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DESIGN AND SYNTHESIS OF NEW LEADS FOR PKC BISUBSTRATE INHIBITORS.

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Abstract : In order to obtain selective protein kinase C (PKC) inhibitors competitive toward ATP, an efficient synthetic process of a series of monoindolylmaleimide derivatives was achieved. As expected from the PKC/PKA homology, the PKC selectivity was promoted by addition of amine chains on the maleimide ring. The most active derivative could be used as starting molecule for the preparation of specific PKC isotype inhibitors according to the bisubstrate concept.

The mammalian protein kinase C (PKC) family consists of twelve related isotypes that play a pivotal role in the signal transduction pathways of a variety of hormones, cytokines, neurotransmitters and growth factors[1-5]. Recently, potentially different functions of PKC isotypes in proliferation and differentiation of various cell types have been addressed[6]. Therefore, specific PKC isotype inhibitors could be very useful tools in the understanding of the different PKC dependent cellular processes and in the search for drugs in diseases such as human cancers, disorders of the immune system and other metabolic systems.

All PKC isotypes display the same structural organization consisting in two domains : a regulatory domain and a catalytic domain which possesses two sites binding respectively ATP and peptidic substrates. Blocking of ATP or peptidic substrate site is a means to inhibit PKC. Until now, most of described inhibitors interacting with the catalytic domain were ATP-competitive compounds, including isoquinoline sulfonamides[7] and microbial metabolites possessing an indolocarbazole unit as staurosporine[8] and K252 a,b[9]. Unfortunately, these compounds show little selectivity for PKC over other kinases. In the search for selective and potent PKC inhibitors, several groups have chosen staurosporine as a structural lead to derive a series of bisindolylmaleimides with a significantly improved selectivity for PKC[10-13]. Following a bisubstrate approach, we have previously reported[14] the design of PKC inhibitors suitable to interact simultaneously with the ATP and the protein substrate binding domains. In our initial design, we had tried to achieve PKC specificity using recognition rules[15-19] at the peptidic substrate level as at this time no PKC specific ligand for the ATP binding site was known.

Although very potent inhibitors were obtained, their lack of specificity could be attributed to the use of a non specific isoquinoline or naphtalenesulfonamide derivative as ATP mimic. Therefore our efforts have been focused on the synthesis of a more specific PKC ATP mimic which could be linked to a peptidic sequence. Eventually, this peptidic sequence might be chosen in the pseudosubstrate[20-21] region of the different PKC isotypes in order to lead to an isotypic specificity.

As no structural data was available for PKC, we used the crystal structure[22] of the catalytic domain of c-AMP dependent protein kinase (PKA)[23-25] recently accessible from the Protein Data Bank and the high sequence homology (40 %) between the PKA and PKC catalytic domains to extrapolate a model of the catalytic domain of PKC. According to the PKA-PKC sequence alignment, a major difference was observed in a region close to the ATP binding site and to the C-terminal part of the peptide inhibitor PKI(5-24) bound to PKA : the

amino acids leucine 82, lysine 83 and glutamine 84 of PKA are replaced by three adjacent negatively charged aspartic acids in PKC (Figure 1).

Taking advantage of this information, we describe in this paper an efficient synthetic route to a series of monoindolylmaleimides possessing different amine chains on the maleimide ring. These functions were introduced in order to interact with the previously described acidic region and to allow the extension with a peptidic sequence according to the bisubstrate concept (figure 2).

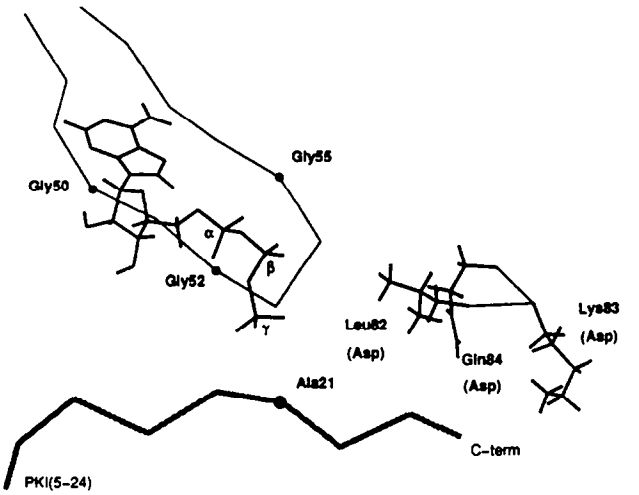


Figure 1. Schematic representation of the PKA catalytic site according to the crystal structure of the ternary complex PKA-PKI(5-24)-ATP[22] showing : the ATP molecule in its glycine-rich binding site, the C-terminal region of the peptide inhibitor PKI(5-24) and the three amino acids Leu 82, Lys 83 and Gln 84 replaced in PKC by three aspartic acids (in brackets).

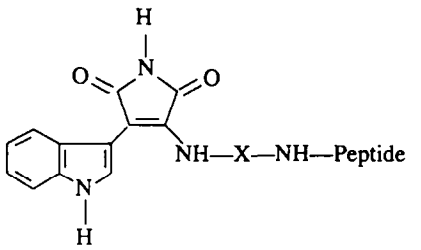
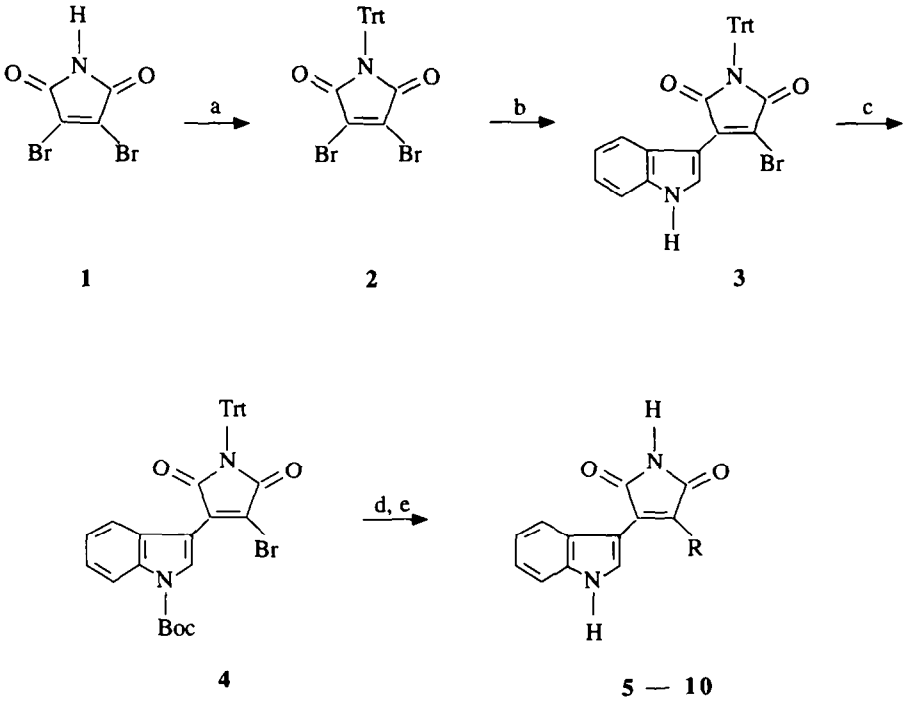


Figure 2.

The monoindolylmaleimide derivatives **5-10**[35] were synthesized as shown in Scheme 1 : addition of indolylmagnesium bromide to a 2,3-dibromo-N-protected maleimide, protection of the indolic nitrogen and finally substitution of the bromine atom by an amine. Protecting groups on the maleimidylimine and the indolic nitrogen were used to avoid side reactions. Although various protecting groups have been described in the literature for the protection of the imino function including 4-methoxybenzyl, anisyl, allyl, benzyloxymethyl, 1-oxy-2-picolyl, benzyl or methyl[26], the use of these different N-protecting groups was not possible in the synthesis of staurosporinone, the imino analogue of staurosporine[26].

Besides, while N-methylation was successfully employed in the synthesis of arcyriarubin B structurally related to the aglycon of staurosporine[27] and GF 109203X another bisindolylmaleimide PKC inhibitor[13], in our series the final removal of the methyl protecting group employing the usual conditions (i : refluxed aqueous or ethanolic KOH. ii : HCl 2N and ammonium acetate, 140°C) did not yield the desired amines.

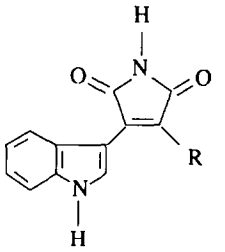


Scheme 1. a) tritylCl, TEA, $CHCl_3$, O°C ; b) indolylMgBr, toluene-THF, 50°C ; c) Boc_2O, DMAP, THF, O°C ; d) amine, CH_2Cl_2, room temperature ; e) pure TFA.

This led us to investigate for the imino protection, the use of acidic labile groups which would offer the advantage of allowing a single final deprotection step[28]. Tritylchloride $(C_6H_5)_3C$-Cl and its analogues 4-methoxytrityl and 4,4'-dimethoxytrityl were thus introduced on 2,3-dibromomaleimide **1**[34] in the presence of triethylamine. Only the first reagent gave a N-protected product **2** stable enough to allow an easy purification with a good yield. Compound **3** obtained by reaction of **2** with indolylmagnesium bromide was converted into its N-tert-butyloxycarbonyl derivative **4** by treatment with di-tert-butyl dicarbonate in the presence of 4-(dimethylamino)pyridine[29]. The nucleophilic substitution of **4** by different primary diamines or polyamines taken in excess was performed in dichloromethane. The different products of substitution were purified by thick layer chromatography, deprotected by TFA and then precipitated by dropwise addition of the TFA solution into diethyl ether. The inhibitory capacities of compounds **5-10** towards PKC (β isoform) and PKA were tested using

histones III S and histones II A respectively as substrates. IC_{50} determinations for the two protein kinases are given in Table 1, along with the non-specific inhibitor H-7[7] and GF 109203X[13] for comparison.

Table 1. Inhibitory potencies for compounds **5-16**[35, 36]



compounds	R	PKC IC_{50} (μM)	PKA IC_{50} (μM)
5	NH-$(CH_2)_2$-NH_2	90	100
6	NH-$(CH_2)_5$-NH_2	8	300
7	NH-$(CH_2)_6$-NH_2	40	50
8	NH-$(CH_2)_7$-NH_2	5	150
9	NH-$(CH_2)_2$-NH-$(CH_2)_2$-NH_2	120	310
10	NH-$(CH_2)_3$-NH-$(CH_2)_3$-NH_2	100	390
11	NH-$(CH_2)_5$-NH-Arg	60	no inh.
12	NH-$(CH_2)_5$-NH-Phe	120	no inh.
13	NH-$(CH_2)_5$-NH-Glu	105	390
14	NH-$(CH_2)_5$-NH-$(CH_2)_2$-NH_2	110	> 500
15	NH-$(CH_2)_5$-NH-$(CH_2)_2$-NH-Arg	8	210
16	NH-$(CH_2)_5$-NH-$(CH_2)_2$-NH-Phe	95	120
GF 109203X		0,01	2
H-7		6	3

With the exception of **5** and **7**, the different amines were selective toward PKC versus PKA. 1,5 diaminopentyl **6** and 1,7 diaminoheptyl **8** derivatives showed the best inhibitory potencies in the micromolar range while compounds **9-10** with an alkyl chain including three amino groups were less active.

In order to investigate its mechanism of PKC inhibition, the most selective compound **6** was tested for its competitive effect toward ATP and toward histones IIIS. The results clearly indicated that this compound was competitive toward ATP with a K_i value of 10 µM and not competitive toward histones. As the PKC and PKA ATP binding sites are very similar, PKC specificity can be due as expected to the interaction between its terminal amine group and the acidic region found only in PKC.

To the suitable ATP mimic **6**, we decided to couple the first amino acid which would serve as a cornerstone for the subsequent addition of a peptidic sequence. Arginine, phenylalanine and glutamic acid were thus chosen for their different polarities and linked to the protected form of **6** either directly or after elongation with an aminoethylene group to preserve the terminal basicity. In the last case, the protected form of **6** was first treated with 2-bromoethylamine to yield the protected form of **14**. Addition of arginine, phenylalanine or glutamic acid was attempted by the DCC/HOBt method with respectively Fmoc-Arg(Pmc)-OH[33], Boc-Phe-OH[33] and Fmoc-Glu(OtBu)-OH[33]. Whatever the conditions, the protected form of **14** did not react with the glutamic derivative.

The resulting protected derivatives were purified by thick layer chromatography and submitted to the final TFA deprotection to give **11-13** and **15-16**. The Fmoc protecting group of glutamic acid and arginine derivatives required a cleavage step with piperidine 20 % in DMF before the final acid deprotection. In the case of the arginine derivatives the transfer of the Pmc group to the indolic nitrogen was observed as described in peptide solid phase synthesis[30] but could be avoided by the use of ethanedithiol and thioanisole as scavengers. Simultaneous removal of the protecting groups Boc and Pmc was thus achieved by treatment with TFA (85 %), water (5 %), ethanedithiol (5 %) and thioanisole (5 %). The trityl protecting group proved to be resistant and required a final treatment with pure TFA to be completely removed as checked by mass spectrometry or ^1H NMR. Each deprotected product was finally precipitated by dropwise addition of the TFA solution into diethyl ether.

As shown in Table 1, acylation of **6** with any of the three amino acids leads to less active compounds **11, 12, 13** demonstrating the importance of the terminal primary amine for recognition by PKC. When **6** was first elongated with an aminoethylene group, acylation with phenylalanine yields a less active and non selective compound **16** ; on the contrary, acylation with arginine whose side chain is known to be involved in the recognition of protein substrates by PKC leads to the potent and selective inhibitor **15** (IC_{50} : 8 µM for PKC versus 210 µM for PKA).

In conclusion, we have developed a convenient synthesis of a series of monoindolylmaleimide derivatives functionalized with various amine chains ; an original maleimide protection by the trityl group has been achieved. Compound **6** is the most PKC selective inhibitor and interacts with the ATP binding site. Compound **15** which includes an additional aminoethylene chain and an arginine residue proves to be the best derivative. It can be a good lead for the design of specific PKC isotype inhibitors in a bisubstrate approach ; additional amino acids should be added in order to increase the interaction with the peptidic recognition site.

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33. Abbreviations : Boc, tert-butyloxycarbonyl; Boc_2O, di-tert-butyl dicarbonate; DCC, dicyclohexylcarbodiimide; DMAP, 4-(dimethylamino)pyridine; Fmoc, fluorenylmethoxycarbonyl; HOBt, 1-hydroxybenzotriazole; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; TEA, triethylamine; TFA, trifluoroacetic acid; THF, tetrahydrofuran.
34. 3,4 dibromo pyrrole 2,5 dione **1** was synthesized as previously described[31-32].
35. All new compounds gave ^1H NMR and MS consistent with their structure and elemental analytical data were satisfactory for all protected derivatives.
36. For a detailed description of PKA-PKC inhibition tests see reference 14.
Molecular modeling was performed using the Insight II module of the BIOSYM software (BIOSYM Technologies, San Diego) implemented on a INDIGO R4000 Silicon Graphics workstation.

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