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NON-GLYCOSIDIC/NON-AMINOALKYL-SUBSTITUTED INDOLOCARBAZOLES AS INHIBITORS OF PROTEIN KINASE C

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Abstract: Non-glycosidic indolocarbazoles lacking aminoalkyl side-chains were synthesized and optimized with respect to potent and selective inhibition of protein kinase C as compared to other serine/threonine and tyrosine-specific protein kinases.

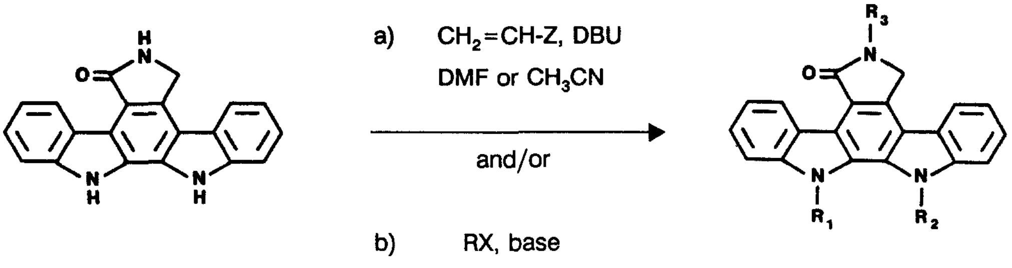
The phospholipid-dependent protein kinase (protein kinase C; PKC), which comprises a family of at least 8 isozymes, has been found to play a key role in the regulation of many cellular processes, such as growth, differentiation, contraction and secretion.[1] Thus, inhibitors of this enzyme, or one of its subtypes, might be useful for therapeutic application.[2]

The most potent inhibitor of PKC identified thus far is the microbial alkaloid staurosporine.[3] The therapeutic use of this compound, however, is limited since it lacks selectivity, i.e. it inhibits other protein kinases with comparable potency as found for inhibition of PKC.[4] In search for PKC inhibitors with higher selectivity, compounds structurally related to staurosporine were isolated from microbial origin, e.g. K-252a[5] (identical with SF-2370[6]) and UCN-01[7], or were obtained by derivatisation of natural products (CGP 41251[8]; NA-0344, NA-0345, NA-0346[9]). This indeed resulted in the discovery of PKC inhibitors with improved selectivity as compared to staurosporine.[7,8]

Another approach towards the development of more selective inhibitors that we and other groups have pursued is aimed at the identification of substructures of staurosporine. From these studies, the series of bisindolylmaleimides[10] emerged. For these compounds to be sufficiently potent inhibitors, they have to be substituted at one of the indole nitrogen atoms by an aminoalkyl side chain, which appears to mimic the aminoalkyl function present in the sugar moiety of staurosporine. Therefore, it has been postulated that this side chain by providing a cationic binding site, is essential for sufficient inhibitory activity. Contrary to this notion, we have found that non-glycosidic indolocarbazoles lacking aminoalkyl side chains can be potent and selective PKC inhibitors.

Synthesis of Compounds

Staurosporine aglycone **2**, identical with K-252c of microbial origin[11], was synthesized by a Clemmensen reduction[12] of the corresponding imide (arcyriaflavin A).[13] Indolocarbazoles **3** were prepared a) by Michael type reactions or b) by alkylation of aglycone **2**.[14]



2 **3**

a) Compounds **3h** - **3j** were obtained by reacting aglycone **2** with an excess of t-butyl acrylate, acrylamide or acrylonitrile and catalytic amounts of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in dimethylformamide or acetonitrile at room temperature followed, in the case of **3h**, by acidic cleavage of the t-butyl ester group. A regioselectivity of about 9:1 was observed for cyanoethylation of **2** at the indole nitrogen atom in para position to the carbonyl group of the lactam ring. The main regioisomer could be isolated in a pure form by crystallization.

b) Monoalkylations of **2** with one equivalent of sodium hydride and RX in dry dimethylformamide at room temperature gave indolocarbazoles, e.g. **3a** or **3f**, as a mixture of regioisomers, where the main regioisomer was derived from alkylation of the more acidic indole nitrogen atom in para position to the carbonyl group of the lactam ring.

Compound **3g** was synthesized by alkylation of **2** with 2,2-dimethyl-4-(p-toluenesulfonyl-oxymethyl)-1,3-dioxolane[15] and heating the acetal intermediate with 1N HCl in alcohol.

Dialkylations of **2** or a second alkylation of monosubstituted compounds with stoichiometric amounts of sodium hydride and RX gave indolocarbazoles **3b** - **3e**, where both indole nitrogen atoms were substituted. Trisubstituted compounds formed by additional alkylation of the nitrogen of the lactam ring were observed only in small amounts but predominate, when an excess of NaH/RX is employed. They could be separated by chromatography.

The second alkylation step to indolocarbazoles **3k** - **3n** was preferably carried out under phase transfer conditions, refluxing with potassium carbonate, potassium hydroxide, 18-crown-6, and RX in acetone.[16]

Assays for Testing of Protein Kinases

Test Compounds. All PKC inhibitors were dissolved in 100% DMSO at a concentration of 20 mM. Compounds were 10-fold diluted by addition to the assay mixture from stock solutions in 10% DMSO. The final DMSO concentration in the assay was 1% for all experiments.

PKC (C-Kinase). The enzyme was purified form rat brain according to the procedure described by Inagaki et al.[17] This resulted in a homogenous, mixed preparation of the 4 Ca^{2+}-dependent PKC isozymes as shown by western blotting with isozyme-specific antibodies. Enzyme activity was determined in a reaction cocktail of 200 µl which contained 50 mM HEPES-NaOH (pH 7.5), 5 mM $MgCl_2$, 1 mM EDTA, 1.25 mM EGTA, 1.32 mM $CaCl_2$, 1 mM DTT, 1 µg phosphatidylserine, 0.2 µg 1,2-diolein, 40 µg histone III-S (Sigma), 10 µM ATP (0.3 µCi [γ-^{32}P]ATP), 10 units (pmol P_i/min) of enzyme and test compound. Incubation was started by the addition of enzyme and performed for 5 min at 30° C. Reaction was stopped by addition of 2 ml of 8.5% H_3PO_4. Subsequently samples were filtrated through nitrocellulose filters and incorporation of $^{32}P_i$ was determined by liqid scintillation counting.

cAMP-dependent Protein Kinase (A-Kinase). The assay was performed with the commercially available catalytic subunit from bovine heart (Sigma). The incubation mixture of 200 µl contained 50 mM PIPES-NaOH (pH 6.8), 10 mM $MgCl_2$, 1 mM DTT, 50 µg histone VII-S (Sigma), 40 µM ATP (0.3 µCi [γ-^{32}P]ATP), 20 units of enzyme and test compound. Assay procedure was as described for PKC.

cGMP-dependent Protein Kinase (G-Kinase). The enzyme was purified from bovine lung as described.[18] The incubation mixture of 200 µl contained 20 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 1 mM DTT, 40 µg BSA, 2% glycerol, 10 µM cGMP, 10 µg histone II-A (Sigma), 10 µM ATP (0.3 µCi [γ-^{32}P]ATP), 10 units of enzyme and test compound. Assay procedure was as described for PKC.

Myosin Light-chain Kinase (M-Kinase). Myosin light-chain and myosin light-chain kinase were purified from chicken gizzard as described.[19] The incubation mixture of 200 µl contained 50 mM MOPS-NaOH (pH 7.2), 5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 1 mM DTT, 0.1 µM calmodulin, 12.5 µM myosin light-chain, 250 µM ATP (0.3 µCi [γ-^{32}P]ATP), 40 units of enzyme and test compound. Assay procedure was as described for PKC.

Tyrosine-specific Protein Kinase (T-Kinase). Small resting B cells from murine spleen cells were prepared as described.[20] The supernatant fraction (100,000 x g for 60 min) was used for measuring cytosolic tyrosine kinase activity. The incubation mixture of 50 µl contained 50 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 20 µM vanadate, 10 mM $MnCl_2$, 0.1% NP40, 0.3 mM Poly(Glu,Tyr; 4:1) (Sigma), 60 µM ATP (0.3 µCi [γ-^{32}P]ATP), 10 units of enzyme and test compound. The assay was performed as described for PKC.

Table 1. Inhibition of Protein Kinases by Non-glycosidic Indolocarbazoles in *in vitro* Enzyme Assays (Mean Values of IC_{50}; n=2-3)

Compound*	$R^1(R^2)$	$R^2(R^1)$	R^3	Ratio of Regioisomers	IC_{50} (μM)				
					C-Kin.	A-Kin.	G-Kin.	M-Kin.	T-Kin.
1		Staurosporine			0.009	0.040	0.018	0.010	0.400
2	H	H	H	-	0.68	>10	0.65	2.8	n.d.
3a	H	Me	H	7:1	0.52	>10	0.4	1.1	n.d.
3b	Me	Me	H	-	0.45	>100	5.5	5.8	n.d.
3c	Et	Et	H	-	0.20	>10	0.7	1.2	n.d.
3d	Me	n-Pr	H	1:1	0.10	>30	5.1	11	>10
3e	n-Pr	n-Pr	H	-	8.1	n.d.	n.d.	n.d.	n.d.
3f	H	$-CH_2-CH=CMe_2$	H	2:1	3.5	n.d.	n.d.	n.d.	n.d.
3g	H	$-CH_2-CHOH-CH_2OH$	H	95:5	0.099	4.8	0.28	0.39	n.d.
3h	H	$-(CH_2)_2CO_2H$	H	96:4	0.590	>100	21	1.8	n.d.
3i	H	$-(CH_2)_2CONH_2$	H	4:1	0.110	n.d.	n.d.	0.082	n.d.
3j	H	$-(CH_2)_2CN$	H	single isomer	0.075	>10	0.32	0.5	n.d.
3k**	Me	$-(CH_2)_2CN$	H	single isomer	0.020	>100	6.2	5.8	>10
3l	Et	$-(CH_2)_2CN$	H	9:1	0.027	6.3	1.3	1.6	>10
3m	Me	$-CH_2CHF_2$	H	3:1	0.032	2.4	0.71	0.44	>10
3n	Me	$-(CH_2)_3N_3$	H	9:2	0.025	n.d.	n.d.	0.45	n.d.
3o	Me	$-(CH_2)_2CN$	Me	single isomer	>30	>30	>30	>10	>10

* Spectroscopic characterization see reference 21

** Gö 6976

n.d.: not determined

Structure Activity Relationships

Some controversy exists in the literature regarding the inhibitory activity of staurosporine aglycone **2**. An IC_{50} of 0.214 μM was reported for the product isolated along with staurosporine from natural sources[11]. More recently, synthetic aglycone **2** was described to be devoid of any significant activity[10b]. The authors of that paper concluded that the activity reported for the natural product may have been due to the presence of impurities consisting of active fermentation products. Our data for **2** (IC_{50} = 0.68 μM) confirm the previously reported activity and indicate that the inhibitory activity resides in the indolocarbazole moiety.

Monomethylation of one of the indolyl nitrogens of **2** led to no improvement in IC_{50}s (**3a**). N,N'-dimethylation brought about a slight increase both in potency and selectivity (**3b**). Diethyl derivative **3c** was about 3 times more potent than aglycone **2** with only marginal increases in selectivity. The most potent and selective compound among the simple alkyl derivatives was found to be **3d**. Substitution with larger alkyl or alkenyl residues (**3e,3f**) resulted in a marked decrease in potency.

Further improvements in potency were obtained when the alkyl residues themselves were substituted by a polar group such as hydroxy (**3g**) or cyano (**3j**). Of the various carboxylic acid derivatives, nitrile (**3j**) proved to be the best representative. Substitution of the second indole nitrogen with small alkyl groups resulted in a further increase in selectivity (**3k,3l**). For compound **3k** (**Gö 6976**) selectivity ratios calculated from IC_{50} values were all above 100. Additional alkylation of the lactam nitrogen abolished activity (e.g. **3o**).

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21. All new compounds gave IR, ^{1}H NMR, MS and elemental analytical data consistent with their structures. The structural assignments of regioisomers in isomeric mixtures of monoalkylated compounds were derived from ^{1}H NMR, where the downfield indole NH was attributed to the regioisomers with the free indole NH in para position to the carbonyl group of the lactam ring (in general, the minor regioisomer). This assignment has additionally been confirmed by a regioselective synthesis of compound **3k** and the minor regioisomer of compound **3a** (J.Aranda, unpublished results). ^{1}H NMR (DMSO-D6/TMS) of compound **3k**:
δ=2.87 (t, 2H, J=7Hz, CH_2CN); 4.19 (s, 3H, NMe); 4.97 (s, 2H, NCH_2 5-Ring); 5.15 (t, 2H, J=7, Indol-NCH_2); 7.32 (t, 1H, ArH); 7.42 (t, 1H, ArH); 7.5-7.65 (m, 2H, ArH); 7.72 (d, 1H, ArH); 7.96 (d, 1H, ArH); 8.07 (d, 1H, ArH); 8.65 (broad s, 1H, NH); 9.39 (d, 1H, ArH).