1368 (OH); MS (EI): *m/z* 291 (100) [M^+]; ^{1}H NMR (400 MHz, [D_6]dmso): δ = 3.84 (s, 3H, 3-OCH_3), 6.47 (d, ^4J (H/H) = 2.5 Hz, 1H, 5-H), 6.91 (dd, ^3J (H/H) = 8.8 Hz, ^4J (H/H) = 2.5 Hz, 1H, 7-H), 7.49 (d, ^3J = 8.8 Hz, 1H, 8-H), 7.49–7.51 (m, 2H, 2′-, 6′-H), 7.53–7.61 (m, 3H, 3′-, 4′-, 5′-H), 8.25 (s, 1H, 2-H), 9.34 ppm (s, 1H, OH); elemental analysis calcd (%) for $C_{18}H_{13}NO_3$: C 74.22, H 4.50, N 4.81; found: C 73.89, H 4.45, N 4.95.

3-Methoxy-4-(4-methylphenyl)-benzo[4,5]furo[2,3-*b*]pyridine-6-ol (13): Yield: 12%; pink colored powder; mp: 237–239 °C; IR (KBr): $\tilde{\nu}$ = 3421 (OH), 1367 (OH), 1274 (C-O-C); MS (EI): *m/z* 305 (100) [M^+]; ^{1}H NMR (400 MHz, [D_6]dmso): δ = 2.45 (s, 3H, 4′-CH_3), 3.82 (s, 3H, 3-OCH_3), 6.56 (d, ^4J = 2.5 Hz, 1H, 5-H), 6.91 (dd, ^3J (H/H) = 8.8 Hz, ^4J (H/H) = 2.5 Hz, 1H, 7-H), 7.39 (s, 4H, aromat. H), 7.48 (d, ^3J (H/H) = 8.8 Hz, 1H, 8-H), 8.23 (s, 1H, 2-H), 9.33 ppm (s, 1H, OH); elemental analysis calcd (%) for $C_{19}H_{15}NO_3$: C 74.74, H 4.95, N 4.59; found: C 74.57, H 5.01, N 4.66.

3-Methoxy-4-(4-methoxyphenyl)-benzo[4,5]furo[2,3-*b*]pyridine-6-ol (14): Yield: 11%; orange powder; mp: 242–245 °C; IR (KBr): $\tilde{\nu}$ = 3401 (OH), 1367 (OH), 1275 (C-O-C); MS (EI): *m/z* 321 (100) [M^+]; ^{1}H NMR (400 MHz, [D_6]dmso): δ = 3.83 (s, 3H, 4′-OCH_3), 3.87 (s, 3H, 3-OCH_3), 6.61 (d, ^4J (H/H) = 2.5 Hz, 1H, 5-H), 6.91 (dd, ^3J (H/H) = 8.8 Hz, ^4J (H/H) = 2.5 Hz, 1H, 7-H), 7.13–7.15 (m, 2H, 3′-, 5′-H), 7.43–7.46 (m, 2H, 2′-, 6′-H), 7.48 (d, ^3J (H/H) = 8.8 Hz, 1H, 8-H), 8.22 (s, 1H, 2-H), 9.34 ppm (s, 1H, OH); elemental analysis calcd (%) for $C_{19}H_{15}NO_4$: C 71.02, H 4.71, N 4.36; found: C 71.11, H 4.82, N 4.32.

3-Methoxy-4-(2-methoxyphenyl)-benzo[4,5]furo[2,3-*b*]pyridine-6-ol (15): Yield: 11%; brownish powder. mp: 210–214 °C; IR (KBr): $\tilde{\nu}$ = 3428 (OH), 1367 (OH), 1276 (C-O-C); MS (EI): *m/z* 321 (100) [M^+]; ^{1}H NMR (400 MHz, [D_6]dmso): δ = 3.66 (s, 3H, 2′-OCH_3), 3.82 (s, 3H, 3-OCH_3), 6.33 (d, ^4J (H/H) = 2.6 Hz, 1H, 5-H), 6.90 (dd, ^3J (H/H) = 8.8 Hz, ^4J (H/H) = 2.6 Hz, 1H, 7H), 7.13 (dt, ^3J (H/H) = 7.5 Hz, ^4J (H/H) = 0.9 Hz, 1H, 5′-H), 7.25 (dd, ^3J (H/H) = 8.5 Hz, ^4J (H/H) = 0.9 Hz, 1H, 3′-H), 7.29 (dd, ^3J (H/H) = 7.5 Hz, ^4J (H/H) = 1.7 Hz, 1H, 6′-H), 7.46 (d, ^3J = 8.8 Hz, 1H, 8-H), 7.55 (ddd, ^3J (H/H) = 8.5 Hz, ^3J (H/H) = 7.5, ^4J (H/H) = 1.7 Hz, 1H, 4′-H), 8.22 (s, 1H, 2-H), 9.32 ppm (s, 1H, OH); elemental analysis calcd (%) for $C_{19}H_{15}NO_4$: C 71.02, H 4.71, N 4.36; found: C 70.88, H 4.76, N 4.50.

3-Benzyloxy-4-phenyl-benzo[4,5]furo[2,3-*b*]pyridine-6-ol (16): Yield: 13%; orange powder. mp: 220–224 °C; IR (KBr): $\tilde{\nu}$ = 3401 (OH), 1359 (OH), 1276 (C-O-C); MS (EI) *m/z* 367 (39) [M^+], 276 (11) [$M^+ - C_7H_7$], 91 (100) [C_7H_7]; ^{1}H NMR (400 MHz, [D_6]dmso): δ = 5.17 (s, 2H, O$CH_2C_6H_5$), 6.51 (d, ^4J (H/H) = 2.7 Hz, 1H, 5-H), 6.92 (dd, ^3J (H/H) = 8.9 Hz, ^4J (H/H) = 2.7 Hz, 1H, 7-H), 7.26–7.31 (m, 5H, aromat. H), 7.49 (d, ^3J (H/H) = 8.9 Hz, 1H, 8-H), 7.54–7.60 (m, 5H, aromat. H), 8.32 (s, 1H, 2-H), 9.34 ppm (s, 1H, OH); elemental analysis calcd (%) for $C_{24}H_{17}NO_3$: C 78.46, H 4.66, N 3.81; found: C 78.95, H 4.59, N 3.76.

3-Methoxy-4-phenylpyridine (17): Yield: 55%; mp: 41–44 °C; IR (ATR): $\tilde{\nu}$ = 1243 (C-O-C); MS (EI): *m/z* 185 (100) [M^+]; ^{1}H NMR (400 MHz, [D_6]acetone): δ = 3.93 (s, 3H, 3-OCH_3), 7.30 (dd, ^3J (H/H) = 4.8 Hz, ^5J (H/H) = 0.4 Hz, 1H, 5-H), 7.37–7.47 (m, 3H, aromat. H), 7.58–7.61 (m, 2H, aromat. H), 8.26 (d, ^3J (H/H) = 4.8 Hz, 1H, 6-H), 8.43 ppm (d, ^5J (H/H) = 0.4 Hz, 1H, 2-H); elemental analysis calcd (%) for $C_{12}H_{11}NO$: C 77.81, H 5.99, N 7.56; found: C 77.55, H 6.27, N 7.47.

3-Methoxy-4-(4-methylphenyl)-pyridine (18): Yield: 51%; colourless crystals; mp: 52–54 °C; IR (ATR): $\tilde{\nu}$ = 1237 (C-O-C); MS (EI): *m/z* 199 (100) [M^+]; ^{1}H NMR (400 MHz, [D_6]acetone): δ = 2.36 (s, 3H, 4′-CH_3), 3.92 (s, 3H, 3-OCH_3), 7.24–7.26 (m, 2H, 3′-, 5′-H), 7.28 (d, ^3J (H/H) = 4.8 Hz, 1H, 5-H), 7.48–7.51 (m, 2H, 2′-, 6′-H), 8.24 (d, ^3J (H/H) = 4.8 Hz, 1H, 6-H), 8.40 ppm (s, 1H, 2-H); elemental analysis calcd (%) for $C_{13}H_{13}NO$: C 78.36, H 6.58, N 7.03; found: C 78.28, H 6.86, N 7.01.

3-Methoxy-4-(4-methoxyphenyl)-pyridine (19): Yield: 62%; colourless plates; mp: 93–98 °C; IR (ATR): $\tilde{\nu}$ = 1249 (C-O-C); MS (EI): *m/z* 215 (100) [M^+]; ^{1}H NMR (400 MHz, [D_6]acetone): δ = 3.84 (s, 3H, 4′-OCH_3), 3.93 (s, 3H, 3-OCH_3), 6.98–7.02 (m, 2H, 3′-, 5′-H), 7.28 (d, ^3J (H/H) = 4.8 Hz, 1H, 5-H), 7.55–7.59 (m, 2H, 2′-, 6′-H), 8.22 (d, ^3J

(H/H) = 4.8 Hz, 1H, 6-H), 8.39 ppm (s, 1H, 2-H); elemental analysis calcd (%) for $C_{13}H_{13}NO_2$: C 72.54, H 6.09, N 6.51; found: C 72.13, H 6.35, N 6.21.

3-Methoxy-4-(2-methoxyphenyl)-pyridine (20): Yield: 45%; colourless plates; mp: 115–120 °C; IR (ATR): $\tilde{\nu}$ = 1242 (C-O-C); MS (EI): *m/z* 215 (100) [M^+]; ^{1}H NMR (400 MHz, [D$_6$]acetone): δ = 3.76 (s, 3H, 2′-OCH$_3$), 3.85 (s, 3H, 3-OCH$_3$), 6.99 (dt, ^3J (H/H) = 7.4, ^4J (H/H) = 1.0 Hz, 1H, 5′-H), 7.08 (dd, ^3J = 8.3 Hz, ^4J (H/H) = 1.0 Hz, 1H, 3′-H), 7.16 (dd, ^3J = 4.7 Hz, ^5J (H/H) = 0.5 Hz, 1H, 5-H), 7.20 (dd, ^3J = 7.4 Hz, ^4J (H/H) = 1.7 Hz, 1H, 6′-H), 7.36 (ddd, ^3J (H/H) = 8.3, 7.4 Hz, ^4J (H/H) = 1.7 Hz, 1H, 4′-H), 8.21 (d, ^3J (H/H) = 4.7 Hz, 1H, 6-H), 8.38 ppm (d, ^5J (H/H) = 0.5 Hz, 1H, 2-H); elemental analysis calcd (%) for $C_{13}H_{13}NO_2$: C 72.54, H 6.09, N 6.51; found: C 72.86, H 6.41, N 6.30.

3-Benzyloxy-4-phenylpyridine (21): Yield: 55%; orange solid; mp: 68–72 °C; IR (ATR): $\tilde{\nu}$ = 1248, 1235 (C-O-C); MS (EI): *m/z* 261 (64) [M^+], 91 (100) [C_7H_7]; ^{1}H NMR (400 MHz, [D$_6$]acetone): δ = 5.27 (s, 2H, 3-OCH_2C$_6$H$_5$), 7.27–7.47 (m, 9H, aromat. H, 5-H), 7.64–7.67 (m, 2H, aromat. H), 8.28 (d, ^3J (H/H) = 4.8 Hz, 1H, 6-H), 8.50 ppm (s, 1H, 2-H); elemental analysis calcd (%) for $C_{18}H_{15}NO$: C 82.73, H 5.79, N 5.36; found: C 82.45, H 6.03, N 5.18.

General procedure C for the synthesis of 3-amide-4-aryl-1-aza-9-oxafluorenes 22, 23: 1-Aza-9-oxafluorene **4** (20 mg, 60 mmol) was dissolved in dry MeOH (5 mL). Then concentrated NH_3 (5 mL) or dimethylamine solution (10 mL, 2 M in THF) were added. The mixture was left standing at −8 °C in the refrigerator until no more starting compound **4** was detectable by TLC. Then the solvent was removed in vacuum and the oily residue was purified by coloumn chromatography using silica gel and an eluent mixture of $CHCl_3$/EtOAc/MeOH (85:15:10). After evaporation to dryness of the united eluent phases the resulting solid residue was recrystallised from diethyl ether.

6-Hydroxy-4-phenylbenzo[4,5]furo[2,3-*b*]pyridine-3-carboxamide (22): Yield: 48%; white solid; mp: 325–332 °C; IR (KBr): $\tilde{\nu}$ = 3448 (OH), 1728, 1684, 1651 (C=O); MS (EI): *m/z* 304 (100) [M^+]; ^{1}H NMR (500 MHz, [D$_6$]dmso): δ = 6.42 (d, ^4J (H/H) = 2.8 Hz, 1H, 5-H), 6.95 (dd, ^3J (H/H) = 8.8 Hz, ^4J (H/H) = 2.8 Hz, 1H, 7-H), 7.42 (s, 1H, 3-CONH_2), 7.48–7.51 (m, 2H, aromat. H), 7.55–7.59 (m, 4H, aromat. H, 8-H), 7.76 (s, 1H, 3-CONH_2), 8.49 (s, 1H, 2-H), 9.47 ppm (br s, 1H, OH); elemental analysis calcd (%) for $C_{18}H_{12}N_2O_3$: C 71.05, H 3.97, N 9.21; found: C 70.85, H 4.05, N 9.15.

6-Hydroxy-*N*,*N*-dimethyl-4-phenylbenzo[4,5]furo[2,3-*b*]pyridine-3-carboxamide (23): Yield: 39%; brownish powder; mp: 260–269 °C; IR (KBr): $\tilde{\nu}$ = 3435 (OH), 1730 (C=O); MS (EI): *m/z* 332 (51) [M^+], 288 (100) [$M^+ - NC_2H_6$]; ^{1}H NMR (400 MHz, [D$_6$]dmso): δ = 2.59 (s, 3H, CON(CH_3)$_2$), 2.75 (s, 3H, CON(CH_3)$_2$), 6.61 (d, ^4J (H/H) = 2.6 Hz, 1H, 5-H), 6.97 (dd, ^3J (H/H) = 8.8 Hz, ^4J (H/H) = 2.6 Hz, 1H, 7-H), 7.50–7.53 (m, 2H, aromat. H), 7.58 (d, ^3J (H/H) = 8.8 Hz, 1H, 8-H), 7.58–7.61 (m, 3H, aromat. H), 8.36 (s, 1H, 2-H), 9.45 ppm (s, 1H, OH); elemental analysis calcd (%) for $C_{20}H_{16}N_2O_3$: C 68.56, H 5.18, N 8.00; found: C 68.16, H 5.32, N 7.88.

Protein kinase affinities. Affinity assays have been carried out with protein kinases which were expressed in Sf9 insect cells as human recombinant GST-fusion proteins. Purification affinity chromatography using GSH-agarose was performed. The specific activities of the kinases used were determined with 4 pmol μg^{-1} *x* min for cdk1/B, 29 pmol μg^{-1} *x* min for cdk2/E, 108 pmol μg^{-1} *x* min for cdk4/D, 16 pmol μg^{-1} *x* min for cdk5/p25, and 2 pmol μg^{-1} *x* min for GSK-3β. Kinase inhibition assays were performed in 96-well FlashPlates™ in a 50 μL reaction volume. The volume consisted of 20 μL assay buffer, 5 μL of ATP solution in water, 5 μL of test compound in a final concentration of 10% DMSO prepared from stock solutions and finally 10 μL of substrate and 10 μL of enzyme premixed solutions. The assays contained HEPES/NaOH (60 mM, pH 7.5), $MgCl_2$ (3 mM), $MnCl_2$ (3 mM), na-orthovanadate (3 μM), DTT (1.2 mM), PEG20000 (50 μg mL^{-1}), [γ-^{33}P]-ATP (1 or 15 μM, ca. 5 × 10^5 cpm or 7.5 × 10^4 cpm per well). Incubation was carried out at 30 °C for 80 min. Reaction was stopped with H_3PO_4 (50 μL, 2%, *v/v*), plates were aspirated and washed twice with H_2O (200 μL) or NaCl (200 μL, 0.9%, *w/v*). Incorporation of $^{33}P_i$ was determined with a microplate scintillation counter. With the residual activities (in %) obtained for each compound IC_{50} values were calculated with proved competional inhibition character for selected compound and GSK-3β. From the IC_{50} values the affinity constants K_i were determined using the equation: $IC_{50} = 1/2\ [E_t] + K_i \times (1 + [S]/K_m)$.

Acknowledgements

This research was supported by the country Saxony-Anhalt within its graduate program to Burkhardt Voigt and by the Alzheimer-Forschung-Initiative (AFI/06825) to Andreas Hilgeroth. We gratefully acknowledge Wolfgang Sippl for providing Figure 2.

Keywords: enzyme inhibitors · medicinal chemistry · protein kinase · structure–activity relationships · tau protein

[1] P. Francotte, E. Graindorge, S. Boverie, P. de Tullio, B. Pirotte, *Curr. Med. Chem.* **2004**, *11*, 1757.
[2] G. G. Glenner, C. W. Wong, *Biochem. Biophys. Res. Commun.* **1984**, *120*, 885.
[3] C. L. Masters, G. Simms, N. A. Weinman, G. Multhaup, B. L. McDonald, K. Beyreuther, *Proc. Natl. Acad. Sci. USA* **1985**, *82*, 4245.
[4] J. Kang, H. G. Lemaire, A. Unterbeck, J. M. Salbaum, C. L. Masters, K. H. Grzeschick, G. Multhaup, K. Beyreuther, B. Moeller-Hill, *Nature* **1987**, *325*, 733.
[5] D. J. Selkoe, *Annu. Rev. Cell Biol.* **1994**, *10*, 373.
[6] J. T. Jarrett, E. P. Berger, P. T. J. Lansbury, *Biochemistry* **1993**, *32*, 4693.
[7] I. Grundke-Iqbal, K. Iqbal, Y. C. Tung, M. Quinlan, H. M. Wisniewski, L. I. Binder, *Proc. Natl. Acad. Sci. USA* **1986**, *83*, 4913.
[8] M. Goedert, M. G. Spillantini, N. J. Cairns, R. A. Crowther, *Neuron* **1992**, *8*, 159.
[9] G. N. Patrick, L. Zukerberg, M. Nikolic, S. De La Monte, P. Dikkes, L. H. Tsai, *Nature* **1999**, *402*, 615.
[10] D. P. Hanger, J. C. Betts, T. L. Loviny, W. P. Blackstock, B. H. Anderton, *J. Neurochem.* **1998**, *71*, 2465.
[11] H. Yamaguchi, K. Ishiguro, T. Uchida, A. Takashima, C. A. Lemere, K. Imahori, *Acta Neuropathol.* **1996**, *92*, 232.
[12] W. Noble, V. Olm, K. Takata, E. Casey, O. Mary, J. Meyerson, K. Gaynor, J. LaFrancois, L. Wang, T. Kondo, P. Davies, M. Burns, A. Veeranna, R. Nixon, D. Dickson, Y. Matsuoka, M. Ahlijanian, L. F. Lau, K. Duff, *Neuron* **2003**, *38*, 555.
[13] F. Plattner, M. Angelo, K. P. Giese, *J. Biol. Chem.* **2006**, *281*, 25457.
[14] C. Schultz, A. Link, M. Leost, D. W. Zaharevitz, R. Gussio, E. A. Sausville, L. Meijer, C. Kunick, *J. Med. Chem.* **1999**, *42*, 2909.
[15] L. Meijer, M. Flajolet, P. Greengard, *Trends Pharmacol. Sci.* **2004**, *25*, 471.
[16] P. Cohen, M. Goedert, *Nat. Rev. Drug Discovery* **2004**, *3*, 479.
[17] S. Leclerc, M. Garnier, R. Hoessel, D. Markos, J. A. Bibb, G. L. Snyder, P. Greengard, J. Biernat, Y.-Z. Wu, E.-M. Mandelkow, G. Eisenbrand, L. Meijer, *J. Biol. Chem.* **2000**, *276*, 251.
[18] K. Brachwitz, B. Voigt, L. Meijer, O. Lozach, C. Schächtele, J. Monár, A. Hilgeroth, *J. Med. Chem.* **2003**, *46*, 876.
[19] Compared to reported cytostatically active 1-aza-9-oxafluorenes which showed some activity in the low micromolar range we found practically no cytostatic activity of our novel compounds with GI_{50} values in screened cell lines > 50 μM.
[20] Protein–ligand interactions have been investigated with GOLD version 2.3[22] by docking first with co-crystallised inhibitors and resulting rmsd values of 0.6–0.7 Å of the docked ligands compared to the co-crystallised ligands. 14 Crystal structures of the enzyme have been used, all of which contain the active conformation. The resulting definite binding

modes of the investigated compounds and the high docking scores strongly support the inhibition of the active form of the kinase.

[21] C. Kunick, Z. Zhihong, R. Gussio, D. Zaharevitz, M. Leost, F. Totzke, C. Schächtele, M. H. G. Kubbat, L. Meijer, Th. Lemke, *ChemBioChem* **2005**, *6*, 541.

[22] G. Jones, P. Willett, R. Glen, A. R. Leach, R. Taylor, *J. Mol. Biol.* **1997**, *267*, 727.

[23] J. Ribas, K. Bettayeb, Y. Ferandin, M. Knockaert, X. Garoffe-Ochoa, F. Totzke, C. Schächtele, J. Mester, P. Polychronopoulos, P. Magiatis, A.-L. Skaltsounis, J. Boix, L. Meijer, *Oncogene* **2006**, *25*, 6304.

Received: July 23, 2007
Revised: September 20, 2007
Published online on November 14, 2007

