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Macrocyclic Bisindolylmaleimides as Inhibitors of Protein Kinase C and Glycogen Synthase Kinase-3

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Abstract—Efficient methods were developed to synthesize a novel series of macrocyclic bisindolylmaleimides containing linkers with multiple heteroatoms. Potent inhibitors (single digit nanomolar IC_{50}) for PKC-β and GSK-3β were identified, and compounds showed good selectivity over PKC-α, -γ, -δ, -ε, and -ζ. Representative compound **5a** also had high selectivity in a screening panel of 10 other protein kinases. In cell-based functional assays, several compounds effectively blocked interleukin-8 release induced by PKC-βII and increased glycogen synthase activity by inhibiting GSK-3β.
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Protein phosphorylation and dephosphorylation are important processes in the regulation of protein function. Phosphorylation occurs on serine, threonine, and tyrosine residues and is catalyzed by protein kinases, thereby mediating various cellular signaling events that can be associated with human diseases.[1] Given the >500 protein kinases identified in the human genome, there is a rich mother lode of drug discovery targets for exploration.[2] We have been interested in finding inhibitors of enzymes in the protein kinase C (PKC) and glycogen synthase kinase-3 (GSK-3) classes as potential therapeutic agents.

PKC represents a family of serine/threonine kinases that play a critical role in intracellular signal transduction, gene expression, and the control of cell differentiation and growth. There are at least 11 isozymes that are classified into three subfamilies on the basis of their cofactor requirements: conventional (α, βI, βII, γ), novel (δ, ε, η, θ, μ), and atypical (ζ, ι). PKC enzymes regulate vascular tone, permeability, and proliferation, and they are involved in cardiovascular disease, cancer, ischemia, inflammation, and CNS disorders.[3] The β isoform, which is induced in response to hyperglycemia in cardiac, aortic, renal, and retinal tissues, is associated with diabetic complications, particularly retinopathy, angiopathy, nephropathy, macular edema, neuropathy, and cardiomyopathy.[4]

GSK-3 is a serine/threonine protein kinase composed of two isoforms (α and β) with high homology (ca. 90%) at the catalytic domain.[5] GSK-3β plays a critical role in glucose homeostasis, CNS function (via the proteins tau and β-catenin), and cancer (via angiogenesis, apoptosis, and tumorigenesis).[6] Inhibition of GSK-3-dependent phosphorylation should activate insulin-dependent glycogen synthesis, thereby mimicking the action of insulin to lower plasma glucose. Thus, inhibitors of GSK-3β would afford a novel mode of treating type II diabetes.[4b,7] Additionally, GSK-3 inhibitors have therapeutic potential for treating neurodegenerative diseases, bipolar disorder, stroke, cancer, and chronic inflammatory diseases.[8]

Several PKC inhibitors, such as UCN-01, CGP-41251 and LY-317615, are now in clinical trials as anticancer agents.[9] Ruboxistaurin (LY-333531) is a selective inhibitor of PKC-β that is in Phase 3 clinical trials for the

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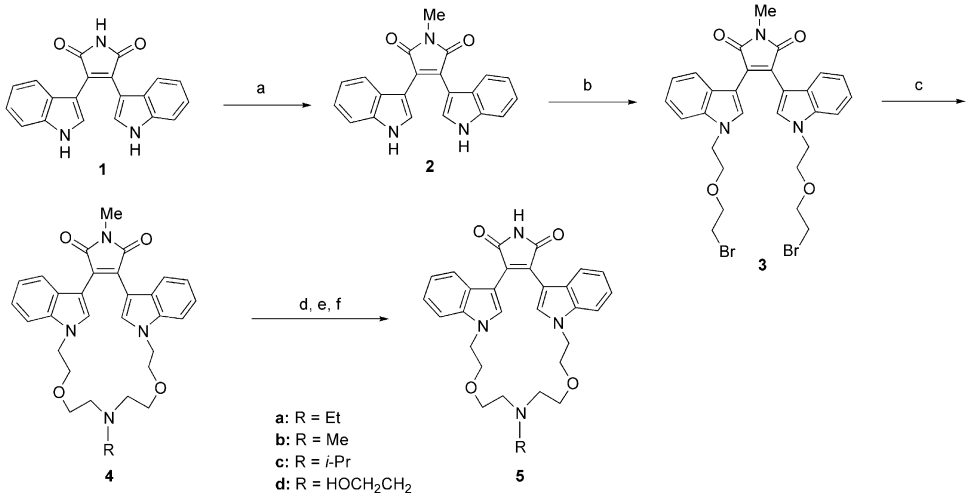
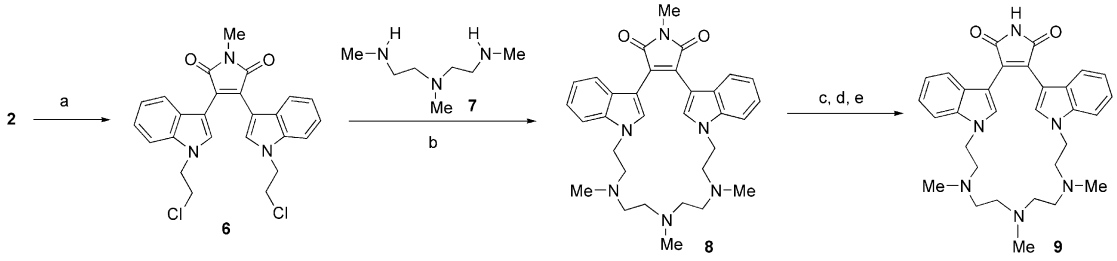


Scheme 1. (a) Me_2SO_4, K_2CO_3, acetone, reflux, 41%; (b) $O(CH_2CH_2Br)_2$ (excess), Cs_2CO_3, DMF, 45 °C, 53%; (c) $EtNH_2$ (excess), *i*-Pr_2NEt, THF, 90 °C, 16 h; then volatiles evaporated, redissolved in dioxane, reflux, 6 h, 40%; (d) KOH, EtOH, reflux; (e) citric acid, H_2O; (f) neat NH_4OAc, 140 °C, 54%.



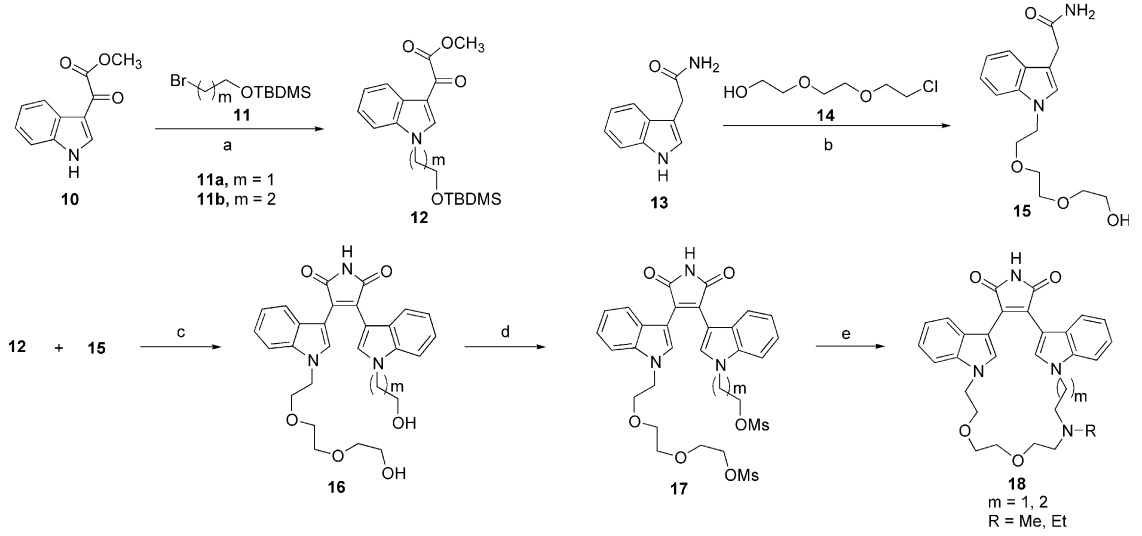
Scheme 2. (a) $ClCH_2CH_2Br$ (excess), Cs_2CO_3, DMF, 45 °C; (b) KI, *i*-Pr_2NEt, THF, 120 °C; (c) KOH, EtOH, 80 °C; (d) citric acid, H_2O; (e) neat NH_4OAc, 140 °C, 41% overall from **2**.

treatment of diabetic retinopathy, macular edema, and neuropathy.[10] However, inhibitors of GSK-3 are limited to preclinical evaluation.[4b,8,11] Herein, we report the synthesis and biological evaluation of a novel series of macrocyclic bisindolylmaleimides[12] that provide interesting dual inhibitors of PKC-β and GSK-3β.

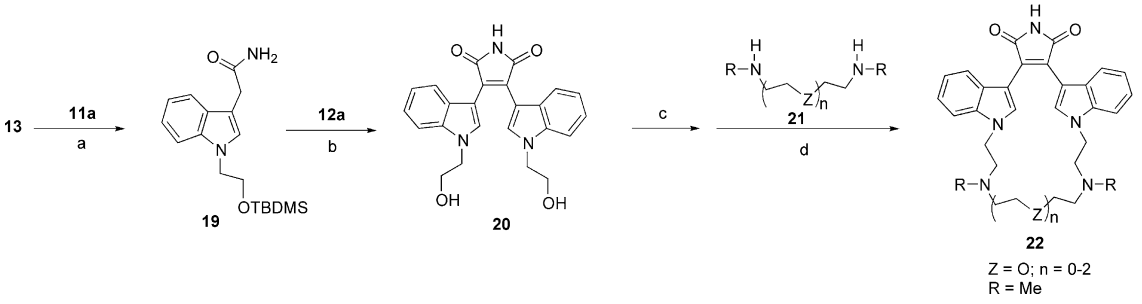
Synthetic Chemistry

Our novel series of macrocyclic bisindolylmaleimides contains linkers with multiple heteroatoms. Efficient syntheses were developed to obtain target compounds in this class. One or more basic nitrogen atoms, usually required for biological and/or physicochemical purposes, were introduced into the linker in the last synthetic step via ring-closing *N*-alkylation. This approach allowed us to introduce diverse amino groups and synthesize both symmetrical and unsymmetrical macrocyclic bisindolylmaleimides. In an approach to monoamine-containing macrocycles, bisindolylmaleimide **1**, readily prepared from indole-3-acetamide and methyl indole-3-glyoxylate in the presence of *t*-BuOK,[13] was treated with Me_2SO_4 and K_2CO_3 in acetone to give *N*-methylated maleimide **2** (Scheme 1), along with some di- and tri-*N*-methylated products. Bis-*N*-alkylation of **2** with the excess of 2-bromoethyl ether using Cs_2CO_3 as a base in DMF afforded dibromide **3**. An attempt to treat dibromide **3** with 1 mol-equiv of ethylamine in the presence of *i*-Pr_2NEt/THF in a pressure tube at 90 °C gave only a trace of the desired cyclized product **4a**. Use of more ethylamine accelerated the reaction, leading to more product **4a** along with some undesired acyclic diamine product. Finally, treatment of **3** with 13 mol-equiv of ethylamine for 16 h resulted in cyclized product **4a**, along with acyclic monoamine (the precursor to the cyclized product), and acyclic diamine side product in a ratio of ca. 2:1:1. To avoid further conversion of acyclic monoamine to the undesired diamine, the excess ethylamine and other volatiles were evaporated in vacuo, and the residue was refluxed in dioxane for 6 h to allow complete conversion of the monoamine to the cyclized product, providing **4a** in 40% isolated yield. Deprotection of the *N*-methyl group in maleimide **4a** was carried out by using a routine process.[14] Thus, **4a** was hydrolyzed with KOH/EtOH and the resulting diacid was treated with citric acid to form a maleic anhydride, which was converted to the desired product **5a** (54% yield from **4a**) by reaction with neat NH_4OAc at 140 °C. By using a similar process, symmetrical amine-containing macrocycles, such as **5b–d**, were prepared. When a less volatile amine was used, such as in the preparation of **4d**, less of an excess was needed.

Through a similar strategy, a symmetrical macrocyclic bisindolylmaleimide with a triamine-containing linker (**9**) was prepared. Thus, bisindolylmaleimide **2** was treated with the excess of 1-bromo-2-chloroethane to give dichloride **6**, which was macrocyclized with triamine **7** (1.2 mol-equiv) in the presence of KI/



Scheme 3. (a) Cs_2CO_3, DMF, 50–75 °C, 68–82%; (b) Cs_2CO_3, DMF, 78 °C, 82%; (c) *t*-BuOK, THF, 0–23 °C; then concd HCl; (c) Ms_2O, pyridine, THF, 50 °C; (d) *i*-Pr_2NEt, DMF, 90 °C, 11–20% overall from **15**.



Scheme 4. (a) NaH, DMF, 60%; (b) *t*-BuOK, THF, 0–23 °C, then concd HCl, 83%; (c) Ms_2O, pyridine, THF, 50 °C, quantitative yield; (d) *i*-Pr_2NEt, DMF, 100 °C, 15–30%.

i-Pr_2NEt/THF at 120 °C to afford **8** (Scheme 2). Deprotection of the *N*-methyl maleimide by the procedure described above provided product **9** in 41% overall yield from **2**.

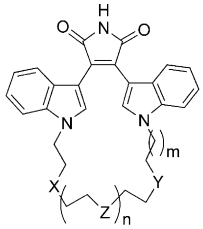
Application of the above processes to an unsymmetrical macrocyclic target, such as **18**, was less successful due to the difficulty of selective *N*-alkylation of **2** with two different alkylating agents. Therefore, we adopted an alternate approach. Methyl indole-3-glyoxylate **10** was *N*-alkylated with **11** by using Cs_2CO_3 as a base to afford **12**, while indole-3-acetamide **13** was *N*-alkylated with **14** by using NaH as a base to give **15** (Scheme 3). Maleimide condensation of **12** and **15** in the presence of *t*-BuOK was followed by treatment of concd HCl to remove the silyl group to derive product **16**. Given that protection and deprotection of the maleimide nitrogen are cumbersome, as seen above, an effort was made to convert diol **16** to cyclized product without protecting the maleimide nitrogen. Thus, treatment of **16** with Ms_2O (4 mol-equiv) in the presence of pyridine/THF at 50 °C selectively produced dimesylate **17** in quantitative yield. Ring-closing of **17** with a primary amine was successful in DMF at 90 °C to afford unsymmetrical macrocycle **18**, although the yield was relatively low (11–20% overall from amide **15**), along with some acyclic diamine that could be easily separated.

By applying similar chemistry, the symmetrical, diamine-containing macrocyclic targets **22** could be prepared in reasonable yields, as shown in Scheme 4. Considering the absence of protection and deprotection of the maleimide nitrogen, this route presents a significant advantage over the previous methods shown in Schemes 1 and 2.

Enzymatic Activity

Given the high homology of PKC isozymes in their catalytic domain, identification of a selective, ATP-competitive inhibitor for a particular isozyme can be challenging. Our original interest was to identify selective PKC-β inhibitors, in the same vein as the promising clinical candidate LY-333531.[10] Thus, we screened our macrocyclic bisindolylmaleimides in enzymatic assays involving the calcium-dependent (conventional) PKC isozymes α, βI, βII, and γ to determine activity and selectivity within the same subfamily (Table 1). In general, the compounds potently inhibited PKC-βI and PKC-βII, while being selective over PKC-α and PKC-γ. The reference compound staurosporine was very potent, but it did not show significant selectivity for PKC-β. Compound **5a**, an amine-containing, symmetrical, 19-membered macrocyclic bisindolylmaleimide, potently

Table 1. Enzymatic activity of macrocyclic bisindolylmaleimdes[a]



Compd	*m*	*n*	X	Y	Z	PKC[b] IC_{50} (μM)							GSK-3β[c]
						PKC-α	PKC-βI	PKC-βII	PKC-γ	PKC-δ	PKC-ε	PKC-ζ	IC_{50} (μM)
5a	1	1	O	O	NEt	0.11±0.02	0.014±0.003	0.009±0.004	0.30±0.04	0.35	0.075	34%	0.025±0.003
5b	1	1	O	O	NMe	0.30±0.04	0.047±0.009	0.041±0.011	28%	40%	0.25	34%	0.071±0.002
5c	1	1	O	O	N(*i*-Pr)	0.17	n.d.	0.017	0.40	n.d.	n.d.	n.d.	n.d.
5d	1	1	O	O	$N(CH_2CH_2OH)$	0.44±0.06	0.068±0.006	0.040±0.013	1.57±0.13	n.d.	n.d.	n.d.	0.040±0.003
9	1	1	NMe	NMe	NMe	0.29±0.11	0.031±0.008	0.027±0.007	1.57±0.02	1.16	0.17	40%	0.043±0.005
18a	2	1	O	NMe	O	0.25±0.08	0.073±0.006	0.046±0.016	0.98	n.d.	n.d.	n.d.	0.004±0.0004
18b	1	1	O	NEt	O	0.29±0.007	0.031±0.01	0.019±0.004	1.00±0.5	0.61±0.05	0.31	4%	0.004±0.0005
22a	1	0	NMe	NMe	None	35%	0.25±0.14	0.20±0.11	23%	n.d.	n.d.	n.d.	0.11±0.003
22b	1	1	NMe	NMe	O	0.49±0.31	0.027	0.041±0.014	1.56	26%	0.61	6%	0.006±0.001
22c	1	2	NMe	NMe	O	0.77	0.14±0.06	0.075±0.010	37%	n.d.	n.d.	n.d.	0.008±0.001
23	1	1	NMe	O	NMe	0.57±0.033	0.010±0.002	0.009±0.001	0.39±0.3	1.50	0.080	7%	0.033±0.002
24	1	1	NEt	O	$–OCH_2CH_2NEt–$	0.91	0.30±0.005	0.11±0.04	36%	n.d.	n.d.	n.d.	0.031±0.001
Staurosporine						0.028±0.0001	0.063	0.010±0.004	0.059±0.007	0.049	0.012	10%	n.d.
SB-216763[d]						n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.007±0.0001

[a]IC_{50} values are expressed as mean±SEM ($n \geq 2$; $n = 1$ for values without error limits). % Inhibition was measured at 1 μM. n.d. denotes not determined.
[b]For PKC-α, -βI, -βII, and -γ isozymes, the assay was performed essentially as previously described[10b] (histone as a substrate) with the following modifications: the reaction volume was reduced to 100 μL, the assay was run in a 96-well format, and [γ-^{33}P]ATP was substituted for [γ-^{32}P]ATP. For δ, ε, and ζ isozymes, the PKC-ε pseudosubstrate hexadecapeptide ERMRPRKRQGSVRRRV was employed in place of histone as a substrate and $CaCl_2$ was eliminated from the reaction mixture.
[c]Performed at Upstate Biotech Inc. using a filtration assay. Recombinant human GSK-3β was used with a peptide derived from glycogen synthase as a substrate.
[d]GSK-3 inhibitor reference compound.[11g]

inhibited PKC-βI and PKC-βII with IC_{50} values of 14 and 9 nM, respectively, and was 12- to 33-fold selective for PKC-βII over PKC-α and PKC-γ. Replacement of the Et group attached to the linker nitrogen in **5a** with Me, *i*-Pr or hydroxyethyl groups (**5b–d**) caused some loss of potency versus PKC-β. An unsymmetrical analogue with the *N*-Et moved from the Z to Y position (**18b**) showed no loss of potency or selectivity. Introduction of diamines (**22b** and **23**) or triamines (**9**) into the linker was well tolerated, but changing the macrocyclic ring size from 19 to 16 (**22a**) or 22 atoms (**22c** and **24**) led to a decrease of potency.

Selected compounds were further tested in PKC isozyme assays representing other subfamilies, such as calcium-independent (novel) isozymes PKC-δ and PKC-ε, and the atypical isozyme PKC-ζ. Most of the tested compounds showed some activity against PKC-ε, but weak inhibition of PKC-δ and PKC-ζ (Table 1).

Compound **5a**, as a representative lead compound, was further screened against a panel of 11 other kinases for assessing kinase selectivity (Table 2). High selectivity was observed across the panel except for CDK1 ($IC_{50} = 0.24$ μM) and GSK-3β ($IC_{50} = 0.018$ μM). The outstanding potency against GSK-3β triggered us to profile other compounds in a GSK-3β assay. Thus, the series generally turned out to show potent dual action against PKC-β and GSK-3β. Certain non-macrocyclic maleimides are able to inhibit GSK-3β.[11f,g,15] However, no GSK data has been disclosed for LY-333531.

Cellular Activity

We developed a stable HEK293 cell line expressing PKC-βII to evaluate the functional activity of inhibitors. When stimulated by the phorbol ester PMA (phorbol-12-myrisate-13-acetate), these cells synthesize and release the cytokine interleukin-8 (IL-8). Selected compounds with good PKC-βII inhibition were studied in this assay to gain information on their ability to penetrate cell membranes and inhibit intracellular PKC-

Table 2. Kinase selectivity, IC_{50} (μM)[a]

Compd	VEGF-R2	CDK1	EGF-R	Protein kinase A	Casein kinase 1	Casein kinase 2	Calmodulin kinase 2	GSK-3β	MAP kinase ERK-2	Insulin receptor kinase-β	PDGF-R
5a	1.41	0.24	>100	14.5	>100	>100	7.8	0.018	>100	>100	4.4
Staurosporine	0.014	0.008	0.049	0.004	1.40	>100	0.006	0.089	1.38	0.20	0.002

[a]The values are the average of duplicate determinations. Assays were performed as described in ref 16.

Table 3. Cellular activity[a]

Compd	IL-8 release[b] IC_{50} (μM)	GS activity[c] EC_{50} (μM)
5a	0.025	0.31
5d	0.058	0.38
9	0.082	0.39
18a	0.045	0.15
23	0.046	0.14
Staurosporine	0.077	n.d.
SB-216763	n.d.	0.20

[a]Values are an average of at least two independent determinations. n.d. denotes not determined.
[b]HEK293 cells stably expressing PKC-βII were preincubated with compounds prior to stimulation with PMA and the amount of IL-8 released in cell supernatant was measured by quantitative ELISA.
[c]HEK293 cells were treated with compounds and GS activity was determined in cell extracts by measuring ^{14}C-UDP glucose incorporation into glycogen.

βII. As presented in Table 3, IL-8 release was effectively inhibited by these molecules, with **5a** being a very potent inhibitor (IC_{50} = 25 nM).

Selected compounds were also tested for their ability to increase glycogen synthase (GS) activity in HEK293 cells, a direct functional assay to measure the cellular activity of GSK-3β inhibitors. The results (Table 3) indicate effective blockade of GSK-3β and increased GS activity within cells. For example, **5a** has an EC_{50} of 0.31 μM in this assay.

Conclusion

We developed several efficient synthetic approaches to novel macrocyclic bisindolylmaleimides containing linkers with multiple heteroatoms. The compounds generally exhibited potent dual inhibition of PKC-β and GSK-3β, and good selectivity over PKC-α, -γ, -δ, -ε, and -ζ. Representative lead compound **5a** had high selectivity in a screening panel of 10 other kinases. Certain compounds were effective intracellularly by blocking PKC-βII-induced IL-8 release and increasing glycogen synthase activity via blockade of GSK-3β.

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