9-Cyano-1-azapaullone (Cazpaullone), a Glycogen Synthase Kinase-3 (GSK-3) Inhibitor Activating Pancreatic β Cell Protection and Replication

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Recently, the serine/threonine kinase glycogen synthase kinase-3 (GSK-3) emerged as a regulator of pancreatic β cell growth and survival. On the basis of the previous observation that GSK-3 inhibitors like 1-azakenpaullone promote β cell protection and replication, paullone derivatives were synthesized including 1-aza-, 2-aza-, and 12-oxapaullone scaffolds. In enzymatic assays distinct 1-azapaullones were found to exhibit selective GSK-3 inhibitory activity. Within the series of 1-azapaullones, three derivatives stimulated INS-1E β cell replication and protected INS-1E cells against glucolipotoxicity induced cell death. Cazpaullone (9-cyano-1-azapaullone), the most active compound in the protection assays, also stimulated the replication of primary β cells in isolated rat islets. Furthermore, cazpaullone showed a pronounced transient stimulation of the mRNA expression of the β cell transcription factor Pax4, an important regulator of β cell development and growth. These features distinguish cazpaullone as a unique starting point for the development of β cell regenerative agents which might be useful in the treatment of diabetes.

Introduction

Diabetes mellitus has become a health and economic burden in recent years, in particular for Western industrial nations.[1] Future prospects are alarming as the prevalence rises. Estimations speak of 300 million adults worldwide suffering from diabetes by 2025.[2] Though a number of new antidiabetic drugs have entered the market within the past decade, many patients still have inadequately controlled blood sugar levels.[3] Apparently, current pharmacological treatments are symptomatic and do not cure the disease. Over time many patients with diabetes develop devastating secondary complications especially affecting the heart, eyes, and kidneys.[4,5] In view of this situation the pharmaceutical industry and academic research groups have undertaken enormous efforts to identify new drug targets and to develop new therapeutic options to face the emerging threat of widespread diabetes.

One promising concept proposes that the preservation and expansion of β cell mass by pharmacological means will improve the blood sugar control in patients with type 1 or type 2 diabetes. In type 1 diabetes almost all β cells are destroyed by an autoimmune reaction.[6,7] In type 2 diabetes, β cells fail to compensate for the increased insulin demand caused by insulin resistance in the liver and muscle. β cell failure is characterized by a secretory dysfunction and a β cell loss of about 40% in glucose intolerant individuals and lean type 2 diabetes patients and to about 60% in obese type 2 diabetes patients compared to the respective nondiabetic control subjects. It appears that the relative decrease in β cell mass in patients with type 2 diabetes results from an increased β cell apoptosis rate.[8,9] Recent findings in preclinical as well as clinical studies with β cell growth or protecting factors have substantiated the regenerative approach. For instance, the combination treatment consisting of the epidermal growth factor (EGF)[a] and the peptide hormone gastrin, which stimulates the expansion of insulin producing β cell mass,[10] demonstrated efficacy in animal models of diabetes.[11,12] Lately, the combination treatment of EGF and gastrin has entered clinical development.[13] Likewise, in a recent clinical study an IL-1 receptor antagonist significantly improved the glycemic control of type 2 diabetes patients, most likely through a direct anti-inflammatory and protective effect on β cells.[14]

Another potential drug target for β cell regenerative approaches is the glycogen synthase kinase-3 (GSK-3), a recently identified regulator of β cell mass.[15–17] GSK-3 is a constitutively active serine/threonine kinase occurring in two forms, GSK-3α and GSK-3β.[18] The kinase is widely expressed and plays a central regulatory role in intracellular signaling pathways such as the Wnt- and the phosphoinositide 3-kinase (PI3K)/Akt pathways, which are most important for β cell function.[19,20] Consistent with these findings, the inactivation of GSK-3 in β cells through RNA interference or small molecular inhibitors was found to protect β cells against experimentally induced cell death.[15–17] Moreover, the suppression of GSK-3 enzymatic activity stimulated the replication of primary rat β cells.[16] Within the past decade, elucidation of the manifold roles of GSK-3 in

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[a] Abbreviations: ATP, adenosine triphosphate; AUC, area under the curve; 6BIO, 6-bromoindirubin-3′-oxime; BrdU, bromodeoxyuridine; BSA, bovine serum albumin; CDK, cyclin dependent kinase; CK1, casein kinase 1; DAD, diode array detector; DCM, dichloromethane; DMEM, Dulbecco's modified Eagle medium; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; dppf, 1,1′-bis(diphenylphosphino)ferrocene; EGF, epidermal growth factor; ER, endoplasmic reticulum; EtOH, ethanol; GSK-3β, glycogen synthase kinase-3β; HPLC, high-performance liquid chromatography; IL-1, interleukin 1; IRS1, insulin receptor substrate 1; NCW, near-critical water; qRT-PCR, quantitative real time polymerase chain reaction; siRNA, small interfering ribonucleic acid; TFA, trifluoroacetic acid; TLC, thin layer chromatography.

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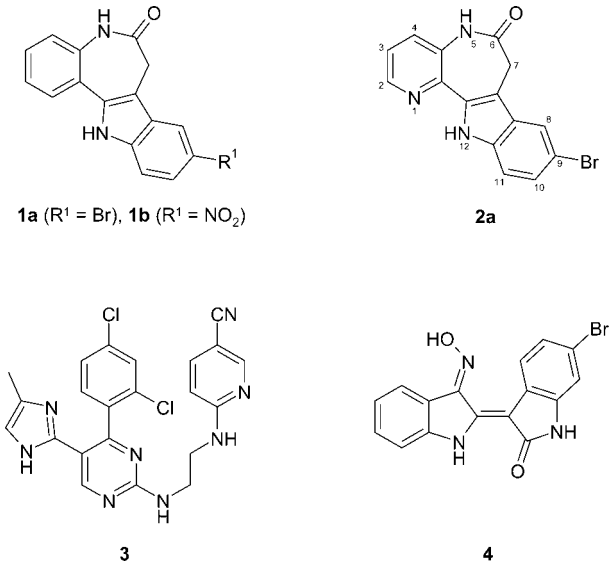


Figure 1. Structures of established GSK-3 inhibitors: kenpaullone (**1a**), alsterpaullone (**1b**), 1-azakenpaullone (**2a**), CHIR99021 (**3**), and 6-bromoindirubin-3′-oxime (6BIO, **4**).

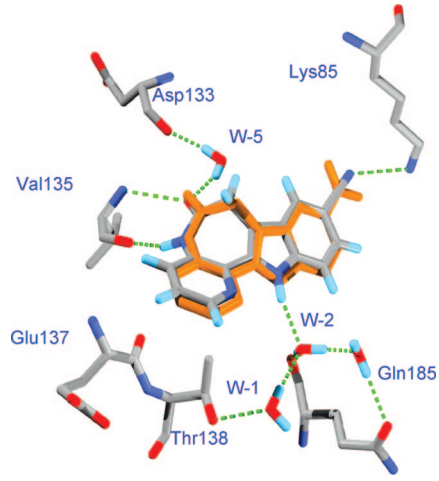


Figure 2. Alsterpaullone (**1b**; light-brown, hydrogen atoms omitted) and 9-cyano-1-azakenpaullone (**2b**, atom color code) bound to the ATP binding site of GSK-3β. Hydrogen bonds involving **2b** are indicated as green dashed lines. The pose of alsterpaullone is based on PDB file 1Q3W.pdb,[34] and the pose of **2b** was generated using the FlexX module in Sybyl.

cellular functions made the enzyme interesting as a putative drug target for several diseases.[21–24]

A variety of small molecules inhibiting GSK-3 have been published during the past decade, including paullones like kenpaullone (**1a**) and alsterpaullone (**1b**),[25,26] indirubins like 6-bromoindirubin-3′-oxime (6BIO, **4**),[27–29] and maleimides (Figure 1).[30–32] While both **1a** and **1b** also inhibit cyclin-dependent kinases (CDKs), the structurally related 1-azakenpaullone (**2a**) displays a 100-fold selectivity for GSK-3 versus CDK1/cyclin B.[33]

Considering the beneficial activity of **2a** in previous investigations, a project was initiated for the development of paullone derivatives with enhanced β-cell protective and proliferation stimulating properties. For this purpose, we first were interested in the design of azakenpaullone congeners with improved GSK-3 inhibitory potency and selectivity. The most promising entities then were to be tested for antiapoptotic and proliferation stimulating activity in cell based models.

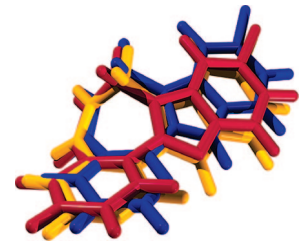


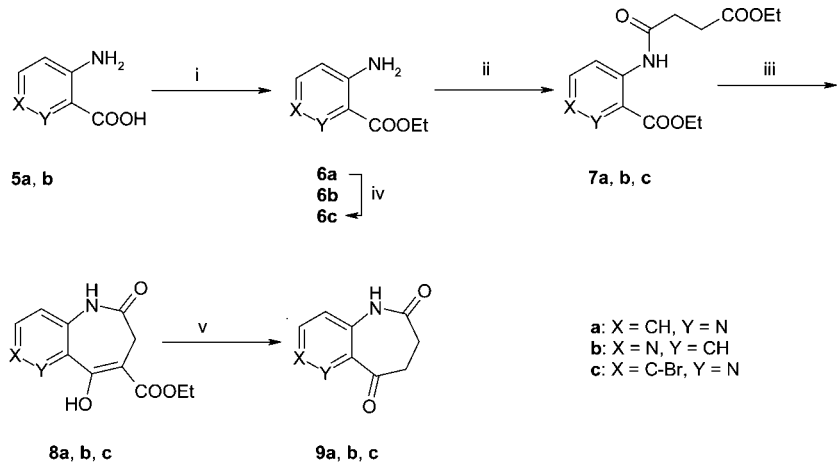
Figure 3. 1-Azakenpaullone (**2a**, blue), 2-azakenpaullone (**13a**, yellow), and 12-oxakenpaullone (**24b**, red) aligned by FlexX in the GSK-3β ATP binding site (generated from the PDB file 1Q3W.pdb).[34]

For a rational structure-based design of improved 1-azakenpaullone congeners, we performed a docking study based on the X-ray structure of an alsterpaullone/GSK-3β complex published recently.[34] In GSK-3β, alsterpaullone is adjusted to the ATP binding pocket by two hydrogen bonds between the lactam group and Val135 of the hinge area. Moreover, the lactam carbonyl oxygen is connected to the backbone carbonyl group of Asp133 by a water molecule (W5) bridge. Another hydrogen bond emanating from the side chain amino group of Lys85 makes contact with the nitro group of the ligand. The indole NH makes a fifth hydrogen bond to a water molecule (W2), which itself is hydrogen-bound to Glu185 and another water molecule (W1). The latter bridges a gap to Thr138 positioned at the edge of the ribose binding pocket[34] (Figure 2). The model derived by docking of 1-azakenpaullone to the ATP binding pocket showed a very similar pose of the ligand in GSK-3β, with the main difference being the missing hydrogen bond to Lys85 (data not shown). Various considerations based on this model provided ideas for a rational structure modification of 1-azakenpaullone. First, the substituent in the 9-position was varied systematically in order to investigate the importance for the hydrogen bond to Lys85. One of the compounds prepared for this purpose was the 9-cyano-1-azapaullone (**2b**), which, similar to alsterpaullone (**1b**), is able to realize the hydrogen bond to Lys85 (Figure 2).

Since the nitrogen in position 1 seems to be responsible for the selectivity of 1-AKP for GSK-3 versus CDKs, we prepared a second series of congeners (**13a**−**f**) in which this nitrogen is shifted to the 2-position. Docking studies showed that 2-azapaullones probably are slightly differently aligned within the ATP binding site compared to the 1-aza congeners (Figure 3). In a third group of compounds (**24a**−**c**), the indole nitrogen of the original (non-aza) paullone scaffold was substituted for oxygen. This modification was considered because in the ATP binding pocket of CDK1/cyclin B, the paullones use the indole NH as hydrogen bond donor aiming the side chain carboxyl function of Asp86, of which a homologue is missing in GSK-3. We speculated that in GSK-3 the depicted water molecule W2 could be reoriented to serve as a hydrogen bond donor enabling a bridge to the benzofuran oxygen of 24. This should make the resulting [1]benzofuro[3,2-*d*][1]benzazepin-6-ones **24** compatible with the ATP binding site of GSK-3 but not with the corresponding site of CDKs, resulting in improved GSK-3 selectivity. Results of docking studies suggested that because of similar molecular shapes, the 12-oxapaullones **24** should fit into the ATP binding site as well as the 1-azapaullones **2** (Figure 3).

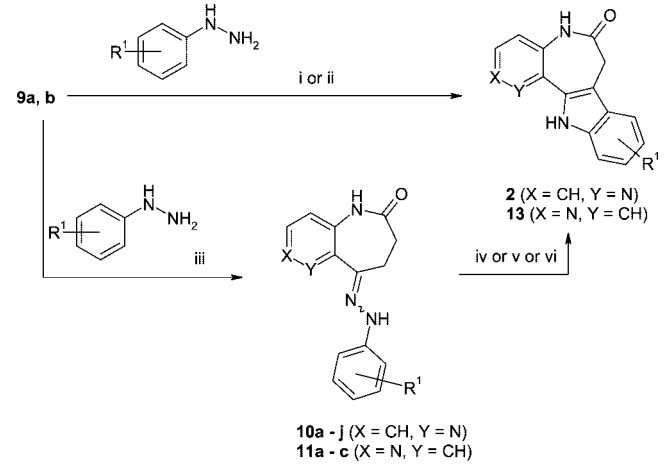
Also, with the aim of designing GSK-3 selective inhibitors, we replaced, in both the 1-aza and in the 2-aza series of compounds, the hydrogen bound to the indole nitrogen by aliphatic or aromatic substituents (**15a**−**e** and **16a**−**c**, respectively). These side chains were intended to displace the water

Scheme 1. Synthesis of Cyclic Ketones **9a**−**c**[a]



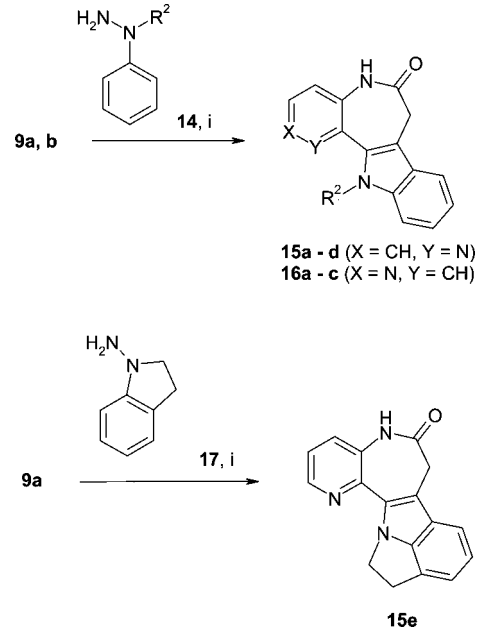
[a] (i) EtOH, concentrated H_2SO_4, 100 °C, 90 h; (ii) ethylsuccinyl chloride, $CaCO_3$, toluene; (iii) KH, toluene, DMF, −10 °C → 60 °C; (iv) Br_2, HOAc, H_2O, H_2SO_4; (v) DMF, H_2O, N_2, 150 °C.

Scheme 2. Synthesis of 1-Azapaullones **2** and 2-Azapaullones **13**[a]



[a] (i) (1) HOAc, NaOAc, 70 °C, (2) concentrated H_2SO_4, HOAc, 90 °C (refers to compounds **13a**,**c**−**f**); (ii) (1) HOAc, NaOAc, 70 °C, (2) EtOH, reflux (refers to compound **2l**); (iii) HOAc, NaOAc, 70 °C; (iv) Ph_2O, reflux, N_2 (refers to compounds **2b-m** and **13b**,**c**); (v) H_2O, MW, 175−215 °C, 0.5 h (refers to compounds **2a**,**b**,**d**,**g** and **13a**); (vi) DMF, MW, 230 °C (refers to compound **2c**). For R^1 refer to Table 1.

Scheme 3. Synthesis of N-Substituted 1-Azapaullones **15** and 2-Azapaullones **16**[a]



[a] (i) HOAc, NaOAc, 70 °C. For R^2 refer to Table 1.

molecule W2 from the ATP binding pocket in GSK-3, a mechanism that was considered not to be applicable to the binding pocket of CDKs, where the corresponding water molecule is missing.

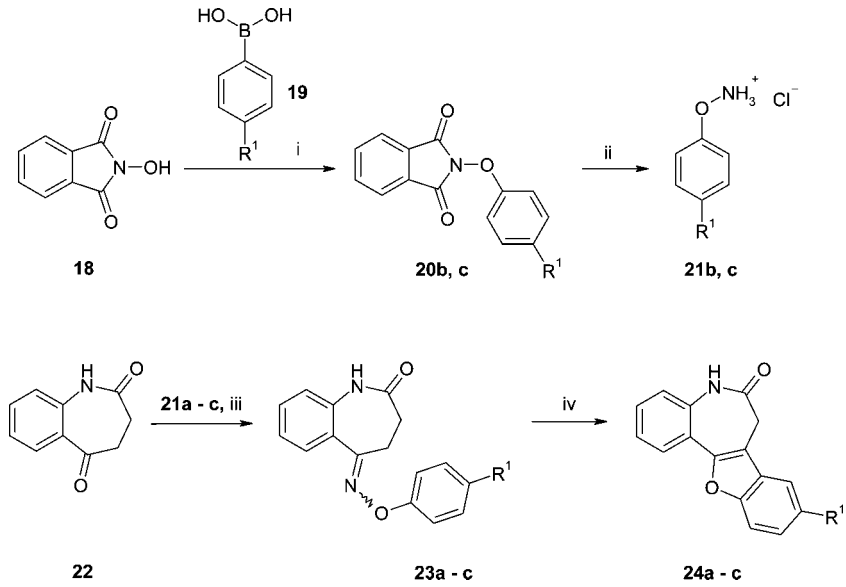
Besides **2j**, two further 1-azapaullones **2n** and **2o** were synthesized to probe the suitability of positions 2 and 11 for halogen atom substitution. In the case of retained GSK-3 inhibitory activity and selectivity, these structures would be useful as intermediates for the attachment of solubilizing side chains by palladium catalyzed reactions. Eventually, the paullone 2,9-dicarbonitrile **30** was designed to study whether the electron-poor pyridine ring of the azapaullones **2** and **13** could be mimicked by a benzene ring bearing a strongly electron-withdrawing substituent.

Chemistry

The new 1-aza- and 2-azapaullones were synthesized by a general method that has been published for the preparation of 1-azakenpaullone, comprising the synthesis of a seven-member cyclic ketone **9** (Scheme 1)[35] and a subsequent Fischer indole ring closure for the annulation of the indole system (Scheme 2).[36] The preparation of the cyclic ketones **9a**−**c** started with an appropriate aminopyridine carboxylic acid **5a** or **5b** which was esterified by prolonged reflux in ethanol catalyzed by sulfuric acid.[37] 3-Aminopicolinic acid ethyl ester **6a** was converted to the corresponding 6-bromo derivative **6c** by reaction with bromine in acetic acid.[38] The esters **6a**−**c** were acylated with ethylsuccinyl chloride, respectively, to yield the amides **7a**−**c**. Subsequent treatment with potassium hydride in a toluene/DMF mixture induced a Dieckmann ring closure reaction leading to the enolized cyclic β oxocarboxylic esters **8a**−**c**. When these esters were heated in wet DMF, the seven-membered cyclic ketones **9a**−**c** were obtained by a dealkoxycarbonylation reaction.

For the indole ring closure, various modifications of the Fischer reaction were employed (Scheme 2). The 2-azapaullones **13a**,**c**−**f** were obtained by a one-pot procedure omitting isolation

Scheme 4. Synthesis of 12-Oxapaullones **24** [a]



[a] (i) CuCl, pyridine, 1,2-dichloroethane, molecular sieves, room temp; (ii) (1) MeOH, $CHCl_3$, hydrazine, room temp, (2) EtOH, HCl; (iii) HOAc, NaOAc, 70 °C; (iv) HCOOH, H_3PO_4, 60 °C. For R^1 refer to Table 1.

of the corresponding phenylhydrazone intermediate. For the reaction, the cyclic ketone **9b** was reacted with an appropriate phenylhydrazine consecutively in acetic acid and an acetic acid/sulfuric acid mixture. In contrast, for the synthesis of the 1-azapaullones **2b**−**l** and the 2-azapaullone **13b** the thermal Fischer indolization method proved to be advantageous. Thus, the crude phenylhydrazone **10** or **11** prepared from **9a** or **9b** were refluxed in diphenyl ether for 2 h to furnish the indolization products in poor to moderate yields. In the case of the 9-donor-substituted 1-azapaullones **2k** and **2l**, the thermal Fischer indolization occurred at an unexpected low temperature in boiling ethanol. This observation was made upon the attempted crystallization of the corresponding phenylhydrazones from ethanol, which instead of purified precursors yielded the desired indole products. To further improve the performance of the thermal Fischer indolization in paullone synthesis, an alternative method was developed in which the phenylhydrazone precursors were heated in the sealed vessel of a monomode microwave reactor in near-critical water (NCW) as reaction medium.[39,40] Application of this procedure for the preparation of **2b** and **2d** increased the yield from 18% to 36% and from 17% to 51%, respectively. In a similar manner 1-azakenpaullone **2a** was obtained employing the microwave assisted method in 73% yield. The 9-hydroxy-1-azapaullone **2m** was prepared by boron tribromide ether cleavage[41] in dichloromethane from the corresponding methoxy derivative **2k**.

The azapaullones **15a**−**d** and **16a**−**c** substituted at the indole nitrogen were easily accessible by heating the cyclic ketones **9a**,**b** with 1-aryl- or 1-alkyl-substituted phenylhydrazines **14** in glacial acetic acid, even in the absence of an additional acidic catalyst. The novel pentacyclic ring system **15e** was obtained from **9a** and 1-aminoindoline (**17**) in a similar reaction (Scheme 3).

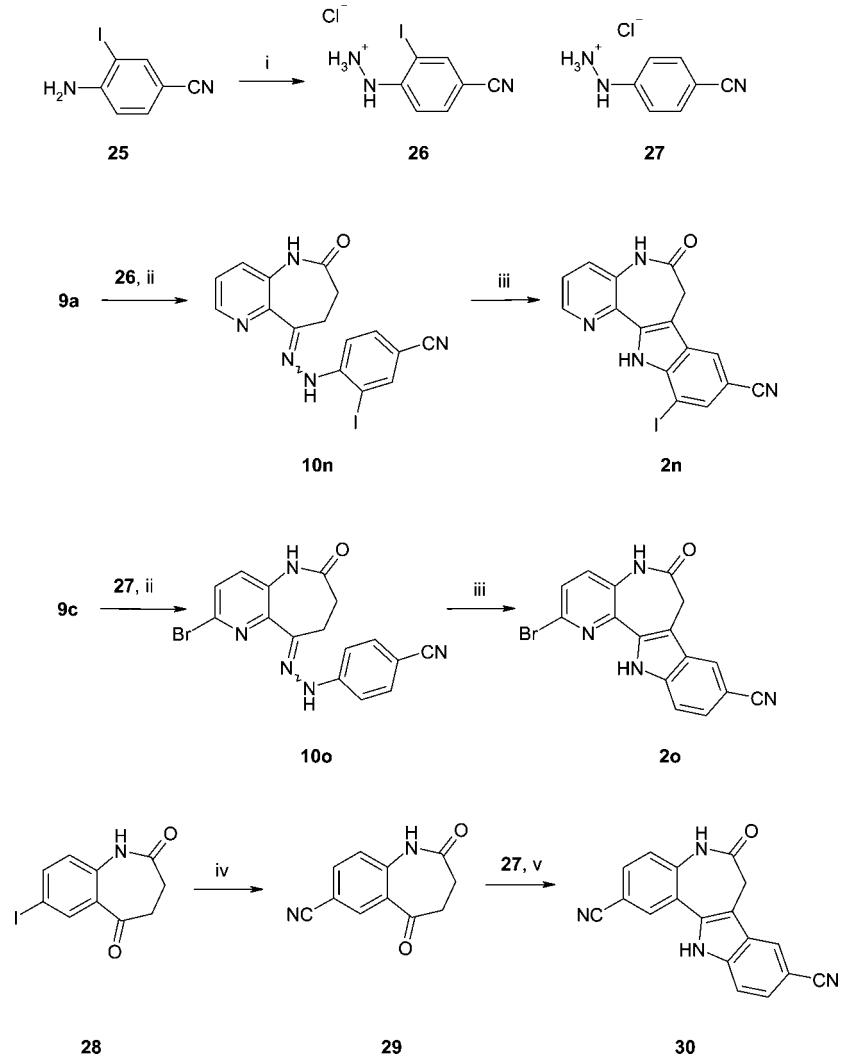
For the preparation of the novel 12-oxapaullones **24a**−**c**, the aryloxyamines **21** were needed as building blocks. While the unsubstituted derivative **21a** was commercially available, the corresponding para-substituted analogues **21b**,**c** were prepared by a copper-mediated coupling of suitable boronic acids **19** and *N*-hydroxyphthalimide at room temperature.[42] Hydrazinolysis of the resulting intermediates **20b**,**c** furnished the desired aryloxyamines which were converted to the corresponding hydrochlorides.[42] The latter were reacted with the cyclic ketone **22**[35] in acetic acid to afford the *O*-aryloximes **23a**−**c** which were subsequently heated in a mixture of formic and phosphoric acid yielding the 12-oxapaullones **24a**−**c** (Scheme 4).[43]

The syntheses of the three special paullone derivatives **2n**, **2o**, and **30** are summarized in Scheme 5. Treatment of 4-amino-3-iodobenzonitrile **25** with sodium nitrite and hydrochloric acid gave a diazonium salt solution, which was not isolated but added to a cold tin chloride solution yielding the hitherto unknown 4-hydrazino-3-iodobenzonitrile hydrochloride **26**.[44] Reaction of **26** with the cyclic ketone **9a** in acetic acid furnished the phenylhydrazone **10n**. The subsequent thermal Fischer indolization procedure mentioned above led to the 11-iodo-1-azapaullone-9-carbonitrile **2n**. A similar sequential procedure involving reaction of ketone **9c** and 4-hydrazinobenzonitrile hydrochloride **27**, isolation, and thermal indolization of the phenylhydrazone **10o** was employed for the preparation of the 2-bromo-substituted 1-azapaullone-9-carbonitrile **2o**. Palladium catalyzed reaction of the iodoarene **28**[45] with zinc cyanide in DMF in the presence of 1,1′-bis(diphenylphosphino)ferrocene (dppf) as ligand gave the 2,5-dioxo-2,3,4,5-tetrahydro-1*H*-1benzazepine-7-carbonitrile **29**.[46] The acid catalyzed one-pot procedure with acetic acid/sulfuric acid at 70 °C proved to be suitable for the indolization of **29** with the 4-hydrazinobenzonitrile hydrochloride **27**, furnishing the paullone-2,9-dicarbonitrile **30**.

Results and Discussion

Kinase Inhibition. Since the aim of the project was the identification of potent and selective new GSK-3 inhibitors in the paullone series, all new paullone derivatives were tested on a set of three protein kinases: first, GSK-3α/β as the main target enzyme of the new structures; second, CDK5/p25, a protein kinase somewhat structurally related to GSK-3; third, casein kinase 1 (CK1), a serine/threonine kinase structurally less related but like GSK-3 involved in the Wnt signaling pathway.[47] CDK5 inhibition has recently been shown to stimulate insulin secretion under high glucose concentration conditions.[48,49] Distinct

Scheme 5. Synthesis of 1-Azapaullones **2n**,**o** and Paullone-2,9-dicarbonitrile **30**[a]



[a] (i) (1) $NaNO_2$, HCl, −10 °C, (2) $SnCl_2$, HCl, −10 °C; (ii) HOAc, NaOAc, 70 °C; (iii) Ph_2O, reflux, N_2; (iv) $Zn(CN)_2$, $Pd_2(dba)_3$, dppf, DMF, N_2, 120 °C; (v) (1) HOAc, NaOAc, 70 °C, (2) HOAc, concentrated H_2SO_4, 70 °C.

derivatives from all series were further checked for inhibition of CDK1/cyclin B, a protein kinase involved in regulating the cell cycle G2/M transition and very similar to CDK5/p25. The antiproliferative activity of paullones like **1a** and **1b**, which exhibit antiproliferative activity for tumor cell lines, is at least partially assigned to CDK1 inhibition.[36,50] For the objectives of the investigation reported here, antiproliferative activity resulting from CDK1 inhibition is explicitly undesirable.

The in vitro test results (Table 1) revealed a strong dependence of the kinase inhibitory properties on subtle structural changes. None of the new entities inhibited CK1 with IC_{50} values below 10 μM. Regarding GSK-3 inhibition, the exchange of the 9-bromo substituent in 1-azakenpaullone for either a cyano (in **2b**) or a trifluoromethyl group (in **2c**) resulted in improved potency. Later experiments showed a strong β cell protection and proliferation stimulation especially by 9-cyano-1-azapaullone **2b**. We will refer to **2b** in the further text using the name "cazpaullone". Compared to the parent 1-azakenpaullone **2a**, the trifluoromethyl derivative **2c** was more selective versus CDK1. Besides cazpaullone (**2b**) and **2c**, the other derivatives of the 1-aza series were less potent than **2a**. Within the series **2**, the relationship between the electronic parameters of 9-substituents and the kinase inhibitory properties described for paullones of the carba analogue series was reproduced: electron-donating substituents (e.g., in **2k**, **2l**, **2m**, and also **2f**, if the carboxylate anion form is assumed) showed poor activity, while high inhibitory activity was found with structures bearing electron withdrawing groups in the 9-position (**2b**−**e**,**i**). Introduction of a second substituent into the 11-position was unfavorable, as the comparison of 9-monosubstituted compounds (**2b**,**d**) and 9,11-disubstituted analogues (**2n**,**j**) revealed. The compounds **13a**−**e** of the 2-aza series showed retained GSK-3 inhibitory activity. Again, compound **13f** with the electron donating substituent in the 9-position was clearly less active. All congeners of the 2-aza series lacked the selectivity shown by the 1-aza analogues. For example, the 9-cyano-2-azapaullone **13b** inhibits GSK-3α/β, CDK1/cyclin B, and CDK5/p25 with IC_{50} values in the two-digit nanomolar range. Noteworthy, alkyl substitutions at the indole nitrogens have different consequences in the 1-aza- and 2-aza-series. While in the 1-aza-series the substitution at the indole nitrogen more or less abrogates the GSK-3 inhibitory activity (entries **15a**−**d**), a similar substitution in the 2-aza series produces derivatives that still are submicromolar GSK-3 inhibitors, showing even some selectivity versus CDK5/p25 (entries **16a**−**c**). The novel pentacyclic paullone derivative **15e** constitutes an exception, being the only submicromolar GSK-3α/β inhibitor in the series of 12-substituted 1-azapaullones. In the 1-aza series, introduction of a 2-bromo

Table 1. Inhibition of GSK-3α/β, CDK5/p25, and CDK1/Cyclin B by Paullone Derivatives

					IC_{50} (μM)		
compd	X	Y	R^1	R^2	GSK-3α/β	CDK5/p25	CDK1/ cyclin B
1a[a]	CH	CH	9-Br		0.023	0.85	0.4
1b[a]	CH	CH	9-NO_2		0.004	0.040	0.035
2a[b]	CH	N	9-Br	H	0.018	4.2	2.0
2b	CH	N	9-CN	H	0.008	0.3	0.5
2c	CH	N	9-CF_3	H	0.008	10	1.65
2d	CH	N	9-F	H	0.080	3	4.5
2e	CH	N	9-Cl	H	0.063	>10	>10
2f	CH	N	9-COOH	H	100	30	2.2
2g	CH	N	8,10-di-Cl	H	0.8	100	>30
2h	CH	N	9-H	H	0.80	9	7
2i	CH	N	9-I	H	0.025	6	1
2j	CH	N	9,11-di-F	H	1.8	>10	>10
2k	CH	N	9-OCH_3	H	0.8	4	2
2l	CH	N	9-CH_3	H	0.13	>10	8
2m	CH	N	9-OH	H	1.40	5	4
2n	CH	N	9-CN, 11-I	H	0.12	2.0	0.41
2o	C−Br	N	9-CN	H	3.8	10	10
13a	N	CH	9-Br	H	0.052	0.18	0.11
13b	N	CH	9-CN	H	0.021	0.032	0.031
13c	N	CH	9-CF_3	H	0.013	0.12	0.19
13d	N	CH	9-F	H	0.051	0.26	1.2
13e	N	CH	9-Cl	H	0.018	0.21	0.12
13f	N	CH	9-OCH_3	H	0.39	0.4	0.2
15a	CH	N	9-H	Me	1	3	>10
15b	CH	N	9-H	Et	13	>10	>10
15c	CH	N	9-H	Bn	2.1	>10	>10
15d	CH	N	9-H	Ph	>10	>10	>10
15e					0.41	>10	>10
16a	N	CH	9-H	Me	0.40	>10	8.0
16b	N	CH	9-H	Et	0.8	>10	3.8
16c	N	CH	9-H	Bn	0.41	>10	>10
24a	CH	CH	9-H		>10	>10	>10
24b	CH	CH	9-Br		>10	>10	>10
24c	CH	CH	9-Cl		>10	>10	>10
30	C-CN	CH	9-CN	H	0.028	0.73	0.12

[a] Data taken from ref 26 (Leost et al., 2000). [b] Data taken from ref 33 (Kunick et al., 2004).

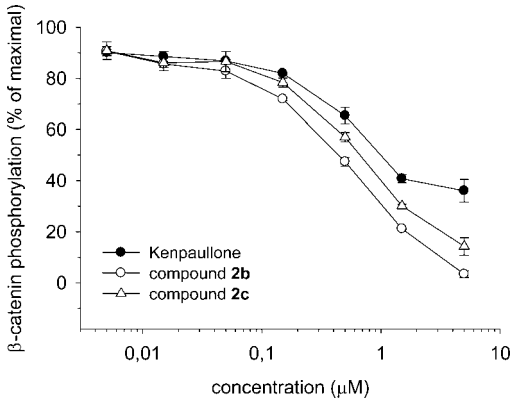


Figure 4. Intracellular inhibition of GSK-3 by kenpaullone (**1a**) and compounds **2b** and **2c**. SH-SY5Y neuroblastome cells were exposed to various concentrations of kenpaullone (**1a**), cazpaullone (**2b**), or **2c** in the presence of a constant level of MG132. The level of GSK-3-phosphorylated β-catenin was estimated by ELISA and was expressed as a percentage of phosphorylated β-catenin in untreated control cells.

substituent produced a spectacular drop in the kinase inhibitory activity. Compared to the 2-unsubstituted cazpaullone (**2b**), the 2-bromo-cazpaullone **2o** is approximately 500-fold less active as a GSK-3α/β inhibitor. The 2,9-dicyanopaullone **30** exhibited an activity pattern similar to analogues of the 2-azapaullone series, inhibiting GSK-3α/β in the two-digit and the CDKs in the three-digit nanomolar concentration range.

The 12-oxapaullone derivatives **24a**−**c** failed to show any kinase inhibitory activity in the set of the four kinases used in this study. This observation disproves our assumption that the ring oxygen of the benzofurane structure could act as a hydrogen bond acceptor for the water molecule W2 that usually accepts the hydrogen bond from the paullone indole NH.

In conclusion, testing of the novel paullone derivatives in kinase assays revealed that only congeners of the 1-aza-series **2** and 12-substituted derivatives of the 2-aza-series **16** exhibited GSK-3 inhibition with sufficient selectivity. While the 12-unsubstituted 2-azapaullones **13** and the paullone 2,9-dicarbonitrile **30** showed potent GSK-3 inhibition, the selectivity against CDKs appeared insufficient for further studies. Both the 12-substituted 1-azapaullones **15** and the 12-oxapaullones **24** failed to exhibit a noteworthy kinase inhibitory activity. We therefore decided to carry out cell-based assays directed to investigate β-cell protection and proliferation with compounds of the series **2**. 12-Substituted derivatives of the 2-aza-series **16** also appear to be useful GSK-3 inhibitors and will be the subject of future studies.

Results of Cell-Based Assays. Confirmation of the Intracellular Inhibition of GSK-3 by Paullones. SH-SY5Y neuroblastoma cells were exposed to various concentrations of kenpaullone (**1a**) or **2b** or **2c** in the presence of a constant level of MG132 (an inhibitor of the proteasome that prevented the rapid degradation of β-catenin once phosphorylated by GSK-3). The level of GSK-3-phosphorylated β-catenin, estimated by an ELISA assay, revealed a dose-dependent inhibition of GSK-3 selective phosphorylation sites on β-catenin, demonstrating that these compounds are able to inhibit GSK-3 in a cellular context (Figure 4).

Selection of Biologically Active GSK-3 Inhibitors of the 1-Azapaullone Series. We used INS-1E β cells, a rat insulinoma cell line widely used to study β cell functions,[51] in combination with a standard viability assay to rapidly identify azapaullone-derived GSK-3 inhibitors with β cell protective potential. 1-Azakenpaullone (**2a**) served as a positive control because this substance has previously demonstrated robust antiapoptotic effects on INS-1E cells.[16] We found that the four new azapaullones **2b**, **2c**, **2d**, and **2i** protected INS-1E cell viability against a toxic glucose/palmitate mixture (parts B, C, D, and E of Figure 5, respectively). In contrast, other congeners of the 1-aza series showed only minor rescuing effects (**2e**, **2h**, **2l**, **2m**) or behaved neutrally in this assay (**2g**, **2k**) (data not shown). Cazpaullone (**2b**) was the most potent compound exhibiting protective activity at concentrations as low as 0.3 μM and with maximal activity at 2 μM (Figure 5B). Significantly higher levels of 1-azakenpaullone (**2a**) or the other azapaullone compounds were needed to achieve effects comparable to cazpaullone. Above 1 μM, however, treatment with cazpaullone slightly suppressed the metabolic activity of otherwise untreated INS-1E cells (Figure 5B), which indicates a narrow concentration range in which cazpaullone confers beneficial effects to β cells. The viability assay turned out to be very informative regarding the biological activity of the tested compounds. Also, the assay is simple, cost effective, and adjustable to a large scale throughput format for the screening of chemical libraries.

We next tested if the newly identified azapaullone GSK-3 inhibitors **2b**, **2c**, **2d** are able to inhibit β cell apoptosis induced by high levels of glucose and palmitate (Figure 6). INS-1E apoptosis was monitored using a DNA fragmentation assay and a caspase activity assay. The antiapoptotic effects of 1-azakenpaullone **2a** were found to be comparable to the activity reported previously.[16] **2c** and **2d** turned out to be as active as

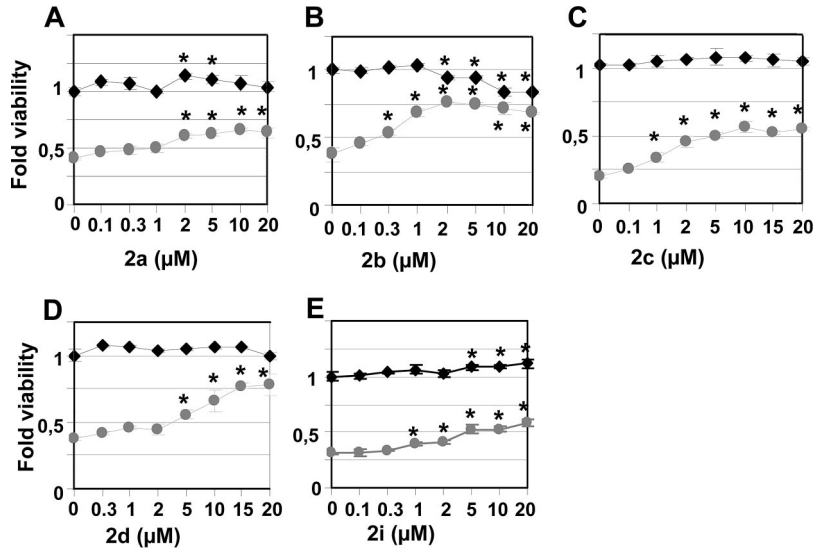


Figure 5. Identification of biological active 1-azapaullone GSK-3 kinase inhibitors. (A) 1-Azakenpaullone **2a** and the new azapaullone GSK-3 inhibitors (B) **2b**, (C) **2c**, (D) **2d**, and (E) **2i** were found to strongly improve the metabolic activity or viability of INS-1E β cells treated with toxic levels of palmitate (0.3 mM palmitate coupled to BSA) and glucose (25 mM) for 24 h (gray lines and filled gray circles). The viability is an indirect measure of the mitochondrial energy production. The effects of the tested compounds on INS-1E cells not exposed to the toxic glucose/palmitate mixture are also shown (black line and filled black diamonds). Shown are representative experiments of at least three independently performed studies. Given values are the mean values of measured data from at least four wells. Values show fold change relative to control ± SD. Asterisks indicate statistically significant differences (Student's *t* test): (*) $P < 0.05$ versus INS-1E cells treated with a mixture of high palmitate and glucose in the absence of the indicated inhibitor.

1-azakenpaullone (**2a**) and moderately suppressed INS-1E cell death. Cazpaullone (**2b**) more strongly protected INS-1E cells against glucolipotoxicity, decreasing the level of apoptosis at 2 μM by more than 50%. In conclusion, our findings substantiate earlier observations that the inactivation of GSK-3 protects INS-1E β cells against glucolipotoxicity. New azapaullone GSK-3 inhibitors have been identified that are able to promote the survival of INS-1E cells. Cazpaullone was found to be the most potent of the new compounds.

New Azapaullone GSK-3 Inhibitors Stimulate the Replication of Pancreatic β Cells in Vitro. 1-Azakenpaullone has previously been shown to activate the replication of INS-1E cells as well as of primary β cells. Likewise, the new azapaullone GSK-3 inhibitors **2b**, **2c**, and **2d** promoted INS-1E cell proliferation in a dose dependent manner (Figure 7A−D). Again, cazpaullone (**2b**) was active at a lower concentration (0.1 μM) than the other compounds and reached maximal activity at 1−2 μM.

A similar result for cazpaullone (**2b**) was observed in the replication experiment with primary pancreatic rat β cells. In subsequent experiments the effects of cazpaullone on replication of primary β cells were compared to those of CHIR99021 (**3**), a highly specific and well characterized small molecular wheight GSK-3 inhibitor.[52,53] For these experiments cazpaullone was selected because of its superior activity in INS-1E cells. CHIR99021 (**3**, 5 μM) activated replication of rat β cells about 2.5-fold, confirming previous observations.[16] At 0.5 μM cazpaullone (**2b**) stimulated primary β cell replication by about 2.5-fold (Figure 8). At 2 μM, however, no positive effect on β cell replication was observed. At higher compound concentrations, off-target effects probably occur that neutralize the proliferative effects resulting from GSK-3 inactivation. In summary, we have identified three new azapaullone GSK-3 inhibitors (cazpaullone (**2b**), **2c**, and **2d**) promoting INS-1E cell replication. For cazpaullone (**2b**) this proliferative effect was confirmed with primary rat β cells.

1-Azakenpaullone (2a) and Cazpaullone (2b) Transiently Activate the Expression of the Pancreatic Transcription Factor Pax4. Through quantitative real-time PCR (qRT-PCR) we found that the above-described β cell protective effects of 1-azakenpaullone (**2a**) and cazpaullone (**2b**) are associated with the ability to activate the expression of the β cell specific transcription factor Pax4 in INS-1E cells and primary rat β cells (Table 2). Pax4 is essential for β cell development during embryogenesis, and its overexpression has been demonstrated to promote the survival and replication of mature rat and human β cells.[54] Also, Pax4 protects INS-1E cells from apoptosis[55] because a ~50% reduction in Pax4 expression level mediated by RNA interference resulted in an increased rate of apoptosis. Interestingly, the prominent anti-apoptotic effects of cazpaullone correlate with a comparatively strong activation of Pax4 mRNA expression in INS-1E cells. It is therefore tempting to speculate that the up-regulation of Pax4 mRNA expression contributes to the observed cytoprotective effects of cazpaullone and 1-azakenpaullone and may explain the superior biological activity of cazpaullone compared to other equally or even more potent GSK-3 inhibitors such as CHIR99021 (**3**) or compound **2c**. Alsterpaullone (**1b**), a paullone with strong antitumor activity known to potently inhibit GSK-3, CDKs, and a number of other kinases, was also found to stimulate Pax4 mRNA expression in INS-1E cells. In contrast, specific GSK-3 inhibitors including CHIR99021 (**3**), 6BIO (**4**), and the new azapaullones **2c** and **2d** did not activate Pax4 mRNA expression in β cells (Table 2A). This indicates that GSK-3 inhibition is not sufficient to activate Pax4 transcription in β cells. 1-Azakenpaullone, cazpaullone. and alsterpaullone probably interact with one or more yet unknown targets besides GSK-3 that are involved in the regulation of Pax4 expression.

Conclusion

Here, we report the design, the synthesis, and the selection of new paullone GSK-3 inhibitors with β cell regenerative capabilities. Cazpaullone appeared to be the most active 1-azapaullone in a number of β cell assays. Compared to other GSK-3 inhibitors, cazpaullone exhibited stronger cytoprotective effects on β cells. Also, cazpaullone, 1-azakenpaullone, and

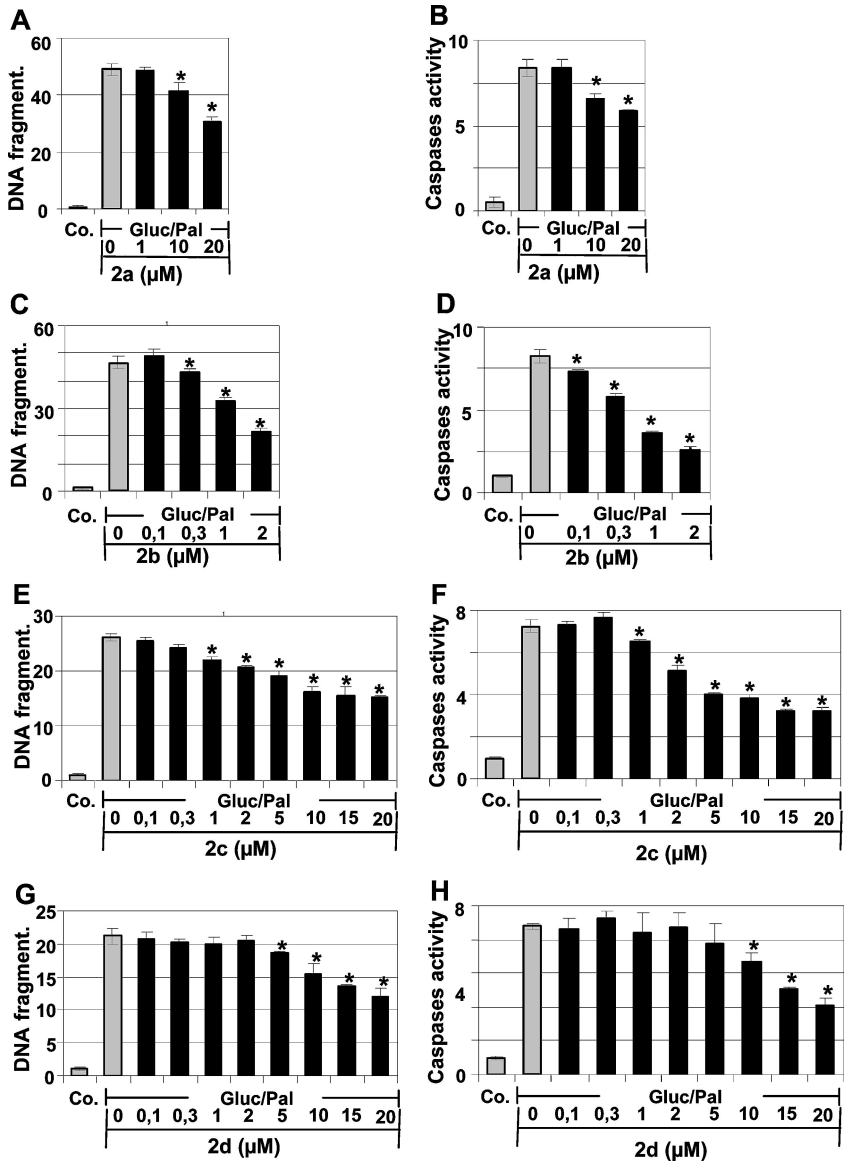


Figure 6. 1-Azapaullone GSK-3 inhibitors **2a**−**2d** protect INS-1E cells against glucolipotoxicity induced cell death. As in the experiments presented in Figure 5, cell death of INS-1E cells was induced by treatment of cells with toxic concentrations of glucose and palmitate (Gluc/Pal) for 24 h. Apoptosis was monitored by assaying the level of cytosolic DNA fragments (DNA fragment) and the activity of caspases versus untreated cells (Co.). At low levels, apoptosis is detectable with these methods in untreated INS-1E cells (Co.). The combination of high glucose and palmitate strongly stimulated the respective apoptotic processes in INS-1E cells. Cells were incubated 1 h before the addition of the toxic mixture with the indicated amounts of the test agents. Compound concentrations are indicated in micromolar. Shown are representative experiments of at least three independently performed studies. Given values are mean values of measured data of at least four wells. Values show fold change relative to control ± SD. Asterisks indicate statistically significant differences (Student's t test): (∗) $P < 0.05$ versus INS-1E cells treated with a mixture of high palmitate and glucose in the absence of the indicated inhibitor.

alsterpaullone, but not any other tested GSK-3 inhibitor, were found to transiently activate the expression of the β cell transcription factor Pax4. The observed increase in Pax4 mRNA levels is remarkable because the ectopic expression of murine Pax4 in human and rat islets induced β cell replication and conferred resistance against cytokine-induced apoptosis.[54] Thus, the inhibition of GSK-3 in parallel with the activation of Pax4 may in a complementary fashion deliver strong survival as well as growth signals to the investigated rat insulin producing β cells.

Further experiments with human islets will help to evaluate the therapeutic potential of Pax4 activating GSK-3 inhibitors. Notably, recent research results with human β cells in culture point to an inherent difference between human and rodent β cells with regard to the regulation of cell replication. Basically, factors able to stimulate replication of human β cells in culture do not robustly work on human islets.[56,57] While little is known about the regulation of human β cell mass in vivo, the observations with cultured human islets may at least in part reflect the situation in the intact human pancreas. One possibility is that human β cells require the stimulation of multiple pathways at the same time for the induction of proliferation. If so, agents like the Pax4 activating azapaullones targeting more than one key regulator of β cell survival and growth may be a superior way to expand human β cell mass in patients with diabetes. At the moment it is unclear how cazpaullone stimulates Pax4 expression and which targets are involved. However, the understanding of the mechanism will be important for further systematic development of Pax4 activating azapaullone GSK-3 inhibitors for β cell regeneration. Also, the Pax4 activating paullones may represent valuable tools for the dissection of Pax4 regulating pathways. Cazpaullone was selected among a series

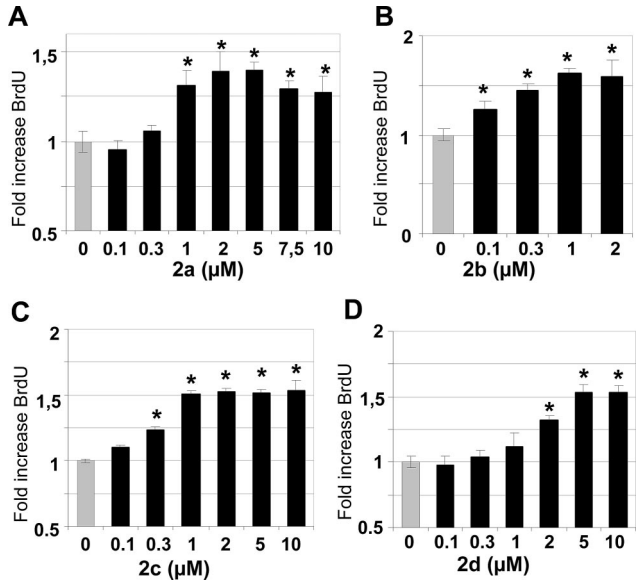


Figure 7. Stimulation of INS-1E β cell replication by azapaullone GSK-3 inhibitors. INS-1E cells were treated with the test compounds 1-azakenpaullone (**2a**), **2b**, **2c**, and **2d** at indicated concentrations for 24 h. Cell replication was determined by BrdU incorporation. The figure illustrates the relative increase in incorporated BrdU in INS-1E cells ("Fold increase BrdU"). Results are presented as fold change relative to control ± SD. Figure shows representative experiments of two independently performed studies. Given values are mean values of measured data of at least four wells. Asterisks indicate statistically significant differences (Student's *t* test): (∗) $P < 0.05$ versus INS-1E cells not treated with the indicated inhibitor.

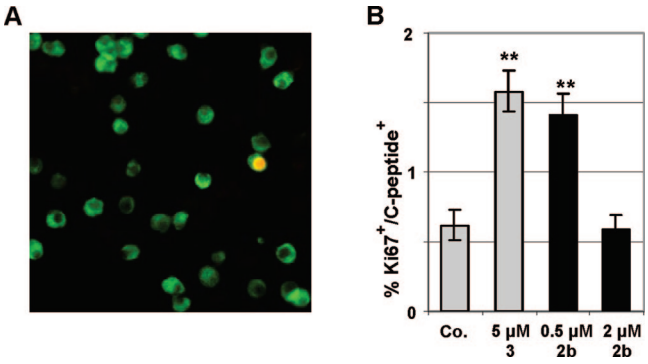


Figure 8. **2b** stimulates replication of primary β cells in isolated rat islets. Replicating β cells in isolated rat islets were identified by double immunofluorescence staining (A) using antibodies against C-peptide (green) and antibodies recognizing the nuclear proliferation marker K_i-67 (yellow). After incubation with **2b** and the positive control CHIR99021 (**3**) for 72 h, islets were disaggregated and the resultant cell suspension was spotted on microscope slides before staining with antibodies and analysis. Islets not treated with any factor were included as controls ("Co."). Part B shows the percentage of K_i-67 expressing cells of all C-peptide positive cells (% K_i-67$^+$/C-peptide$^+$ cells), which corresponds to the fraction of replicating β cells. Part B illustrates a representative experiment of three independent studies, and each data point includes about 20.000 C-peptide positive cells. Error bars are ±SD. Asterisks indicate statistically significant differences (Student's *t* test): (∗) $P < 0.05$; (∗∗) $P < 0.01$ versus control.

of new and rationally designed paullone derivatives using an innovative technological platform consisting of a sequence of biochemical and cellular assays. This platform has proven to be suitable for the detection, characterization, and evaluation of potential β cell regenerative agents. In summary, we have outlined an approach to the development of innovative β cell regenerative agents and identified cazpaullone, a GSK-3 inhibitor with strong cytoprotective and mitogenic activity in β cells.

Experimental Section

Kinase Assays. The kinase inhibition assays were performed as described previously for GSK-3β, CDK5/p25, CDK1/cyclin B, and CK1 (ref 58 and Supporting Information). Tests were carried out in triplicate, and the final ATP concentration was 15 μM. The typical standard error for IC_{50} values in the kinase assays was <5%.

Cell-Based Assays. Culture of INS-1E-cells, isolation of rat islets, viability assays, caspase activity assays, DNA fragmentation assays, in vitro β cell proliferation assays, BrdU labeling and detection assays, and quantitative real-time PCR were performed as described recently.[16]

***β*-Catenin Phosphorylation in SH-SY5Y Human Neuroblastoma Cells.** Nearly confluent SH-SY5Y human neuroblastoma cells were grown in 96-well plates in DMEM (supplemented with 10% fetal calf serum from Invitrogen and antibiotics penicillin-streptomycin from Lonza). Cells were co-treated with tested compounds and 2 μM MG132 (to allow accumulation of phospho-β-catenin). Final DMSO concentration did not exceed 1%. Cells were then subjected to an ELISA assay using antibodies directed against Ser33/Ser37/Thr41-phosphorylated (1:1000) obtained from Cell Signaling Technology. Results are expressed in percentage of maximal β-catenin phosphorylation, i.e., in untreated cells exposed to MG132 only as positive control (100% phosphorylation).

Docking. Docking was performed using the FlexX[59] interface in Sybyl.[60] Before docking, the compounds were preminimized, partial atomic charges were calculated, and they were minimized again. Minimization was done by employing the Tripos force field[61] (Powell conjugate gradient, convergence criterion 0.005 kcal/(mol Å), 1000 iterations), and the charges were calculated using MOPAC 6.0[62] applying the AM1 Hamiltonian. FlexX calculated 30 docking solutions for each compound. The poses depicted in Figure 3 represent the docking solutions with the lowest FlexX total scores. Information of the X-ray crystal structure of alsterpaullone cocrystallized in the GSK-3 (1Q3W)[34] was retrieved from the PDB. To define the active site, a FlexX receptor description file was created, which refers to all atoms of the protein within a distance of 6.5 Å from an atom of alsterpaullone at its crystalline position.

Synthetic Chemistry. The monomode microwave device was a CEM Discover focused microwave synthesis system with ChemDriver software. Melting points (mp) were determined on an electric variable heater (Barnstead Electrothermal IA 9100) and were not corrected. IR spectra were recorded as KBr disks on a Thermo Nicolet FT-IR 200. ^{1}H NMR spectra and ^{13}C NMR spectra were recorded on the following instruments: Bruker Avance DRX-400 and Bruker Avance II-600. The solvent was DMSO-d_6 if not stated otherwise, and the internal standard was tetramethylsilane. Signals are in ppm (δ scale). Elemental analyses were conducted on a CE Instruments FlashEA 1112 elemental analyzer (Thermo Quest). Mass spectra were recorded on a double-focused sector field mass spectrometer Finnigan-MAT 90. Accurate measurements were conducted according to the peak match method using perfluorokerosene (PFK) as an internal mass reference. (EI)-MS ionization energy was 70 eV. TLC parameters are as follows: Polygram Sil G/UV$_{254}$, Macherey-Nagel, 40 mm × 80 mm, visualization by UV illumination (254 nm). Column chromatography parameters are as follows: silica gel 60 (Merck), column width 2 cm, column height 10 cm unless stated otherwise. HPLC parameters are as follows: Elite LaChrom (Merck/Hitachi), pump L-2130, autosampler L-2200, diode array detector L-2450, organizer box L-2000, column Merck LiChroCART 125-4, LiChrosphere 100, RP 18, 5 μm; flow rate 1.000 mL/min, isocratic, volume of injection 10 μL, detection (DAD) at 254 and 280 nm, AUC % method, time of detection 15 min, net retention time (t_s), dead time (t_m) related to DMSO. Preparation of HPLC eluents involved H_2O + TFA (pH 2.11/pH 2.55), and water was adjusted to pH 2.11/2.55 by addition of trifluoroacetic acid. Buffer at pH 2.30 consisted of 980 mL of water/20 mL of triethylamine/242 mg of sodium hydroxide. Adjustment

Table 2. Activation of Pax4 RNA Expression in INS-1E Cells (A) and Primary Rat Islets (B)[a]

(A) In INS-1E Cells

	relative gene expression analyzed by qRT-PCR			
	Pax4		CypB	
compd	4 h	24 h	4 h	24 h
Co.	1.00 ± 0.15	1.08 ± 0.47	1.00 ± 0.11	1.00 ± 0.32
Act-A (1 nM)	3.38 ± 0.34*	4.31 ± 0.12	1.04 ± 0.12	1.12 ± 0.18
1-AKP (**2a**) (10 μM)	1.87 ± 0.14*	0.8 ± 0.16	1.22 ± 0.1	1.14 ± 0.22
alsterpaullone (**1b**) (12.5 μM)	7.1 ± 0.4*	1.00 ± 0.25	1.3 ± 0.14	1.1 ± 0.2
cazpaullone (**2b**) (1 μM)	4.87 ± 1.06*	0.56 ± 0.23*	1.11 ± 0.32	1.42 ± 0.51
2c (10 μM)	0.72 ± 0.22	0.61 ± 0.3	1.2 ± 0.21	1.3 ± 0.32
2d (10 μM)	0.97 ± 0.12	0.45 ± 0.22*	1.14 ± 0.23	0.97 ± 0.16
CHIR99021 (**3**) (10 μM)	0.78 ± 0.24	0.76 ± 0.31	1.31 ± 0.48	1.12 ± 0.03
6BIO (**4**) (1μM)	1.02 ± 0.1	0.81 ± 0.12*	1.04 ± 0.1	1.2 ± 0.2

(B) In Rat Islets

	relative gene expression analyzed by qRT-PCR 4h	
compd	Pax4	CypB
Co.	1.00 ± 0.1	1.00 ± 0.12
1-AKP (**2a**, 10 μM)	2.8 ± 0.1*	1.00 ± 0.35
cazpaullone (**2b**, 2 μM)	2.4 ± 0.4*	0.73 ± 0.15

[a] Expression of Pax4 and cyclophilin B (CypB) genes was analyzed using quantitative RT-PCR (qRT-PCR). Results are presented as fold change relative to the vehicle treated control (Co.). Values are the average of triplicates ± SD. The expression level of the respective gene in vehicle treated controls was defined to be 1. Test agents were activin-A (Act-A), 1-azakenpaullone (1-AKP, **2a**), cazpaullone **2b**, CHIR99021 (**3**), 6-bromoindirubin-3′-oxime (6BIO, **4**), and **2d**. These agents were applied to INS-1E cells (A) or primary rat islets (B) at the indicated concentrations (μM) for 4 h or 24 h. Section A shows representative data, and results were confirmed by at least two independent experiments. Section B shows representative data of four similar experiments. Asterisks indicate statistically significant differences (Student's *t* test): (*) $P < 0.05$ versus control.

to pH 2.30 was done by addition of sulfuric acid. Preparative HPLC parameters are as follows: Merck LaPrep, LaPrep P110 preparative HPLC pump, Knauer injection loop 5 mL, LaPrep P216 fraction collector, LaPrep P311 spectrophotometer, self-packing device Merck NW 25 with a column tube 125 mm, inner diameter 25 mm, silica gel Merck LiChrospher 100 RP-18, 12 μm, flow rate 40 mL/min, detection at 254 nm. The following compounds were prepared according to literature methods: **20b**,**c** and **21b**,**c**,[42] **22**,[35] **28**,[45] and **7a**, **8a**, **9a**, **10a**.[33] Synthetic procedures for the following compounds are available in the Supporting Information: **6a**−**c**, **7b**,**c, 8b**,**c**, **9b**,**c**, **10b**−**j**, **10n**−**o**, **11a**−**c**, **2a**,**c**−**o**, **13b**−**f, 15a**−**e, 16a**−**c**, **23a**−**c**, **24a**,**c**, **26**, **29**, **30**.

General Procedure A for the Synthesis of the Phenylhydrazones 10b−j,n−o and 11a−c. 5*H*-Pyrido[3,2-*b*]azepine-6,9(7*H*,8*H*)-dione (**9a**) (1.0 mmol) or 3,4-dihydropyrido[4,3-*b*]azepine-2,5-dione (**9b**) (1.0 mmol), respectively, and an appropriate substituted phenylhydrazine (1.5 mmol) (respectively, an appropriate substituted phenylhydrazine hydrochloride (1.1 mmol) and sodium acetate (1.1 mmol)) were suspended in glacial acetic acid (10 mL) and stirred for 15–80 min at 70 °C. After cooling to room temperature, the mixture was poured into a 5% aqueous sodium acetate solution. The precipitate was filtered off with suction and washed with 5% aqueous sodium acetate solution and water. The phenylhydrazones **10** and **11** were used after a single crystallization from ethanol for the following synthetic procedures without further purification.

General Procedure B for the Synthesis of the Azapaullones 2b−j,o and 13b,c. An appropriate hydrazine derivative (1.0 mmol), obtained by general procedure A, was refluxed in diphenyl ether (80 mL) under nitrogen for 2 h. The mixture was allowed to cool to room temperature. *n*-Hexane (100 mL) was added, and the forming precipitate was separated by filtration and washed with petroleum ether. Crystallization was carried out from ethanol.

General Procedure C for the Synthesis of the Azapaullones 13a,c−f. 3,4-Dihydropyrido[4,3-*b*]azepine-2,5-dione (**9b**) (1.0 mmol) and an appropriate substituted phenylhydrazine (1.5 mmol) (i.e., an appropriate substituted phenylhydrazine hydrochloride (1.1 mmol) and sodium acetate (1.1 mmol)) were suspended in glacial acetic acid (10 mL), stirred for 1 h at 70 °C, and cooled to room temperature. A few drops of concentrated sulfuric acid were added to the mixture, and stirring was continued at 90 °C for 2–5 h. After cooling to room temperature, the mixture was poured into a 5% aqueous sodium acetate solution. The precipitate was filtered off with suction and washed with 5% aqueous sodium acetate solution and water. Crystallization was carried out from ethanol.

General Procedure D for the Synthesis of the Azapaullones 2a,b,d,f. An appropriate hydrazone derivative (0.1 mmol), obtained by general procedure A, was heated in water (1 mL). The reaction was conducted in a sealed microwave reaction vessel employing the following conditions: ramp time 5 min, reaction time 30 min, reaction temperature 175–215 °C. After the mixture was cooled to room temperature, the precipitate was filtered off and washed with petroleum ether and water.

General Procedure E for the Synthesis of the N-12-Substituted Azapaullones 15a−e and 16c. 5*H*-Pyrido[3,2-*b*]azepine-6,9(7*H*,8*H*)-dione (**9a**) (1.0 mmol) or 3,4-dihydro-pyrido[4,3-*b*]azepine-2,5-dione (**9b**) (1.0 mmol) and an appropriate substituted phenylhydrazine (1.5 mmol) (i.e., an appropriate substituted phenylhydrazine hydrochloride (1.1 mmol) and sodium acetate (1.1 mmol)) were suspended in glacial acetic acid (10 mL) and stirred for 0.5–2 h at 70 °C. After cooling to room temperature, the mixture was poured into a 5% aqueous sodium acetate solution. The precipitate was filtered off with suction and washed with 5% aqueous sodium acetate solution and water. The material was purified by crystallization from ethanol.

General Procedure F for the Synthesis of the Oximes 23a−c. 1*H*-[1]Benzazepine-2,5(7*H*,8*H*)-dione (**22**) (0.50 mmol) and an appropriate *O*-arylhydroxylamine hydrochloride (0.55 mmol) and sodium acetate (0.55 mmol) were suspended in glacial acetic acid (10 mL) and stirred for 1–3 h at 70 °C. After cooling to room temperature, the mixture was poured into a 5% aqueous sodium acetate solution. The precipitate was filtered off with suction and was then washed with 5% aqueous sodium acetate solution and water. The material was purified by crystallization from ethanol.

General Procedure G for the Synthesis of the 12-Oxapaullones 24a−c. An appropriate oxime derivative (0.30 mmol), obtained by general procedure F, was stirred in 5 mL of formic acid (96%) and 0.5 mL of phosphoric acid (85%) at 60 °C. After 20–60 min a gray precipitate appeared. After cooling to room

temperature, the mixture was poured into water (20 mL). The precipitate was separated by filtration and washed with petrol ether and water.

6-Oxo-5,6,7,12-tetrahydropyrido[3′,2′:2,3]azepino[4,5-*b*]indole-9-carbonitrile (Cazpaullone) (2b). Preparation of **2b** was either accomplished according to general procedure B or D. Preparation according to general procedure B from **10b** (260 mg, 0.89 mmol) yielded 18% of a brown powder, mp >330 °C. ^{1}H NMR δ 3.76 (s, 2H, azepine-CH_2), 7.46 (dd, 1H, 8.2/4.5 Hz, ArH), 7.53 (dd, 1H, 8.5/1.5 Hz, ArH), 7.62 (dd, 1H, 8.5/0.5 Hz, ArH), 7.65 (dd, 1H, 8.2/1.4 Hz, ArH), 8.38 (s, 1H, ArH), 8.51 (dd, 1H, 4.5/1.4 Hz, ArH), 10.34 (s, 1H, NH), 12.30 (s, 1H, NH); ($C_{16}H_{10}N_4O$) HRMS (EI) (*m*/*z*) [M^+] calcd 274.0855, found 274.0174. Preparation according to general procedure D from **10b** (29 mg, 0.10 mmol) yielded 36% of a brown powder. ^{1}H NMR data were consistent with the data obtained for **2b** prepared by general procedure B.

9-Bromo-7,12-dihydropyrido[4′,3′:2,3]azepino[4,5-*b*]indol-6(5*H*)-one (13a). Preparation according to general procedure C from **9b** (56 mg, 0.32 mmol), (4-bromophenyl)hydrazine hydrochloride (78.1 mg, 0.35 mmol), and sodium acetate (28.6 mg, 0.35 mmol) afforded 20% of a gray solid, mp >330 °C. ^{1}H NMR δ 3.66 (s, 2H, azepine-CH_2), 7.19 (d, 1H, 5.5 Hz, ArH), 7.31 (dd, 1H, 8.6/1.9 Hz, ArH), 7.42 (d, 1H, 8.6 Hz, ArH), 7.96 (d, 1H, 1.8 Hz, ArH), 8.45 (d, 1H, 5.5 Hz, ArH), 8.92 (s, 1H, ArH), 10.53 (s, 1H, NH), 11.96 (s, 1H, NH); ($C_{15}H_{10}BrN_3O$) HRMS (EI) (*m*/*z*) [M^+] calcd 327.0007, found 326.9990.

9-Bromo-5,7-dihydro-6*H*-[1]benzofuro[3,2-*d*][1]benzazepin-6-one (24b). Preparation according to general procedure G from **23b** (104 mg, 0.30 mmol) yielded 38% of a gray solid, mp >330 °C. ^{1}H NMR (DMSO-d_6 + TFA, 600 MHz) δ 3.69 (s, 2H, azepine-CH_2), 7.30–7.34 (m, 2H, ArH), 7.46–7.49 (m, 1H, ArH), 7.54 (dd, 1H, 8.7/2.0 Hz, ArH), 7.65 (d, 1H, 8.6 Hz, ArH), 7.87 (dd, 1H, 7.8/1.4 Hz, ArH), 8.12 (d, 1H, 2.0 Hz, ArH), 10.41 (s, 1H, NH). Anal. ($C_{16}H_{10}BrNO_2$) C, H, N.

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Supporting Information Available: Details for the synthesis of **6a**−**c**, **7b**,**c**, **8b**,**c**, **9b**,**c**, **10b**−**j**,**n**−**o**, **11a**−**c**, **2a**,**c**−**o**, **13b**−**f**, **15a**−**e**, **16a**−**c**, **23a**−**c**, **24a**,**c**, **26**, **29**, **30**, spectroscopic data, HPLC purity data, and data from elemental analyses. This material is available free of charge via the Internet at http://pubs.acs.org.

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