extracted with ether/hexane (v/v = 1/1). The combined organic layers were washed several times with water, dried over anhydrous sodium sulfate, and concentrated in vacuo. The residue was chromatographed (hexane) to obtain **5r** (1.03 g, 54%) as a colorless oil: NMR δ 7.92 (d, J = 1 Hz, 1 H), 7.72-7.94 (m, 1 H), 7.43 (t, J = 7.5 Hz, 1 H), 7.14-7.32 (m, 1 H), 2.59 (s, 3 H).

3-Fluoro-7-methylbenzo[*b*]thiophene (5u). 3-Bromo-7-methylbenzo[*b*]thiophene (**5p**; 9.05 g, 40 mmol) was dissolved in dry ether (35 mL), and *n*-BuLi (25 mL, 40 mmol, 1.6 M solution in hexane) was added under argon at -78 °C. After stirring for 20 min at this temperature the mixture was charged slowly with perchloryl fluoride (**Caution! This reagent is extremely hazardous.**[13]) (4.5 g, 44 mmol) and the temperature was maintained below -60 °C. After an additional 30 min at -78 °C, the temperature was allowed to rise to 0 °C during 1 h. The reaction mixture was quenched with water, the separated organic layer was dried over magnesium sulfate, and the solvent was removed in vacuo. The crude product was chromatographed (hexane) to give **5u** (4.6 g, 35%) as a colorless oil.

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Design of Potent Protein Kinase Inhibitors Using the Bisubstrate Approach

A. Ricouart, J. C. Gesquiere, A. Tartar, and C. Sergheraert*

Faculté de Pharmacie de Lille, URA CNRS 1309, Institut Pasteur de Lille, rue Calmette, 59019 Lille, France.
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A new class of serine/threonine protein kinase inhibitors was designed by associating, in the same structure, mimics of both the ATP binding site and a protein substrate. Among the several potent antagonists which were obtained, the most active consists of isoquinoline-5-sulfonamide, as ATP mimic, and Ser-Arg_6, as peptidic moiety, bound by a $-NH(CH_2)_2NH(CH_2)_2CO-$ linker. This compound, with a K_i of 0.1 μM toward protein kinase C (PKC) and 0.004 μM toward cyclic AMP dependent protein kinase (PKA), is respectively 60- and 750-fold more active than the commercial inhibitor H-7.

The physiological importance of protein kinase C (PKC) activation is widely appreciated and well-documented; a number of recent reviews has covered various aspects of the enzymology of this kinase and its role as a signal-transducing protein in a plethora of physiological responses.[1-5] Several discrete subspecies have been defined: all these proteins, derived from multiple genes or from alternative splicing, have in common the presence of a regulatory and of a catalytic domain.

Activators of PKC such as phospholipids or diacylglycerol interact with the regulatory domain, while both ATP and the protein substrate interact with the catalytic domain.[6,7] The different inhibitors or activators of PKC reported to date are known to interact with a single of these four binding sites. We wish to report in this paper our efforts to design new PKC inhibitors able to interact simultaneously with two of these sites according to the bisubstrate concept. In this approach, the combination of two substrates required by the enzyme to form a single molecule allows the advantage of not only at least partially additive binding energies but also a significant entropic contribution.

We have focused our efforts on the two entities which are known to interact with the catalytic domain: the peptidic substrate and ATP.

In order to prepare such inhibitors, it is necessary not only to design structures which are suitable to interact with both binding sites but also to covalently link these two moieties with a spacer which could reproduce the distance between the binding sites in the enzyme structure.

(i) **Substrate Mimics.** The substrate specificity of PKC has been studied by many groups using, in general, a series of synthetic peptides.[8-12] These studies have shown the importance of basic residues close to the phosphorylated serine (or threonine) either on its C or N terminus side or both. We have therefore decided to use a cluster of arginine residues (Arg_4 or Arg_6) to interact with the peptidic recognition site. A second question was whether a phosphorylable residue should or should not be included. In favor of the former is the fact that when serine is substituted for alanine in a high-affinity substrate, the corresponding peptide obtained, which is devoid of phosphorylable residue, proves to be a poor inhibitor.[13] On the basis that a high-affinity substrate can become an inhibitor, provided the simultaneous binding of an ATP mimic prevents its phosphorylation, a serine residue was added in some analogues to the cluster of arginines.

(ii) **ATP Mimics.** No X-ray structural data are available for protein kinases; however, the crystal structure of phosphoglycerate kinase with bound ATP has been determined by Bryant et al.[14] This structure shows the presence of many hydrophobic side chains surrounding the adenine ring of ATP. The presence of such an hydro-

* To whom correspondance should be addressed.

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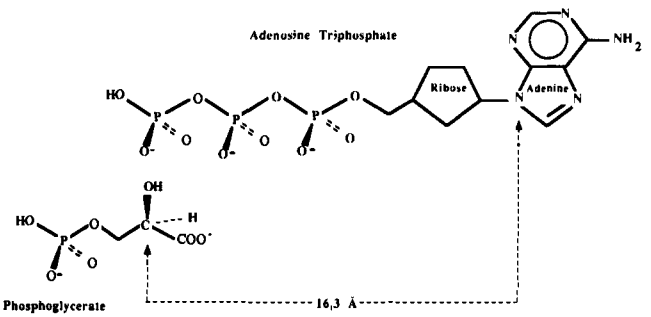


Figure 1. Relative substrates disposition in the active site of phosphoglycerate kinase from X-ray diffraction data.

phobic pocket, if common to other kinases, could account for the fact that hydrophobic bicyclic molecules even distantly related to ATP can act as potent inhibitors. Thus naphthalenesulfonamides inhibit calmodulin-dependent protein kinase while isoquinolinesulfonamide derivatives, among which is H-7,[7] are potent inhibitors of PKC and cyclic nucleotide dependent protein kinases. Kinetic studies indicate that these inhibitors compete with ATP for a free enzyme. We have thus used [(dimethylamino)naphthalenyl]sulfonyl (dansyl) and 5-isoquinolinylsulfonyl groups as competitors with the ATP binding site of PKC.

(iii) Design of a Spacer. The last problem which remained to be solved was the distance between the two moieties. We made the assumption that the data obtained with phosphoglycerate kinase could be extended to protein kinases; using this model, it was possible to determine a 16.3-Å distance between the carbon atom bearing the phosphorylated hydroxyl and the N-9 nitrogen atom of adenine, which is linked to the ribose moiety (Figure 1). A flexible linker consisting of two β-alanine residues was thus used between the two moieties. Molecular modeling of this linker shows that, in an extended conformation, it allows a proper spacing between the two moieties, corresponding to the distance determined previously. To test this hypothesis, a larger linker consisting of four β-alanine residues was also used in one case.

Chemistry

5-Isoquinolinesulfonic acid was synthesized as previously described.[16] It was reacted with phosphorous pentachloride or thionyl chloride to yield 5-isoquinolinesulfonyl chloride.[17,18] Peptides were synthesized by using the Boc/TFA/HF solid-phase methodology. After completion of the peptide chain, two (or four) Boc-β-alanines were sequentially coupled. After deprotection of the last Boc β-alanine group, 5-isoquinolinesulfonyl chloride or dansyl chloride was coupled (compounds **1–6**, **8**, Table I). In each case, compounds proved to be resistant to the treatment by anhydrous HF used to deprotect and to cleave the peptide from the resin.

Compounds **9–17** were obtained in several steps (Scheme I). Compound **9** was obtained by coupling 5-isoquinolinesulfonyl chloride with β-alanine methyl ester. Compounds **11** and **12** were respectively synthesized by the addition of methyl 3-bromopropionate or methyl 5-bromovalerate on 1-(5-isoquinolinylsulfonyl)piperazine (**10**). Addition of methyl 3-bromopropionate on 5-isoquinoline sulfonylethylenediamine **13** gave **14**. The carboxylic acid, obtained after hydrolysis of **14** was coupled to the heptapeptide Ser-Arg_6 still linked to the polymer.

After HF cleavage and deprotection, purification of the peptides was achieved by gel filtration and preparative HPLC. All compounds were controlled by analytical HPLC and FAB-mass spectrometry.

Table I. Structure of Compounds 1–8

ATP mimic–linker–substrate mimic

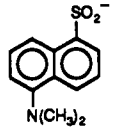
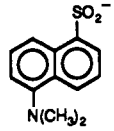
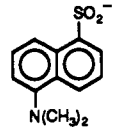
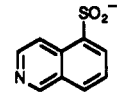
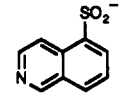
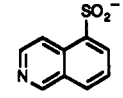
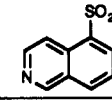
no.	ATP mimic	linker	substrate mimic
1		β-Ala_2	Ala-Arg_4
2		β-Ala_2	Ser-Arg_4
3		β-Ala_4	Ser-Arg_4
4		β-Ala_2	Ala-Arg_4
5		β-Ala_2	Ser-Arg_4
6		β-Ala_4	Ser-Arg_4
7		β-Ala	Ser-Arg_6
8		β-Ala_2	Ser-Arg_6

Table II. Inhibition Potencies for Compounds 1–17 Compared to That of H-7

	IC_{50},[a] μM		K_i,[a] μM	
no.	PKC	PKA	PKC	PKA
1	>75	>75		
2	>75	>75		
3	>75	>75		
4	48	1.2		
5	48	1.2		
6	>75	1.2		
7	very weak inh	no inh		
8	7	0.3	8	0.170
9	no inh	no inh		
10	12	6		
11	no inh	no inh		
12	no inh	no inh		
13	7	2		
14	20	2		
15	no inh	no inh		
16	no inh	no inh		
17	0.3	0.003	0.1	0.004
H-7	6	3.5	6	3

[a] Each reported value represents the means of at least three separate experiments.

Results and Discussion

The inhibitory capacities of compounds (Table II) toward PKC- and cAMP-dependent protein kinase (PKA) were tested with histones H1 III_S and II_A, respectively, as

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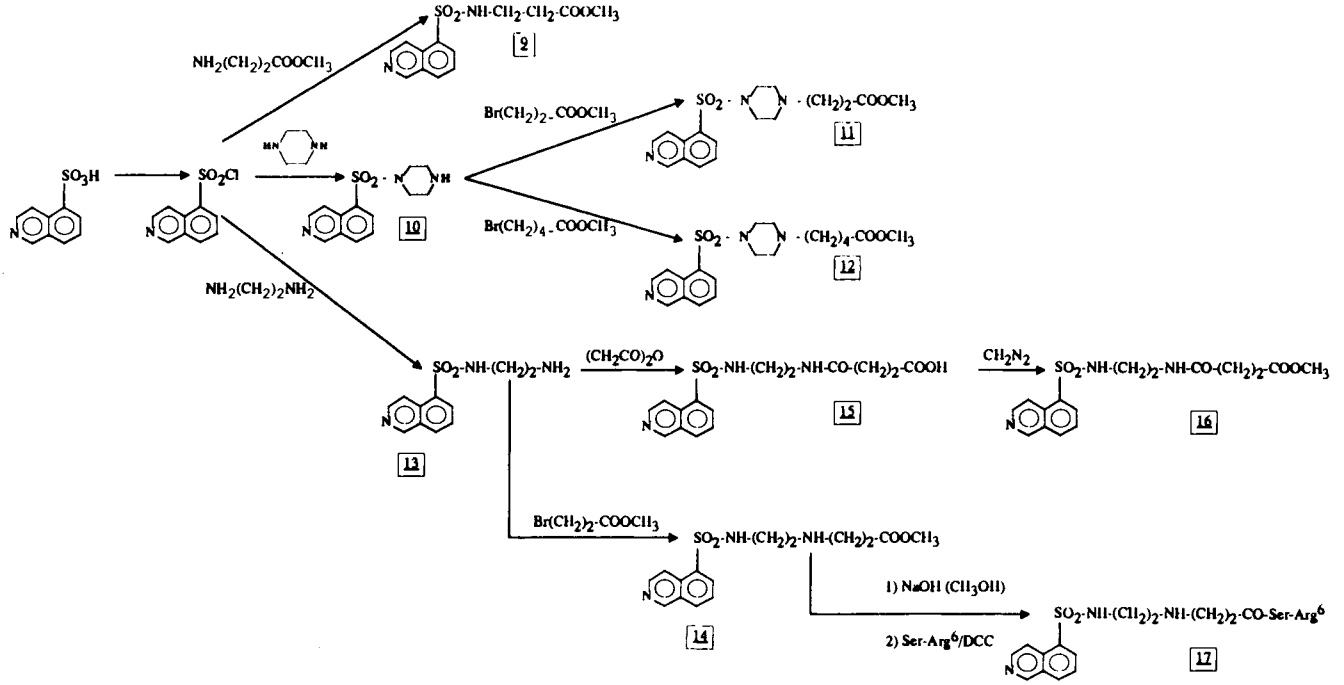
Scheme I



substrates. In this procedure, Histones are precipitated at the end of the phosphorylating reaction with trichloroacetic acid and bovine serum albumin as a carrier. Under these conditions, small molecules remain soluble so that their possible phosphorylation is not detected. IC_{50} values are compared to those obtained by Hidaka et al.[7] for the highly potent PKC and PKA inhibitor 2-(5-isoquinolinylsulfonyl)methyl]piperazine (H-7).

A first series of derivatives (1–6, Table I) was prepared to allow comparison of the efficiency of dansyl versus isoquinoline derivatives.

IC_{50}, determined both for PKA and PKC, are shown in Table II. Compound 1 is a weak inhibitor of both PKA and PKC; addition of a phosphorylable serine residue to the arginine cluster in 2 or increasing the length of linker to four β-alanine groups in 3 does not modify the inhibition potency. On the contrary, isoquinolinylsulfonyl derivatives exhibited moderate PKC inhibition capacities but were able to inhibit PKA in the micromolar range. In the isoquinoline series, no effect was detected following the replacement of Ala by Ser. Interestingly, increasing the distance between the two moieties by incorporating two additional β-alanine residues to the linker led to a decrease in the inhibiting capacity of 6 toward PKC.

Having established the superiority of isoquinoline derivatives, a first attempt was made to increase the inhibiting capacity of 5 by increasing the length of the peptide moiety in compound 8 from four to six arginine residues.

This modification led to an important decrease in IC_{50} both for PKC (7 μM) and PKA (0.3 μM). Compound 8 is thus almost as active as H-7 toward PKC but 10 times more active than H-7 toward PKA. As a control, the two separate components of 8 (i.e.: 7 and 9) were prepared and tested separately: 9 proved to be totally devoid of activity while 7 had only a very weak inhibiting capacity toward PKC (Table II). Even when used as a mixture, 7 and 9 proved to be almost inactive, thus indicating that the covalent combination of both moieties in a same molecule is necessary to achieve a high inhibiting capacity.

Another important observation was that 9, corresponding to the ATP-interacting moiety in 8, was devoid of activity on its own. This result was not surprising as compounds such as H-7 need an additional amino group in the sulfonamide side chain to be active.

In order to improve the efficiency of inhibitor 8, it was thus important to design an isoquinoline moiety incorporating the necessary features to be active by itself as an ATP mimic and to preserve this activity when covalently bound to the peptidic structure. It was also important that the length of the spacer be compatible with the distance between the binding sites.

We have thus prepared several structures shown in Scheme I. On the basis of the observations of Hidaka et al.[7], that the inhibitory potencies of isoquinoline sulfonamide analogues depend on the presence of an additional amino group on the sulfonamide, we prepared several new derivatives of the previously described 1-(5-isoquinolinyl sulfonyl)piperazine (10)[17] to inhibit PKC and PKA through their interaction with the active site.

In order to allow the coupling with the amino group of the peptidic chain, an alkyl chain consisting of two or four methylene groups bearing a carboxylic function was added to the piperazine amino group, respectively, leading to 11 and 12. When tested as methyl esters, both compounds 11 and 12 proved to be totally inactive. This result was unexpected as the basicity of the terminal nitrogen, known to be an essential factor influencing activity in the H-7 series, was preserved. This led us to suspect that the presence of hydrogen atom on the amino group was also necessary to maintain good inhibiting capacity.

A second series of derivatives based on 5-isoquinoline sulfonylethylenediamine 13, another known PKA and PKC inhibitor,[7] was thus prepared. Consistent with our hypothesis requiring the presence of a secondary amine, compound 14 proved to inhibit both PKC and PKA, although it was 10-fold more active toward the latter. Compounds 15 and 16 were inactive, indicating that the terminal nitrogen must not only bear a hydrogen atom but also remain basic.

When compound 14 was coupled to $Ser\text{-}Arg_6$, which had been previously shown as being the best substrate mimic, compound 17 was obtained, which proved to be a highly potent inhibitor of PKC and an even more potent inhibitor of PKA (Table II).

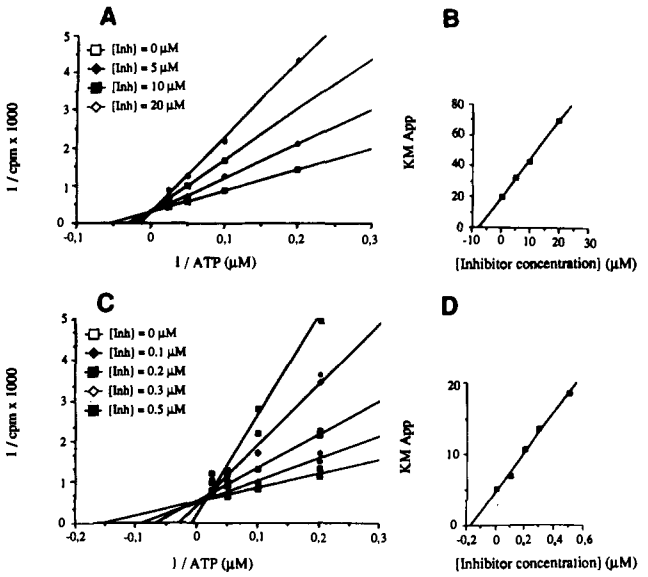


Figure 2. (A) Bisubstrate 8/ATP competition, Lineweaver–Burk plot for PKC; (B) K_i value for PKC deduced from the secondary plot, apparent K_m versus inhibitor concentration; (C) bisubstrate 8/ATP competition, Lineweaver–Burk plot for PKA; (D) K_i value for PKA deduced from the secondary plot, apparent K_m versus inhibitor concentration.

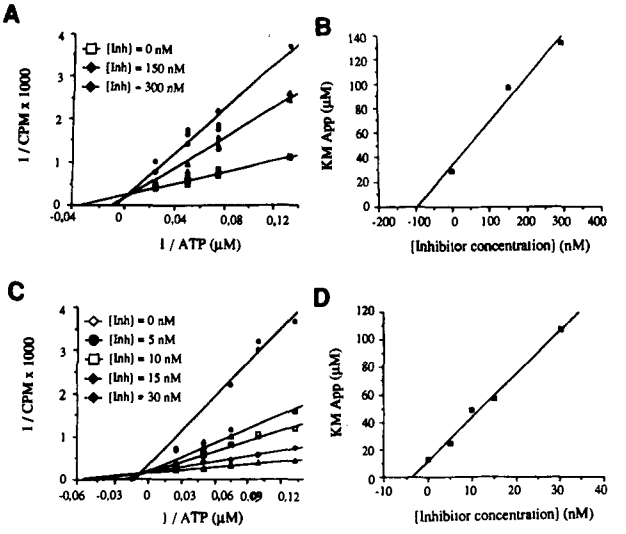


Figure 3. (A) Bisubstrate 17/ATP competition, Lineweaver–Burk plot for PKC; (B) K_i value for PKC deduced from the secondary plot, apparent K_m versus inhibitor concentration; (C) bisubstrate 17/ATP competition, Lineweaver–Burk plot for PKA; (D) K_i value for PKA deduced from the secondary plot, apparent K_m versus inhibitor concentration.

In order to validate the bisubstrate mechanism of inhibition, 8 and 17 were tested for their competitive effect toward ATP and peptidic substrates (glycogen synthase (GS) 1-12 for PKC,[15] kemptide for PKA). These studies indicated that 8 and 17 are competitive toward ATP; K_i values are, respectively, as follows: 8 and 0.1 μM for PKC (Figures 2 and 3, parts A and B) and 170 and 4 nM for PKA (Figures 2 and 3, parts C and D). However, when 8 and 17 were tested for their competing capacity toward peptidic substrates, no competitive effect could be detected even at ATP concentration as high as 50 μM. This result probably indicates an ordered mechanism of inhibition in which interaction with the ATP binding site is first required to allow the interaction of the peptidic moiety. There are two points in favor to this hypothesis: (i) the inhibition was more potent toward PKA while the cluster of basic residues is located on the C-terminus side of the phosphorylable residue and should therefore induce a PKC versus PKA specificity;[13] (ii) exchange of the serine residue in **5** for an alanine in **4** had no effect, while in the case of isolated peptides, it is generally associated with a decrease in affinity.

Conclusion

We have prepared a series of inhibitors of protein kinases which, in contrast with all inhibitors known to date, are designed to interact simultaneously with the two binding sites known to be present in the catalytic domain.

The most potent antagonist obtained is compound **17**, which is about 60 times more active than H-7 toward PKC and 750 times more active toward PKA. In this compound, the ATP mimic consists of an isoquinolinylsulfonyl group, which proved to be superior to the dansyl group.

The substrate mimic consists of a cluster of six arginine residues. The two moieties are covalently bound by a $-NH(CH_2)_2NH(CH_2)_2CO-$ linker, which reproduces the distance determined from the structure of phosphoglycerate kinase. This linker also maintains a secondary amine group in the central position, which was shown to be necessary to preserve the inhibiting potency in the isolated ATP mimic **14**.

The competitive tests suggest an ordered mechanism in which interaction with the ATP binding site is first required to allow the interaction of the peptidic mimic. We are now focusing our efforts on a better design of this peptidic moiety in order to improve the selectivity toward various protein kinases.

Experimental Section

Melting points were taken on a Gallenkamp apparatus in open capillary tubes. Elemental analyses were performed by the service de Microanalyses du CNRS (Vernaison, France). FAB ionization spectra of peptidic derivatives and isoquinoline intermediates were performed at the Université des Sciences (Lille, France): all found $(MH)^+$ values agreed with the calculated ones. Mass spectra were recorded on a four-sectors Kratos Concept II HH. The FAB ionization was obtained with a FAB field source (Ion Tech Ltd., Teddington, U.K.) operated with xenon at 8 kV and 1 mA. Cesium iodide was used for calibration. Accelerating voltage was at 6 kV. Thin-layer chromatography was carried out with Merck F254 silica gel using the solvent system EtOAc–MeOH (2:1).

The peptides were synthesized with a solid-phase peptide synthesizer (Applied Biosystems 430A). All protected amino acids were from Novabiochem. Solvents (Aldrich) were of analytical grade. Pam resin was from ABI. Amino acid protecting groups were as follows: arginine, tosyl; serine, benzyl. The removal of the peptides from the resin and simultaneous deprotection of the side chains was achieved by treatment with anhydrous HF (10 mL/g of resin-bound peptide) containing *p*-cresol (0.1 g/mL of HF) for 60 min at 0 °C. Crude peptides were lyophilized and chromatographed on Fractogel TSK HW50 (S). The purity of the final peptides was checked by HPLC (Gilson 302, Nucleosil 5-μm 100-Å C18 column, 250 × 4.6 mm), the analytical conditions were as follows: solvents, H_2O, 0.05% TFA; linear gradient, 3–50% CH_3CN (15 min) at a flow-rate of 0.7 mL/min with UV (210 nm) detection. Amino acid analyses were carried out with a Beckman 7300 analyzer after hydrolysis with 6 N HCl, at 110 °C for 24 h: all values were within ±5% of the theoretical values.

The following abbreviations have been used: Boc, *tert*-butyloxycarbonyl; TFA, trifluoroacetic acid; HF, hydrogen fluoride; DMF, dimethylformamide, HoBt, 1-hydroxybenzotriazole; DIEA, *N,N*-diisopropylethylamine; DCC, *N,N*-dicyclohexylcarbodiimide; FAB, fast atom bombardment.

Histone III_S and II_A, phosphatidylserine, and diolein were purchased from Sigma, [γ^{32}P]ATP was from Amersham, and Aqualyte reagent was from Baker. Protein kinase A was purchased from Sigma; it was the catalytic subunit prepared from bovine heart. Protein kinase C was kindly provided by Dr. T. Grand-

Perret (Glaxo Laboratories, Les Ulis, France). It was purified from bovine brain on DEAE Sepharose then on phenyl Sepharose and consisted chiefly of the β subspecies; the activity was 96% strictly Ca^{2+} and phospholipid dependent.

5-Isoquinolinesulfonic acid was prepared as previously described.[16] To 22 mL of concentrated sulfuric acid was added 47.5 mL of practical-grade isoquinoline. The product was broken up into lumps, which were added to 110 mL of 65% fuming sulfuric acid with swirling in an ice bath. The solution was allowed to stand at room temperature for 48 h and poured onto 700 g of ice. The product separated as white needles to give 50 g (65%).

(5-Isoquinolinylsulfonyl)-β-alanine Methyl Ester (9). *N*-α-(*tert*-Butyloxycarbonyl)-β-alanine. Di-*tert*-butyl dicarbonate (2.3 g, 10 mmol) was added dropwise within 1 h to a well-stirred solution of β-alanine (0.89 g, 10 mmol) and NaOH (0.4 g, 10 mmol) in water (5 mL) and *tert*-butyl alcohol (10 mL). The mixture was stirred at room temperature for 12 h, diluted with water (25 mL), and then extracted with pentane (3 × 30 mL). The pH was adjusted to 2.5 by addition of potassium hydrogenocarbonate and the solution was extracted with ethyl acetate (2 × 40 mL). The combined extracts were dried (Na_2SO_4) and evaporated under vacuum to give white crystals (1.7 g, 90%). Mp: 81 °C.

***N*-α-(*tert*-Butyloxycarbonyl)-β-alanine Methyl Ester.** *N*-α-(*tert*-Butyloxycarbonyl)-β-alanine (4.19 g, 22.2 mmol) was dissolved in C_2H_5OH (200 mL), and water (20 mL) was added. The pH was adjusted to 7.0 by addition of aqueous cesium carbonate solution (10%). The mixture was evaporated to dryness; the residue was stirred with CH_3I (3.8 g, 26.7 mmol) in DMF (15 mL) for 12 h. Upon removal of the solvent, the residue was taken up in ethyl acetate, washed with aqueous sodium bicarbonate solution (5%, 30 mL) and water (2 × 30 mL), dried (Na_2SO_4), and evaporated to an oil which was used directly in the next step.

(5-Isoquinolinylsulfonyl)-β-alanine Methyl Ester (9). The oily ester was stirred in a mixture TFA–CH_2Cl_2 (40 g, 60 mmol) for 45 min. The solution was evaporated to dryness; anhydrous ether was added (three times) and removed. The residue was dissolved in CH_2Cl_2 and 1 equiv of triethylamine and 5-isoquinolinesulfonyl chloride was added. After 2 h the mixture was evaporated, and water (50 mL) and ethyl acetate were added. The organic phase was dried (Na_2SO_4) and evaporated to give crystals of **9** (4.5 g, 75%). Mp: 131 °C. NMR ($CDCl_3$, δ ppm): 2.5 (2 H, t, CH_2), 3.3 (2 H, t, CH_2), 3.6 (3 H, s, methyl ester), 5.8 (1 H, m, NH), 7.7 (1 H, t, isoquinoline), 8.2–8.7 (4 H, n, isoquinoline), 9.3 (1 H, s, isoquinoline). Anal. ($C_{13}H_{14}N_2O_4S$): C, H, N, S.

1-(5-Isoquinolinylsulfonyl)piperazine (10). To a solution of 0.688 g (8 mmol) of piperazine and 1.1 mL (8 mmol) of triethylamine in CH_2Cl_2 (30 mL) was added dropwise 20 mL of a CH_2Cl_2 solution containing 0.908 mg (4 mmol) of 5-isoquinolinesulfonyl chloride under ice cooling. The solution was stirred at room temperature for 3 h; water (50 mL) was added. The organic layer was dried (Na_2SO_4) and evaporated to an oily residue which crystallized from light petroleum to give 0.96 g (87%). Mp: 162 °C. R_f: 0.15. NMR ($CDCl_3$, δ, ppm): 2 (1 H, s, NH), 2.8–3.3 (4 H, d, CH_2CH_2), 7.7 (1 H, t, isoquinoline), 8.2–8.7 (4 H, m, isoquinoline), 9.3 (1 H, s, isoquinoline). Anal. ($C_{13}H_{15}N_3O_2S$): C, H, N, S.

Methyl 1-(5-Isoquinolinylsulfonyl)piperazinepropionate (11). To a solution of 0.36 g (1.3 mmol) of **10** and 0.361 mL (2.6 mmol) of triethylamine in CH_3OH (20 mL) was added 0.26 g (1.43 mmol) of methyl 3-bromopropionate. The solution was allowed to react at reflux for 4 h and was evaporated to dryness; water (30 mL) was added and the organic layer was dried and evaporated to an oily residue, which was chromatographed on silica gel preparative layers (ethyl acetate) to yield 11 (0.35 g, 75%). Mp: 140 °C. R_f: 0.80. NMR ($CDCl_3$, δ, ppm): 2.5 (8 H, m, CH_2 piperazine), 3.2 (4 H, m, CH_2CH_2CO), 3.7 (3 H, s, methyl ester), 7.7 (1 H, t, isoquinoline), 8.2–8.7 (4 H, m, isoquinoline), 9.3 (1 H, s, isoquinoline). FAB-MS: $(MH)^+$ = 364 *m/z*. Anal. ($C_{17}H_{21}N_3O_4S$): C, H, N, S.

Methyl 1-(5-Isoquinolinylsulfonyl)piperazinevalerate (12). Compound **10** was allowed to react as above but with methyl 5-bromovalerate. The same treatment yielded **12** (0.3 g, 60%). Mp: 64 °C. R_f: 0.75. Anal. ($C_{19}H_{25}N_3O_2S$): C, H, N, S.

***N*-(2-Aminomethyl)-5-isoquinolinesulfonamide (13).** To a mixture of crude isoquinoline-5-sulfonyl chloride[18] (5 g, 19 mmol) and water (150 mL) was added slowly $NaHCO_3$ (1.5 g, 19 mmol) with stirring and cooling on ice. The resulting solution was extracted with CH_2Cl_2 (50 mL × 2). The organic layer was dried (Na_2SO_4) and added dropwise to a solution of ethylenediamine (5.7 g, 95 mmol) and CH_2Cl_2 (50 mL) with stirring and ice cooling. After 2 h at room temperature the reaction mixture was extracted with 10% aqueous HCl solution. The pH of the aqueous layer was adjusted to 10 with a 10% aqueous NaOH solution and extracted with $CHCl_3$. Upon removal of the solvent, an oily residue was obtained which crystallized from light petroleum to yield 2.85 g (60%). Mp: 104 °C. R_f: 0.15. NMR ($CDCl_3$, δ, ppm): 2.6–3.2 (4 H, dd, CH_2CH_2), 7.7 (1 H, t, isoquinoline), 8.2–8.7 (4 H, m, isoquinoline), 9.3 (1 H, s, isoquinoline). Anal. ($C_{11}H_{13}N_3O_2S$): C, H, N, S.

Methyl *N*-(2-Isoquinolinesulfonamidoethyl)-3-aminopropionate (14). To a solution of 0.71 g (2.83 mmol) of **13** and 0.787 mL (5.65 mmol) of triethylamine in CH_3OH (30 mL) was added 0.519 g (3.11 mmol) of methyl 3-bromopropionate. The reaction mixture was refluxed for 4 h and then evaporated to dryness; water (30 mL) and CH_2Cl_2 (30 mL) were added. The organic layer was dried and evaporated to an oily residue which crystallized from light petroleum (0.9 g, 95%). Mp: 105 °C. R_f: 0.30. NMR ($CDCl_3$, δ, ppm): 2.4–3.2 (6 H, m, CH_2NH), 3.5 (2 H, m, CH_2CO), 7.7 (1 H, t, isoquinoline), 8.2–8.7 (4 H, m, isoquinoline), 9.3 (1 H, s, isoquinoline). FAB-MS: $(MH)^+$ = 338 *m/z* Anal. ($C_{15}H_{19}N_3O_4S$): C, H, N, S.

***N*-(2-Aminoethyl)-5-isoquinolinesulfonamide 15 and 16.** A mixture of 0.24 g (0.95 mmol) of **13** and 0.1 g (1 mmol) of succinic anhydride in toluene (40 mL) was warmed at 60 °C for 10 min. Upon removal of the solvent, the residue was triturated with CH_3OH to yield white crystals of the carboxylic derivative **15** (0.2 g, 60%). Mp: 175 °C. R_f: 0.50. Anal. ($C_{15}H_{17}N_3O_5S$): C, H, N, S.

To a solution of 0.12 g of **15** in anhydrous ether was slowly added a solution of diazomethane in ether prepared by basic decomposition of nitrosomethylurea. After 10 min, the solvent was removed to give an oily residue that was purified by chromatography on an Al_2O_3 column (10% v/v MeOH–$CH_3COOC_2H_5$) to give orange crystals of **16** (0.11 g, 90%). Mp: 152 °C. R_f: 0.85. NMR ($CDCl_3$, δ, ppm): 2.2–2.7 (4 H, dd, CH_2CH_2N), 3–3.4 (4 H, dd, CH_2CH_2CO), 3.7 (3 H, s, methyl ester), 7.7 (1 H, t, isoquinoline), 8.2–8.7 (4 H, m, isoquinoline), 9.3 (1 H, s, isoquinoline). Anal. ($C_{16}H_{19}N_3O_5S$): C, H, N, S.

Incorporation of the Heterocyclic Moieties on the NH_2-Terminus Group of the Peptides. Isoquinoline-5-sulfonyl chloride or dansyl chloride (Aldrich) was incorporated on the NH_2-terminus group of the peptides still linked to the resin with a 2.5-fold excess and 1 equiv of triethylamine in CH_2Cl_2. The completeness of the coupling was monitored with ninhydrin and the resulting product was treated with anhydrous HF (see above). For incorporation of **14**, the ester group was first saponified (NaOH, CH_3OH, reflux for 30 min). Upon removal of the solvent, water addition, and acidification (pH = 3), the aqueous solution was evaporated to dryness. The oily residue crystallized from acetone and was directly coupled with a 2.5-fold excess and 1 equiv of DCC, HOBt, and DIEA in DMF.

Phosphorylation Assays. Protein kinase C phosphorylation assays were performed in a reaction (80 μL) mixture containing histone III_S (60 μg/mL), $MgCl_2$ (5 mM), $CaCl_2$ (0.5 mM), phosphatidylserine (50 μg/mL), diolein (5 μg/mL), [γ^{32}P]ATP (10 μM, 2000–4000 cpm/pmol), Tris/HCl buffer (pH 7.5), protein kinase C (0.5 μg), and inhibitors at different concentrations.

The chosen order of addition of various components of the reaction was as follows: inhibitor, radioactive mixture containing $CaCl_2$, $MgCl_2$, substrate, then phospholipids and finally PKC. From a practical aspect, a different order of addition is more difficult to assess (PKC stability) but leads to the same results. The reaction was studied in the initial rate conditions; for a given inhibitor concentration, between about $t = 0$ and $t = 10$ min, the inhibition was time dependent; it was also linear with respect to the amount of enzyme present. In the absence of diolein and phospholipids, the basal activity was much too weak to measure the effect of inhibitors.

Protein kinase A phosphorylation assays were performed in a reaction mixture (80 μL) containing Histone II_A (60 μg/mL), $MgCl_2$ (5 mM), [γ^{32}P]ATP (10 μM, 2000–4000 cpm/pmol), Tris/HCl buffer (pH 7.0), protein kinase A (1 μg), and inhibitor at different concentrations.

For each kinase, when histones were used as substrates, reactions were run at 30 °C for 7 min and stopped by trichloroacetic acid (12% w/v) in presence of bovine serum albumin (0.9 mg) as a carrier. After centrifugation (10 min at 3000 rpm) supernatant containing [γ^{32}P]ATP and unprecipitable inhibitors were discarded, and the pellet was dissolved in 1 M NaOH and precipitated a second time by trichloroacetic acid. Radioactivity incorporated into histones was counted by scintillation spectrometry with Aqualyte reagent. All experiments were carried out in triplicate.

When peptides (GS 1-12 or kemptide) were used as substrates, the procedure differed as follows: after the reactions were stopped with trichloroacetic acid, [γ^{32}P]ATP was removed as described by Glass et al.[19] by spotting an aliquot (50 μL) of each sample onto phosphocellulose paper which was washed three times in H_3PO_4 (75 mM). Squares were dried and the radioactivity was quantified by scintillation spectrometry.

Registry No. 1, 129786-26-1; **2**, 129786-27-2; **3**, 129786-28-3; 4, 129786-29-4; **5**, 129786-30-7; **6**, 129786-31-8; **7**, 129786-32-9; **8**, 129786-33-0; **9**, 129786-34-1; **10**, 84468-24-6; 11, 129786-35-2; **12**, 129786-36-3; **13**, 84468-17-7; **14**, 129786-37-4; **15**, 129786-38-5; **16**, 129786-39-6; **17**, 129786-40-9; PKC, 89800-68-0; PKA, 9026-43-1; H-β-Ala-OH, 107-95-9; BOC-β-Ala-OH, 3303-84-2; BOC-β-Ala-OMe, 42116-55-2; $BrCH_2CH_2COOMe$, 3395-91-3; $Br(CH_2)_4COOMe$, 5454-83-1; $H_2NCH_2CH_2NH_2$, 107-15-3; piperazine, 110-85-0; succinic anhydride, 108-30-5; 5-isoquinolinesulfonyl chloride, 84468-15-5; dansyl chloride, 605-65-2.

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(Acyloxy)alkyl Carbamate Prodrugs of Norfloxacin

Jose Alexander,* Robert A. Fromtling,† Judith A. Bland,† Barbara A. Pelak,† and Evamarie C. Gilfillan†

INTERx Research Corporation, Merck Sharp and Dohme Research Laboratories, 2201 W. 21st Street, Lawrence, Kansas 66047, and Merck Sharp and Dohme Research Laboratories, Rahway, New Jersey 07065-0900. *Received May 2, 1990*

As a new prodrug approach to norfloxacin (NFLX) we prepared the acetoxyalkyl carbamates of the type NFLX–CO–OCHR–OAc by the reaction of sodium or mercuric acetate on NFLX α-chloroalkyl carbamates. These prodrugs did not have the bitter taste of NFLX. In vitro, the acetoxyethyl carbamate exhibited activity only against *Staphylococcus* spp. and was inactive against Gram-negative organisms. However, in the presence of serum and intestinal homogenate, esterase-catalyzed hydrolysis of the ester bond in these modified carbamates led to a cascade reaction resulting in the rapid regeneration of NFLX. At high oral doses of the prodrug, the acetaldehyde produced as a side product in the breakdown of the promoiety caused a slight decrease in alcohol metabolism in a mouse model. The bioavailability of NFLX from the acetoxyethyl carbamate was lower compared to an equivalent dose of NFLX when given as an oral suspension in rhesus monkeys, presumably because of the lower aqueous solubility of the prodrug.

Norfloxacin (**1a**) is a synthetic second generation quinolone antibacterial agent which exhibits remarkable activity against a broad spectrum of Gram-positive and Gram-negative bacteria.[1] Since only 35–40% of a typical oral dose of the drug is absorbed in human subjects, attempts were made to improve the therapeutic efficacy of the drug by several different methods of prodrug modification.[2] The desirability of masking the somewhat disagreeable taste of norfloxacin was an added impetus. Both the carboxylic acid group[3a,4] and the secondary nitrogen of the piperazino substituent[3a,4-6] have been used as loci for latentiation. The carboxylic acid esters were ineffective, either because they were too unstable chemically or because they failed to liberate norfloxacin after absorption. Reduction of the carboxylic acid to the corresponding formyl group was reported[4] to result in improved absorption in mice, leading to increased serum levels of norfloxacin after oxidation in vivo. The N-blocked norfloxacin prodrugs,[5-7] viz., *N*-[(5-methyl-2-oxo-1,3-dioxol-4-yl)methyl] and *N*-oxoalkyl derivatives, release the parent drug in vivo by oxidative dealkylation.

In general, for ionizable molecules the rate of transport through biomembranes appears to be proportional to the concentration of undissociated molecules in solution and the lipid solubility. So, a prodrug strategy that involves latentiation of the amino function which will change the zwitterionic nature of norfloxacin is expected to produce a more readily absorbable form. Analogues which carry *N*-alkyl substitution such as pefloxacin (**1b**) have enhanced oral absorbability.[3] A prodrug strategy involving carbamylation of the piperazino nitrogen would have the additional advantage that most carbamates do not ionize under normal physiological pH. However, success with carbamate ester latentiation requires that it must be hydrolyzed to a carbamic acid and an alcohol after penetration through the biological barrier. This is especially true of carbamates of secondary amines, the rate of hydrolysis of which are 10^5 to 10^9 times slower than that of

†Merck Sharp and Dohme Research Laboratories, Rahway, NJ 07065-0900.

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