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2-ARYL-INDOLYL MALEIMIDES - NOVEL AND POTENT INHIBITORS OF PROTEIN KINASE C

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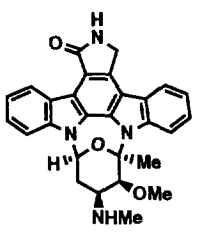
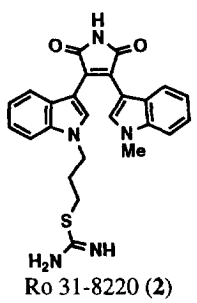
Abstract: A new class of protein kinase C (PKC) inhibitors is described. These inhibitors were derived from the familiar bis-indolyl maleimide series of inhibitors through a structural rearrangement involving transfer of one aryl ring from the maleimide moiety to the C-2 position of the indole ring remaining attached to that moiety. The resulting compounds are among the most potent known inhibitors of PKC and also show good selectivity for PKC in relation to other kinases. The lead compound in this series possesses antitumor activity in several in vitro and in vivo models.

The roughly one dozen isozymes comprising the protein kinase C (PKC) family play crucial roles in a variety of cellular signal transduction events. In particular, they are believed to function as mediators of inflammation and to be involved in the process of malignant transformation.[2] These beliefs arise, in part, from the observation that stimulators of this kinase family (e.g. phorbol esters, aplysiatoxin, and the teleocidins) are strongly inflammatory and also promote the development of tumors in animals treated with sub-carcinogenic doses of mutagens. Given their involvement in such biomedically important processes it is not surprising that a great deal of effort has gone into developing potent and selective inhibitors of these enzymes. Attempts to devise inhibitors rationally - as, for example, by targeting the consensus phosphorylation sequence[3] recognized by the PKCs - have yet to yield a viable drug candidate. And the lack of an X-ray crystal structure for any PKC isoform has mostly precluded the use of computer assisted design. Until recently[4] medicinal chemists have had only one family of natural products, the indolocarbazole alkaloids, to turn to in their search for serious leads. While many members of this family, exemplified by staurosporine (**1**) (IC_{50} = 2nM), are potent PKC inhibitors they show poor selectivity for this kinase family over the many others which exist.[5]



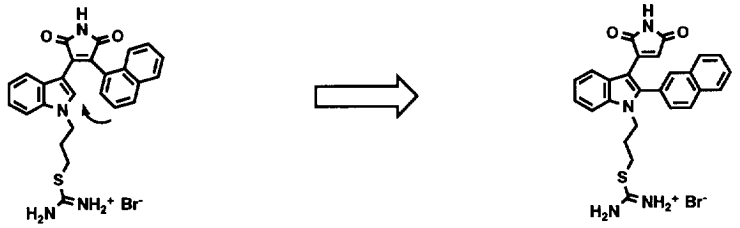
Staurosporine (**1**)



Ro 31-8220 (**2**)

A major step forward was taken by Davis et. al.[6] at Roche who discovered that simplified bis-indolyl maleimide structures such as **2** (IC_{50} = 10nM) strongly inhibit PKC but show little activity against many of the other kinases inhibited by staurosporine. The Roche compounds, as well as staurosporine, inhibit their targets by binding at the site normally occupied by ATP (the enzymes' phosphate donor) and one may speculate that the enhanced selectivity exhibited by the former is a result of the tilting of their two indole rings relative to the plane of the maleimide group. It is reasonable to think that both **1** and **2** bind PKC with their heterocyclic frameworks inserted within a lipophilic cleft common to most protein kinases.[7] And that cleft may be larger in the case of PKC than it is in most other kinases. The flatter staurosporine chromophore could thus enter a large number of kinase ATP sites while the more sterically encumbered Roche compounds would find themselves excluded from all but a few. With these considerations in mind we set out to design molecules which possessed those structural features known to be important for the activity of staurosporine-like PKC inhibitors (e.g. lactam or imide NH) and which also extended themselves three dimensionally in the same manner as **2**. We investigated the effect of relocating one of the aryl substituents of an inhibitor such as **3** to the C-2

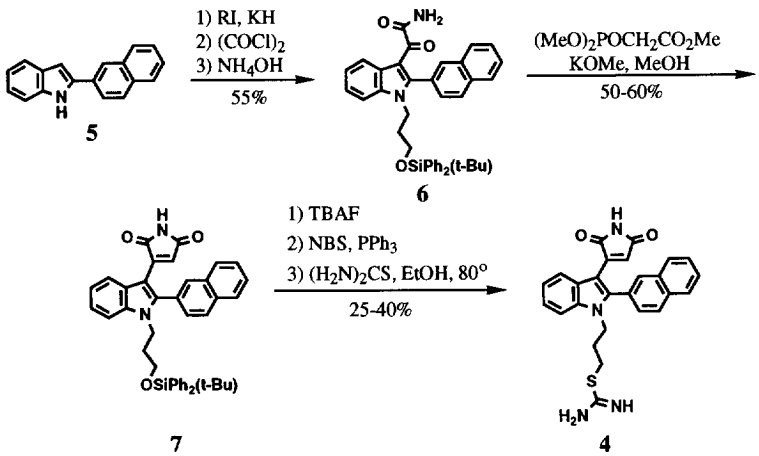
position of the indole ring since modelling had suggested this might give rise to compounds meeting our design criteria. We were pleased to find that **4** was an excellent inhibitor of PKC-ß.[8] In fact, the inhibitor **3** had lost no potency whatsoever as a consequence of this structural modification.



3 (IC_{50} = 10nM) **4** (IC_{50} = 10nM)

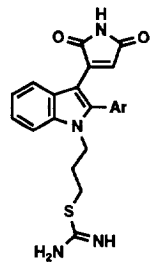
The chemistry used in the synthesis of **4** is outlined below (scheme 1). The starting indoles (e.g. **5**), in most cases known compounds, were prepared from the phenylhydrazones of the appropriate methyl ketones by heating in the presence of $ZnCl_2$. After alkylation of the indole nitrogen and conversion to the glyoxalamide **6**, the maleimide group was assembled in one step using the potassium anion of trimethyl phosphonoacetate in MeOH.[9] Installation of the isothiouronium moiety was accomplished in a straightforward manner.

Scheme 1



5 **6** **7** **4**

Methylation of the imide nitrogen (KH, MeI on **7**) provided an inactive compound and substitution of the propyl connector with a four carbon spacer between the heterocycle and the isothiouronium cation resulted in a compound having ca. 10% the potency of **4** (data not shown). A hydrogen bond donor, such as the isothiouronium moiety, was required for acceptable potency. Also, the corresponding succinamides were devoid of activity. In our early work we elected to leave the isothiouronium moiety and its connector unchanged and to make variations in the C-2 substituent. Several of the analogues prepared to define the SAR at this site are shown in the accompanying table.



4 : Ar = (IC_{50} = 10nM) **8d** : Ar = (IC_{50} = 3nM)

8a : Ar = (IC_{50} = 100nM) **8e** : Ar = (IC_{50} = 20nM)

8b : Ar = (IC_{50} = 80nM) **8f**: Ar = (IC_{50} >> 1μM)

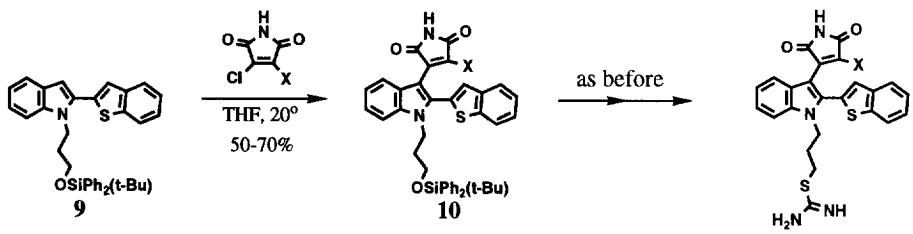
8c : Ar = H (IC_{50} >> 1μM) **8g** : Ar = (IC_{50} = 1000nM)

It is apparent that the geometry of the ATP binding site imposes strict requirements upon the C-2 substituent. That substituent can be neither too broad (**8b**), too thick (**8f**), nor too elongated (**8g**). It is, however, necessary to fill the space occupied by the naphthyl group of **4** since both **8a** and **8c** displayed little activity. Of the numerous C-2 substituents we examined[10] the benzothiophene ring provided the most potent analogue. This compound (**8d**) was chosen for more thorough biochemical evaluation.

Compound **8d** showed selectivity in favor of PKCß over protein kinase A (IC_{50} = 500nM) and the tyrosine kinase p60src (inactive). It did, however, significantly inhibit calmodulin dependent protein kinase (IC_{50} = 10nM). This latter result contrasts with the relative inactivity (IC_{50} = 17μM) reported by the Roche group for Ro 31-8220 towards this enzyme. Compound **8d** also inhibited PKCε (IC_{50} = 10nM) suggesting a lack of isoform selectivity.[11] Although the electron donating character of the indole ring would be expected to render our maleimides fairly unreactive, we were still concerned that **8d** might be inhibiting PKC through covalent modification (alkylation) of the enzyme. A time-course study utilizing data obtained at intervals of from a few minutes to 6h demonstrated this not to be the case; the compound is not a time-dependent PKC inhibitor.

To further explore the SAR within this family of molecules we sought access to compounds substituted at the 4-position of the maleimide ring. We found that reaction of indole **9** with 3-chloro-4-cyano-maleimide[12,13], or with either of its acetyl or benzoyl analogues[14], led smoothly to the substituted systems **10** (scheme 2).

Scheme 2

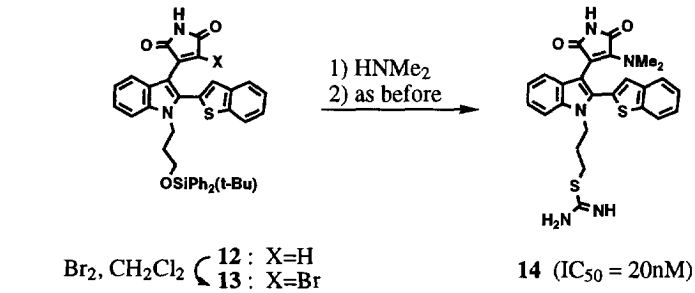


11a : X=CN (IC_{50} = 2nM)
11b : X=$COCH_3$ (IC_{50} = 300nM)
11c : X=COPh (inactive)

The first compound in this series (**11a**) was, if anything, a bit more potent than **8d** itself. Disappointingly, the other two were virtually without activity; in all likelihood these compounds exceed

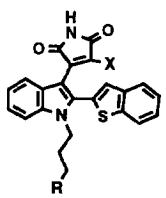
the steric tolerance of the enzyme's active site. (A compound of structure **11** having X = Ph was prepared using the Roche maleimide synthesis.[15] It too was inactive.) It also proved possible to obtain maleimides substituted with electron donating groups. The synthesis of this class of compounds is outlined in scheme 3.

Scheme 3



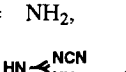
Treatment of an unsubstituted maleimide such as **12** with bromine (1eq) at room temperature led almost instantly to its corresponding bromide **13**. It proved possible to trap this rather labile intermediate in 50-75% yield using primary and secondary amines as nucleophiles. Unfortunately, the reaction of NH_3 and alkoxide nucleophiles with **13** failed to afford the desired products. The dimethylaminomaleimide shown above possessed relatively good activity. Indeed, the electronic character of the maleimide moiety seems unimportant in determining potency as **11a**, **8d**, and **14** are all active inhibitors. Steric factors seem to play a much more important role (recall the poor activity of **11b** and **11c**) and may be responsible for the somewhat reduced potency of **14** compared with its unsubstituted and cyanomaleimide counterparts.

We were also interested in determining what sorts of replacements for the isothiouronium cation would be tolerated since we were not very optimistic regarding the prospect of these salts as drugs. The following table summarizes a few of our results:

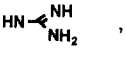


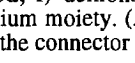
15a : R = NMe_2, X = H (IC_{50} = 100nM)

15b : R = OOCNHMe, X = CN (inactive)

15c : R =  , X = H (IC_{50} = 30nM)

15d : R = NH_2, X = CN (IC_{50} = 10nM)

15e : R =  , X = CN (IC_{50} = 33nM)

15f : R =  , X = CN (IC_{50} = 20nM)

Although several compounds (e.g. **15d, f**) demonstrated respectable potency, no superior replacement could be found for the isothiouronium moiety. (A similar problem was encountered by the Roche workers who overcome it by rigidifying the connector that linked their heterocycle to the cationic group.[6d])

We undertook a study of the *in vivo* efficacy of our most potent compounds, **8d** and **11a**. Two CHO (Chinese hamster ovary) cell lines were constructed one of which overexpressed PKCß and the other of which overexpressed PKCε. It was found that both cell lines gave rise to aggressive tumors when injected i.p. into previously irradiated nude mice.[16] The cell line from which the transformants were derived was non-tumorigenic in the same mice. Compound **8d**, given once daily (i.p., 10mg/kg), extended the median survival time of animals bearing PKCß-overexpressing tumors from 18 days to 24

days. The median survival time of animals bearing PKCε-overexpressing tumors was increased from 14 days to 21 days. Animals injected s.c. with human small-cell lung carcinoma H82 developed tumors whose diameter could be measured easily. Compound **8d**, given as above, slowed the growth of these tumors by ca. 50% in experiments running 40 days. These results were statistically significant. Interestingly, **11a** showed no *in vivo* activity.

Since it is unknown which (if any) human cell lines owe their malignant phenotype to PKC overexpression or overactivity we sent samples of **8d**, **15a**, and **11a** to the National Cancer Institute for screening against their 60 tumor cell line panel.[17] The inactive compound **8c** (which contains both the maleimide and isothiouronium moieties) was submitted as a negative control. Compound **8d** was the most potent with an average GI_{50} of 800nM against the panel. It showed the greatest activity against breast tumor lines as a group (e.g. GI_{50}<10nM against MDA-N). Although less potent (average GI_{50}=2μM), **15a** displayed a pattern of selectivity similar to that of **8d**. The adriamycin-resistant breast tumor line MCF-7/ADR-RES was likewise resistant to **8d** and **15a** (GI_{50}=20μM vs. 100nM against MCF-7 for **8d**). The negative control, **8c**, had little potency (average GI_{50}=20μM) and displayed no selectivity between the various cell lines. The same was true for **11a** which is surprising in light of its potency in the enzyme assay. At this time we can only speculate that **11a** has difficulty entering cells or that it is destroyed by reaction with intracellular nucleophiles once it does enter.

In conclusion, we have shown that a new class of PKC inhibitors results from transposition of the aryl substituent in Roche-type inhibitors such as **3** to C-2 of their indole ring. These inhibitors possess, in some cases, excellent potency towards, and reasonable selectivity for, PKC. Moreover, they have demonstrated anti-cancer activity in several *in vitro* and *in vivo* models. It is to be hoped that compounds such as **8d** will prove useful as research tools in unravelling the biology of PKC and perhaps also as drugs for the management of human disease.

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8. Assay procedure: The reaction mixture contained 25mM Tris-HCl, pH 7.5, 2.5mM $Mg(NO_3)$, 1.0mM EGTA, 20 μM peptide substrate, 1 μg/mL phosphatidylserine (PS), 5 x 10^{-6} M diacylglycerol (di-C8), 50 μM ATP spiked with γ-^{32}P ATP (>5,000 Ci/mmol) to provide approximately 10^6 CPM per reaction and 0.08 μg/mL PKC in a 50 μl volume. The assay was run with or without test compounds according to protocol. After a five minute incubation at room temperature, the reaction was stopped by the addition of 0.2V of a 50% TCA solution. 30 μl of sample is then spotted onto Whatman P-81 ion exchange chromatography paper. The ^{32}P incorporated was then measured using a scintillation counter.
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16. We thank John Knopf and Lisa A. Sultzmann of Genetics Institute for the engineered CHO cell lines.
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