# Isoquinolinesulfonamides, Novel and Potent Inhibitors of Cyclic Nucleotide Dependent Protein Kinase and Protein Kinase C†

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ABSTRACT: Naphthalenesulfonamides such as *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) are potent calmodulin (CaM) antagonists and act upon several protein kinases at higher concentration. When the naphthalene ring was replaced by isoquinoline, the derivatives were no longer CaM antagonists but retained the ability to inhibit protein kinases, and some of the derivatives exhibited selective inhibition toward a certain protein kinase. cAMP-dependent, cGMP-dependent, and $Ca^{2+}$–phospholipid-dependent (protein kinase C) protein kinases were inhibited significantly by addition of $10^{-6}$ M *N*-[2-(methylamino)ethyl]-5-isoquinolinesulfonamide (H-8) and 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H-7). H-8 was the most active of the inhibitors in this series and inhibited more markedly cyclic nucleotide dependent protein kinases, than other kinases, while the derivative with the sulfonylpiperazine residue (H-7) was the most potent in inhibiting protein kinase C. Apparent $K_i$ values of H-8 were 0.48 and 1.2 $\mu$M for cGMP-dependent and cAMP-dependent protein kinases, respectively, and the $K_i$ value of H-7 for protein kinase C was 6 $\mu$M. Both the holoenzyme and the catalytic subunit (or fragment), which is active without an enzyme activator, are susceptible to these compounds with a similar concentration dependency, thereby indicating that the inhibitory effect is attributed to the direct interaction of the compound with the active center of the enzyme but not with the enzyme activator. The inhibitions were freely reversible and of the competitive type with respect to ATP and of the noncompetitive type with respect to the phosphate acceptor. Kinetic studies with the catalytic subunit of cAMP-dependent protein kinase indicated that isoquinolinesulfonamides, structurally unrelated to ATP, compete with ATP for free enzyme but do not interact with the same enzyme form as does the phosphate acceptor (i.e., enzyme–ATP complex). The inhibitory potency of these derivatives seems to be dependent on the position and the strength of the plus charge contributed by the terminal nitrogen in the isoquinolinesulfonamide molecule. This report is concerned with the preparation and inhibitory mechanisms of isoquinolinesulfonamide derivatives, as possible tools for clarifying the in vitro and in vivo functions of protein kinases.

Protein phosphorylation is an established major general mechanism by which intracellular events in mammalian tissues are controlled by external physiological stimuli (Rubin & Rosen,1975; Krebs & Beavo, 1979; Krueger et al., 1977). Cyclic nucleotides, $Ca^{2+}$–calmodulin (CaM), and diacylglycerol are universal regulators neither tissue nor species specific and have an effect on a large number of cellular functions (Greengard, 1978; Nishizuka, 1983). Biological functions of cyclic nucleotides, $Ca^{2+}$–CaM, and diacylglycerol seem to be manifest through protein phosphorylations by cyclic nucleotides, $Ca^{2+}$–CaM-dependent protein kinases, and protein kinase C.[1]

Pharmacological studies using CaM antagonists such as phenothiazines and naphthalenesulfonamides suggest that $Ca^{2+}$–CaM-dependent protein phosphorylation may play an important role in the function of various tissues including human platelets (Nishikawa et al., 1980) and tracheal smooth muscle contraction (Silver & Stull, 1983). On the other hand, potent and selective inhibition of cyclic nucleotide dependent protein kinases and protein kinase C by synthesized compounds has not been reported. We then synthesized derivatives of sulfonamides and tested their inhibition toward various protein kinases. Naphthalenesulfonamides act as potent and specific inhibitors of CaM-dependent protein kinase by inactivating CaM (CaM antagonists) (Hidaka et al., 1980) but weak inhibitors of other protein kinases (Tanaka et al., 1982; Schatzman et al., 1983). The weak inhibition of several $Ca^{2+}$–CaM-independent protein kinases by W-7 and its derivatives was found to be produced by direct interaction of W-7 with protein kinases and not by effects on activating processes. These results prompted us to synthesize more potent and specific inhibitors of protein kinase. When the naphthalene nucleus was replaced by isoquinoline, the resulting compounds, "isoquinolinesulfonamides", were found to be more potent inhibitors of protein kinase. These newly synthesized compounds, isoquinolinesulfonamides, should serve as useful pharmacological tools for elucidating the biological significance of protein kinase mediated reactions.

## Experimental Procedures

Histones H1 and H2B were purchased from Boehringer-Mannheim Biochemicals. Phosphatidylserine (pig liver) was purchased from Serdary Research Laboratories, Inc. Chloroform was removed from this phospholipid by a stream of nitrogen, and the phospholipid was sonicated in water for 1 min to produce a suspension of 0.5 mg/mL. [$\gamma$-$^{32}$P]ATP was obtained from Amersham, England.

Trypsin [(TRTPCK) treated with L-1-(tosylamido)-2-phenylethyl chloromethyl ketone to inhibit contaminant chymotryptic activity according to Kostka & Carpenter (1964)] was from Worthington Biochemical Corp., and soybean trypsin inhibitor was from Sigma Chemical Co.

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[1] Abbreviations: H-8, *N*-[2-(methylamino)ethyl]-5-isoquinolinesulfonamide; H-7, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine; W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide; cAMP, cyclic AMP; cGMP, cyclic GMP; AMP-PNP, 5′-adenylyl imidodiphosphate; EGTA, ethylene glycol bis($\beta$-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; MLCK, myosin light chain kinase; protein kinase C, $Ca^{2+}$-activated, phospholipid-dependent protein kinase; Tris-HCl, tris(hydroxymethyl)-aminomethane hydrochloride; ATPase, adenosinetriphosphatase; PMSF, phenylmethanesulfonyl fluoride.

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Myosin light chain was prepared by the method of Perrie & Perry (1970). The partially purified holoenzyme of cAMP-dependent protein kinase I (second DE-52 step) and its purified catalytic subunits were prepared from rabbit skeletal muscle, by the method of Beavo et al., (1974). cGMP-dependent protein kinase from pig lung was partially purified by the method of Kuo & Greengard (1974). $Ca^{2+}$-activated, phospholipid-dependent protein kinase (protein kinase C) was prepared from rabbit brain, as described by Inoue et al. (1977). This preparation was confirmed to be free of other interfering enzymes, endogenous phosphate acceptor proteins, cAMP, cGMP, and $Ca^{2+}$–calmodulin. Myosin light chain kinase was purified from chicken gizzard by the method of Adelstein & Klee (1981). Casein kinase I from rat liver was prepared by the method of Meggio et al. (1979). Casein kinase II from rabbit skeletal muscle was prepared according to Huang et al. (1982). Actomyosin from rabbit skeletal muscle was prepared according to Ebashi & Ebashi (1964) and erythrocyte membranes according to Luthra et al. (1976).

*Enzyme Assay and Determinations.* cAMP-dependent protein kinase activity was assayed in a reaction mixture containing, in a final volume of 0.2 mL, 50 mM Tris-HCl (pH 7.0), 10 mM magnesium acetate, 2 mM EGTA, 1 $\mu$M cAMP or absence of cAMP, 3.3–20 $\mu$M [$\gamma$-$^{32}$P]ATP ($4 \times 10^5$ cpm), 0.5 $\mu$g of the enzyme, 100 $\mu$g of histone H2B, and each compound, as indicated. cGMP-dependent protein kinase activity was assayed in a reaction mixture containing, in a final volume of 0.2 mL, 50 mM Tris-HCl (pH 7.0), 50 mM magnesium acetate, 2 mM EGTA, 1 $\mu$M cGMP or absence of cGMP, 3.3–20 $\mu$M [$\gamma$-$^{32}$P]ATP ($4 \times 10^5$ cpm), 100 $\mu$g of histone H2B, 2.4 $\mu$g of the enzyme, and each compound, as indicated. Protein kinase C activity was assayed in a reaction mixture containing, in a final volume of 0.2 mL, 50 mM Tris-HCl (pH 7.0), 10 mM magnesium acetate, 0.5 mM calcium chloride or 1 mM EGTA, 10 $\mu$g of phosphatidylserine, 3.3–20 $\mu$M [$\gamma$-$^{32}$P]ATP ($4 \times 10^5$ cpm), 100 $\mu$g of histone H1, and 0.5 $\mu$g of the enzyme. Myosin light chain kinase activity was assayed under the conditions described earlier (Tanaka et al., 1980), in a reaction mixture containing, in a final volume of 0.2 mL, 50 mM Tris-HCl (pH 7.0), 10 mM magnesium acetate, 0.1 mM calcium chloride or 1 mM EGTA, 100 ng of calmodulin, 5–100 $\mu$M [$\gamma$-$^{32}$P]ATP ($4 \times 10^5$ cpm), 20 $\mu$M smooth muscle 20 000-dalton myosin light chain, and 0.6 $\mu$g of myosin light chain kinase. Casein kinase I and casein kinase II activities were assayed in a reaction mixture containing, in a final volume of 0.2 mL, 50 mM $\beta$-glycerol-P (pH 7.0), 10 mM magnesium acetate, 0.5 mM EGTA, 1 mM dithiothreitol, 3.3–20 $\mu$M [$\gamma$-$^{32}$P]ATP ($4 \times 10^5$ cpm), 3.0 (casein kinase I) or 2.8 $\mu$g (casein kinase II) of the enzyme, and 800 $\mu$g of casein (Hammersten quality). Myosin light chain kinase and protein kinase C were treated with trypsin as described by Tanaka et al. (1980) and Inoue et al. (1977), respectively. The incubation was carried out at 30 °C for 5 min. The reaction was terminated by the addition of 1 mL of ice-cold 20% trichloroacetic acid following addition of 500 $\mu$g of bovine serum albumin as a carrier protein. The sample was centrifuged at 3000 rpm for 15 min, the pellet was resuspended in ice-cold 10% trichloroacetic acid solutions, and the centrifugation–resuspension cycle was repeated 3 times. The final pellet was dissolved in 1 mL of 1 N NaOH, and radioactivity was measured by a liquid scintillation counter.

Actomyosin ATPase assay was carried out at 25 °C in a volume of 0.2 mL containing 40 mM Tris–maleate buffer (pH 7.0), 60 mM KCl, 1 mM $MgCl_2$, 100 $\mu$M $CaCl_2$, and 0.2–1.0 mM ATP. Erythrocyte ($Ca^{2+}$–$Mg^{2+}$)-ATPase assay used here was essentially the same as that described by Gopinath & Vincenzi (1977). Reaction mixtures, in a final incubation volume of 1 mL, contained 18 mM histidine–imidazole buffer, pH 7.0, 0.1 mM EGTA, 3 mM $MgCl_2$, 80 mM NaCl, 15 mM KCl, 0.1 mM ouabain, 110 $\mu$g of erythrocyte ghost proteins, and 0.2–1.0 mM ATP. $CaCl_2$ (0.2 mM) was present in all tubes except in those for the determination of $Mg^{2+}$-ATPase. The reaction was started by the addition of ATP and terminated by the addition of 1 mL of 20% trichloroacetic acid. Inorganic phosphate liberated after 20 min of incubation was determined by the colorimetric method of Fiske & SubbaRow (1925).

Protein was determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard.

The $pK_a$ value of each compound was determined by a potentiometric titration method.

*Preparation of Isoquinolinesulfonamides.* 1-(5-Isoquinolinylsulfonyl)-2,3-dimethylpiperazine was synthesized as follows; in 30 mL of methylene chloride were dissolved 1.75 g of 2,3-dimethylpiperazine and 1.53 g of triethylamine, and to the solution was added dropwise 20 mL of a methylene chloride solution containing 1.73 g of 5-isoquinolinesulfonyl chloride, under cooling with ice. The mixed solution obtained was stirred at a temperature of 5–10 °C for 3 h, and the reaction product was washed with water and dried with anhydrous magnesium sulfate. After the methylene chloride was distilled therefrom, the residue obtained was subjected to alumina column chromatography (alumina 50 g; solvent chloroform) to give 1.38 g of 1-(5-isoquinolinylsulfonyl)-2,3-dimethylpiperazine. Procedures similar to those described above were repeated by using the compounds 1-(5-isoquinolinylsulfonyl)-3,5-dimethylpiperazine and 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H-7).

*N*-(2-Aminoethyl)-5-isoquinolinesulfonamide was synthesized as follows: in 200 mL of chloroform was dissolved 8.8 g of 1,2-diaminoethane, and to the solution was added dropwise 100 mL of a chloroform solution containing 4.55 g of 5-isoquinolinesulfonyl chloride, under cooling with ice. After the dropwise addition of the chloroform solution, the mixed solution was stirred at a temperature of 20–25 °C for 2 h after which the reaction solution was extracted with a 10% aqueous hydrochloric acid solution. The pH of the aqueous layer was adjusted to 10 with a 10% aqueous sodium hydroxide solution, and the aqueous layer was extracted with chloroform. The chloroform layer extracted was washed with water and dried with anhydrous potassium carbonate. Then the chloroform was distilled from the chloroform layer, and the residue obtained was subjected to column chromatography [silica gel 200 g; developing solvent 2% methanol/chloroform (v/v)] to give 3.46 g of *N*-(2-aminoethyl)-5-isoquinolinesulfonamide. The same procedures as described above were repeated by using compounds with various lengths of hydrocarbon chain and *N*-(2-guanidinoethyl)-5-isoquinolinesulfonamide.

*N*-[2-(Methylamino)ethyl]-5-isoquinolinesulfonamide (H-8) was synthesized as follows: in 50 mL of a chloroform solution containing 1.4 g of 2-(methylamino)ethylamine and 1.4 g of triethylamine was added dropwise 30 mL of a chloroform solution containing 2.6 g of 5-isoquinolinesulfonyl chloride, under cooling with ice. After the dropwise addition of the chloroform solution, the mixture was stirred at a temperature of 2–10 °C for 4 h, and the reaction product was then washed with water and dried with anhydrous magnesium sulfate. After the chloroform was distilled from this, the residue obtained was subjected to column chromatography (silica gel 70 g; solvent chloroform) to yield 2.38 g of *N*-[2-(methyl-

Table I: Effects of Isoquinolinesulfonamides and Related Compounds on cGMP-Dependent, cAMP-Dependent, $Ca^{2+}$–Phospholipid-Dependent, and $Ca^{2+}$–CaM-Dependent Protein Kinases

| compounds | $K_i$ (μM) cGMP-dependent protein kinase | cAMP-dependent protein kinase | MLC kinase | protein kinase C |
|---|---|---|---|---|
| *N*-[2-(methylamino)ethyl]-5-isoquinolinesulfonamide (H-8) | 0.48 | 1.2 | 68 | 15 |
| *N*-(2-aminoethyl)-5-isoquinolinesulfonamide | 0.87 | 1.9 | 70 | 18 |
| *N*-(2-guanidinoethyl)-5-isoquinolinesulfonamide | 1.3 | 2.3 | 150 | 40 |
| *N*,*N*-dimethyl-5-isoquinolinesulfonamide | 1.7 | 4.7 | 120 | 29 |
| 1-(5-isoquinolinylsulfonyl)-2,3-dimethylpiperazine | 7.7 | 1.8 | 170 | 75 |
| 1-(5-isoquinolinylsulfonyl)-3,5-dimethylpiperazine | 8.4 | 2.9 | 100 | 45 |
| 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H-7) | 5.8 | 3.0 | 97 | 6.0 |

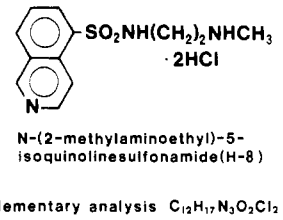


FIGURE 1: Chemical structure of *N*-[2-(methylamino)ethyl]-5-isoquinolinesulfonamide (H-8).

amino)ethyl]-5-isoquinolinesulfonamide. The structure is shown in Figure 1.

*Enzyme–Immunoassay Procedure.* The levels of H-8 were examined by using a sensitive enzyme–immunoassay system. Various amounts of the standard H-8 or sample solution were incubated with pieces of silicone rubber (3 × 4 mm; Sankoh Plastic Co., Osaka, Japan) coated with anti-H-8 antibody, at 30 °C for 5 h in a final volume of 0.5 mL with 0.01 M sodium phosphate buffer (pH 7.0) containing 0.3 M NaCl, 1 mM $MgCl_2$, 0.5% gelatin (Difco), 0.1% bovine serum albumin (fraction V; Armour), 0.1% $NaN_3$, and 1 mM calcium acetate. After the incubation, the reaction medium was removed by aspiration and the residual silicone rubber pieces were washed twice with 1 mL of chilled 0.01 M sodium phosphate buffer (pH 7.0) containing 0.1 M NaCl, 1 mM $MgCl_2$, 0.1% bovine serum albumin, and 0.1% $NaN_3$ (buffer A). The pieces were then incubated with 3 milliunits of the β-D-galactosidase-labeled anti-H-8 antibody at 4 °C overnight in 0.2 mL of buffer A containing 1 mM calcium acetate. The pieces were washed twice with buffer A as above, and the β-D-galactosidase activity bound to the piece was assayed with 15 μmol of 4-methylumbelliferyl β-D-galactoside (Sigma) as substrate. Anti-H-8 antiserum was raised in New Zealand white rabbits by injecting human albumin conjugated *N*-(2-aminoethyl)-5-isoquinolinesulfonamide.

## Results

*Inhibition of Protein Kinase Activity by Isoquinolinesulfonamides.* All the isoquinolinesulfonamides tested were found to inhibit protein kinases such as cAMP-dependent protein kinase, cGMP-dependent protein kinase, protein kinase C, and MLC kinase to variable extents. The inhibitory effect of the compounds on each protein kinase could be overcome by increasing the amounts of ATP added. Figure 2 shows a typical inhibitory effect of H-8 on cAMP-dependent protein kinase activity. Essentially the same results were obtained with other protein kinases or with other isoquinolinesulfonamide derivatives. These findings indicate that the interaction between isoquinolinesulfonamide and the enzyme is freely reversible, and this compound provides an apparent increase in the $K_d$ value for ATP. Kinetic analysis by double-reciprocal plots revealed that the inhibition of each protein kinase produced by each of the compounds was competitive with respect

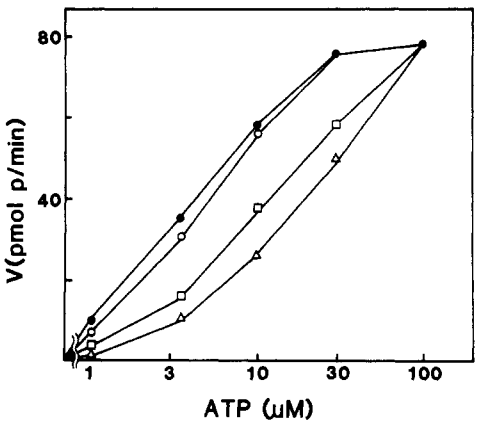


FIGURE 2: Effect of H-8 on cAMP-dependent protein kinase as a function of ATP concentration. The protein kinase, from rabbit skeletal muscle, was assayed as described under Experimental Procedures, except for the varying concentrations of ATP and in the absence (●) or presence of H-8: 0.5 (○), 3 (□), or 5 μM (△), respectively.

Table II: Effect of H-7 and H-8 on Protein Kinases and ATPases

| enzymes | H-8 $K_i$ (μM) | H-7 $K_i$ (μM) |
|---|---|---|
| cGMP-dependent protein kinase | 0.48 | 5.80 |
| cAMP-dependent protein kinase | 1.20 | 3.00 |
| protein kinase C + $Ca^{2+}$–PS | 14.4 | 6.0 |
| trypsin treated + EGTA | 13.0 | 6.0 |
| MLC kinase + $Ca^{2+}$–CaM | 68.0 | 97.0 |
| trypsin treated + EGTA | 65.0 | 97.0 |
| casein kinase I | 133 | 100 |
| casein kinase II | 950 | 780 |
| actomyosin ATPase | 750 | >1000 |
| ($Ca^{2+}$–$Mg^{2+}$)-ATPase | 980 | ND[a] |

[a] ND, not determined.

to ATP and noncompetitive with respect to phosphate acceptor. The $K_i$ values estimated by Dixon plots are given in Table I. As can be seen, H-8 was the most active of the inhibitors in this series and inhibited more markedly cGMP-dependent and cAMP-dependent protein kinases than did the other kinases. The $K_i$ values of H-8 for cGMP-dependent and cAMP-dependent protein kinases were 0.48 μM and 1.2 μM, respectively. When the aminoethylsulfonamide residue in the H-8 molecule was replaced by sulfonylpiperazine, derivatives such as H-7 became more potent inhibitors of protein kinase C, compared to other derivatives. The $K_i$ value of H-7 for protein kinase C was 6 μM. Since the isoquinolinesulfonamides compete with ATP in the protein kinase reaction, we investigated the effects of these compounds on a wider range of protein kinases and other ATP-utilizing enzymes such as ($Ca^{2+}$–$Mg^{2+}$)-ATPase in erythrocyte ghosts and actomyosin ATPase in skeletal muscle. Table II summarizes the results obtained with the representative derivatives of isoquinolinesulfonamide, H-7 and H-8. Both compounds had weak effects

Table III: Effect on Protein Kinases of H-8 and Its Analogues with Various Lengths of Alkyl Chain

| compound | $pK_a$ value | | $K_i$ ($\mu$M) | |
|---|---|---|---|---|
| | terminal nitro atom | nitro atom of isoquinoline | cGMP-dependent protein kinase | cAMP-dependent protein kinase |
| *N*-[2-(methylamino)ethyl]-5-isoquinolinesulfonamide | 8.9 | 3.4 | 0.48 | 1.2 |
| *N*-(2-aminoethyl)-5-isoquinolinesulfonamide | 8.7 | 3.4 | 0.87 | 1.9 |
| *N*-(2-aminopropyl)-5-isoquinolinesulfonamide | | | 1.7 | 4.3 |
| *N*-(2-aminobutyl)-5-isoquinolinesulfonamide | | | 2.1 | 5.4 |
| *N*-(2-aminohexyl)-5-isoquinolinesulfonamide | | | 16 | 12 |

on casein kinases I and II as well as on MLC kinase. The ATPase activities were affected by these compounds, only to a weak extent. Thus, it has been more clearly demonstrated that isoquinolinesulfonamides potently and selectively inhibit cyclic nucleotide dependent protein kinases and/or protein kinase C. Among them, H-8 was a relatively selective inhibitor of cyclic nucleotide dependent protein kinase, and H-7 was the most potent inhibitor of protein kinase C.

In preceding papers, protein kinase C and MLC kinase were reported to be alternatively activated, in an irreversible manner, by limited proteolysis with trypsin. In this process, the catalytically active fragment produced was entirely independent of $Ca^{2+}$ and enzyme cofactors (phospholipid and calmodulin). These compounds inhibited both the $Ca^{2+}$-dependent and $Ca^{2+}$-independent activities of these two protein kinases with a similar concentration dependency (Table II). Furthermore, these compounds inhibited the activity of the catalytic subunit of cAMP-dependent protein kinase to the same extent as did the holoenzyme of this protein kinase. Therefore, it is likely that they are without effect on the cAMP-dependent dissociation of the catalytic subunit from the regulatory subunit. In light of all these findings, the inhibitory actions of isoquinolinesulfonamides seem to be the result of direct effects on the active site of the enzyme and not due to effects on the enzyme activating process.

*Kinetic Characterization of the Mode of Interaction of H-8 with the Catalytic Subunit of the cAMP-Dependent Protein Kinase.* The mode of interaction of the isoquinolinesulfonamides with protein kinase was investigated by kinetic analysis using the catalytic subunit of the cAMP-dependent protein kinase. When the extent of inhibition by H-8 was examined at two concentrations of ATP (3.3 and 20 $\mu$M), the ability of the compound to block protein kinase activity was less with higher concentrations of ATP. When the degree of inhibition was examined at two concentrations of histone H2B (10 and 100 $\mu$g/0.2 mL), the ability of H-8 to block protein kinase activity was unchanged (Figure 3). Preincubation of the combination of H-8, catalytic subunit, and ATP did not affect the extent of inhibition. These results suggest that the interaction between H-8 and the catalytic subunit of cAMP-dependent protein kinase does not require either ATP or the phosphate acceptor. H-8 cannot be the phosphate donor since it does not have a phosphorus atom. H-8 could not serve as a substrate for protein kinase (data not shown). As indicated in Figure 4, H-8 was a competitive inhibitor vs. ATP and a noncompetitive inhibitor vs. the phosphate acceptor in the protein kinase reaction. This would indicate that, if the reaction is subject to an ordered kinetic mechanism (Gronot et al., 1979, 1980; Whitehouse et al., 1983), then H-8 interacts only with free enzyme, i.e., H-8, structurally unrelated to ATP, competes with ATP for free enzyme, whereas noncompetitive inhibition vs. the phosphate acceptor is observed because the phosphate acceptor and H-8 interact with different enzyme forms (E·ATP and free E, respectively), but that the phosphate acceptor, histone H2B, is the second substrate to bind. It is likely, therefore, that the isoquinolinesulfonamides block

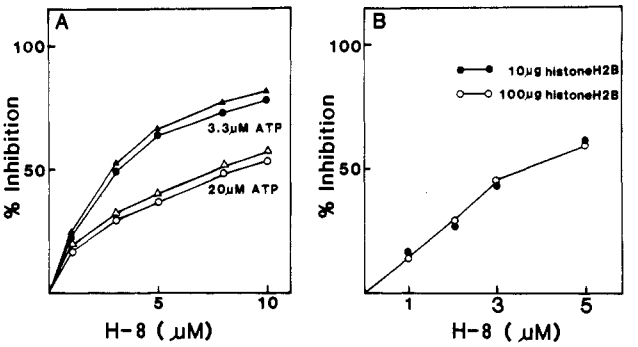


FIGURE 3: Independence of ATP and histone H2B of the inhibition of catalytic subunit by H-8. (A) Catalytic subunit was preincubated in either the absence (○, ●) or presence (△, ▲) of ATP in the reaction mixture for 5 min, at 30 °C. The phosphorylation reaction was then initiated by the addition of ATP and histone H2B, or histone H2B. The ATP concentration was either 3.3 (●, ▲) or 20 $\mu$M (○, △). (B) The assay of protein kinase activity was performed as described under Experimental Procedures with histone H2B at 10 (●) or 100 $\mu$g/0.2 mL (○) and ATP (10 $\mu$M) fixed. Data are expressed as the percent of inhibition of each of these activities.

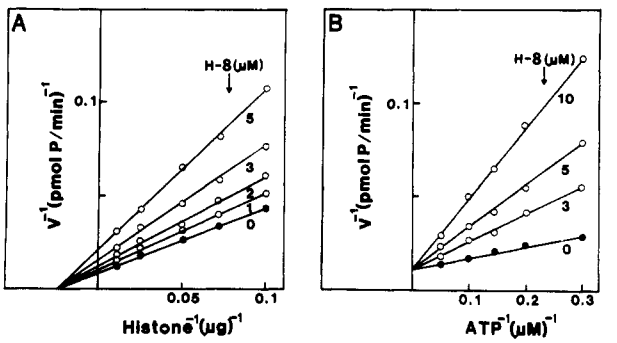


FIGURE 4: Inhibition patterns of catalytic subunit of cAMP-dependent protein kinase by H-8. (A) Reciprocal velocity vs. 1/[histone H2B] at 10 $\mu$M ATP with varying H-7. (B) Reciprocal velocity vs. 1/[ATP] at 100 $\mu$g/0.2 mL histone H2B with varying H-8. All other conditions are as described under Experimental Procedures. The lines are those fit to the data point by using the equations for (A) simple linear noncompetitive and (B) competitive inhibition, respectively.

protein kinase activity by interacting with the free enzyme but not by interacting with enzyme–ATP complex.

*Inhibition Mechanism.* Isoquinolinesulfonamides with various lengths of alkyl chain were examined with regard to inhibition of cGMP-dependent and cAMP-dependent protein kinases (Table III). The $K_i$ values increased in accordance with extension of the carbon chain of the derivatives. Although both *N*-(2-aminopropyl)-5-isoquinolinesulfonamide and H-8 have three carbon atoms at the side chain, *N*-(2-aminopropyl)-5-isoquinolinesulfonamide is a weaker inhibitor than H-8. These results suggest that the affinity of isoquinolinesulfonamide for these kinases cannot be explained by hydrophobicitic and/or hydrophilicitic events. The $pK_a$ value of the terminal nitrogen at the side chain is compared in Table III. H-8, the most potent inhibitor of the kinases, has a more potent charge brought about by the terminal nitrogen at the same distance between the sulfur atom and terminal nitrogen, under

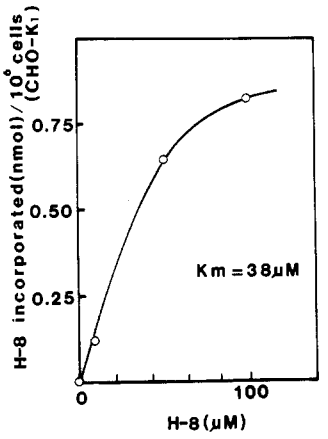


FIGURE 5: Incorporation of H-8 into CHO-$K_1$ cells. The levels of H-8 were examined by using a sensitive enzyme–immunoassay system as described under Experimental Procedures. $2 \times 10^{-6}$ cells per one assay system was used.

physiological assay conditions of pH 7.0. Therefore, the affinity of isoquinolinesulfonamides for cyclic nucleotide dependent protein kinases is probably due to the strength of the plus charge contributed by the terminal nitrogen and the two-carbon atom distance between the sulfur atom and the terminal nitrogen.

*Cell Penetration.* Figure 5 shows a typical uptake curve of H-8 into Chinese hamster ovary $K_1$ (CHO-$K_1$) cells, at various concentrations. The levels of H-8 were examined by using a sensitive enzyme–immunoassay system. After cells were incubated for 1 h in the absence or presence of H-8, the medium was renewed 3 times. The washed cells were homogenized and sonicated in a sodium phosphate buffer (pH 7.4) containing 0.1 mM EGTA, 1 mM PMSF, and soybean trypsin inhibitor (0.2 mg/mL). The soluble fraction containing $2 \times 10^6$ cells per one assay system was used for the enzyme–immunoassay. The antigen detected in the cells increased in accordance with increase of H-8 concentration, and the apparent $K_m$ value was 38 $\mu$M.

## Discussion

The kinetic study with the catalytic subunit of cAMP-dependent protein kinase provides information on the kinetic mechanism of protein kinase reactions. Gronot et al. (1979, 1980) proposed that the kinetic mechanism of protein kinase is either steady-state random or ordered with the nucleotide binding first. The pattern of inhibition obtained with H-8, a compound structurally unrelated to ATP (competitive vs. ATP and noncompetitive vs. phosphate acceptor) was consistent with that obtained with AMP-PNP. AMP-PNP, which is a structural analogue of ATP, binds to the active site of free enzyme and produces dead-end inhibition by formation of an unproductive enzyme–AMP-PNP complex (Whitehouse et al., 1983). The present data seem to support that the reaction mechanism is ordered.

The activity–structure relationships of isoquinolinesulfonamides in inhibition of cyclic nucleotide dependent protein kinases (see Table III) demonstrated that the plus charge contributed by the terminal nitrogen in the isoquinolinesulfonamide molecule may be responsible for the affinity of this compound to cAMP- and cGMP-dependent protein kinases. H-8 has remarkably different $K_i$ values for cAMP- and cGMP-dependent protein kinases, protein kinase C, casein kinase I, and casein kinase II ($0.48 \times 10^{-6}$–$0.95 \times 10^{-3}$ M), despite the finding that all these five enzymes have similar $K_m$ values for ATP [$(0.66–1.5) \times 10^{-5}$ M] and H-8 competes with ATP in all protein kinase reactions. Thus, there are structural differences in active or adjacent sites among the protein kinases.

The extracellular signals produce many of their biological responses by regulating the intracellular concentrations of cyclic nucleotides, diacylglycerol, or $Ca^{2+}$. The actions of these second messengers on biological pathways are achieved through the activation of specific cyclic nucleotide dependent, $Ca^{2+}$–phospholipid-dependent or $Ca^{2+}$–calmodulin-dependent protein phosphorylation. Although pharmacological agents which interact with activators of protein kinases, i.e., cyclic nucleotide, lipid, or calmodulin, demonstrated a certain involvement of these second messenger-mediated systems in cellular responses, the physiological significance of protein phosphorylation in biological systems is elusive. We synthesized a series of isoquinolinesulfonamide derivatives in an attempt to obtain compounds which directly inhibit protein kinase activities. The actions of isoquinolinesulfonamides are independent of enzyme activators such as cAMP or phospholipid. The data given in Figure 5 indicate that H-8 can be incorporated into cells and that intracellular concentrations depend on the amounts of the externally added H-8. Therefore, this compound can be used for in vivo studies. Since there are few ideal inhibitors of protein kinase which have primary effects on the enzyme, studies using these newly developed compounds may promote understanding of the physiological meaning of protein phosphorylation. We found that both cell growth and vascular relaxation are affected by the isoquinolinesulfonamide derivatives. These pharmacological effects are possibly related to their actions on one or more protein kinases. As these compounds compete with ATP, higher concentrations would be required to inhibit protein kinases when cells or tissues contain a large amount of ATP. On the contrary, these compounds may be more effective under conditions with lower concentrations of ATP. Among a series of derivatives, H-8 may be useful for selective inhibitors of cyclic nucleotide dependent protein kinases. H-7 inhibits selectively either protein kinase C or cyclic nucleotide dependent protein kinases, to a similar extent in vitro. Accordingly, H-7 may selectively have the advantage to inactivate protein kinase C, under condition such as low activities of cyclic nucleotide dependent protein kinase.

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**Registry No.** H-7, 84477-87-2; H-8, 84478-11-5; MLCK, 51845-53-5; ATPase, 9000-83-3; 1-(5-isoquinolinylsulfonyl)-2,3-dimethylpiperazine, 84477-75-8; 1-(5-isoquinolinylsulfonyl)-3,5-dimethylpiperazine, 84477-74-7; *N*-(2-aminoethyl)-5-isoquinolinesulfonamide, 84468-17-7; *N*-(2-guanidinoethyl)-5-isoquinolinesulfonamide, 91742-10-8; *N,N*-dimethyl-5-isoquinolinesulfonamide, 84468-22-4; 2,3-dimethylpiperazine, 84468-52-0; 1,2-diaminoethane, 107-15-3; 2-(methylamino)ethylamine, 109-81-9; 5-isoquinolinesulfonyl chloride, 84468-15-5; protein kinase, 9026-43-1; casein kinase, 52660-18-1.

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# Chemical Synthesis of (24*R*)-24,25-Dihydroxy[26,27-$^3$H]vitamin $D_3$ of High Specific Activity†

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ABSTRACT: Chemical synthesis of (24*R*)-24,25-dihydroxy-[26,27-$^3$H]vitamin $D_3$ and its 24-epimer has been devised that allows introduction of $^3$H at the terminal step of the synthesis. The epimeric mixture is derivatized as the tris(trimethylsilyl) ethers and resolved by high-performance liquid chromatography. The product has a specific activity of 178 Ci/mmol and is fully active in binding to the rat plasma vitamin D binding protein and in the elevation of serum calcium levels of vitamin D deficient rats. The synthesis begins with the readily available 3$\beta$-hydroxy-5-cholenic acid methyl ester and involves a Pummerer rearrangement, introduction of the $\Delta$7, irradiation, and isolation of the 26,27-dinor-25-carboxylic acid methyl ester of vitamin $D_3$. This compound is then treated with a Grignard reagent containing $^3$H (80 ± 10 Ci/mmol).

The availability of radioactive vitamin D derivatives, labeled at known sites to high specific activity, has been an important factor in recent successful efforts directed at the elucidation of vitamin D metabolism to its tissue-active hormonal form (Suda et al., 1971; Neville & DeLuca, 1966; Holick et al., 1976; Jones et al., 1975; Tohira et al., 1977; Bell et al., 1973; DeLuca et al., 1968; Yamada et al., 1978). A number of tritiated vitamin D derivatives of high specific activity have been synthesized, and with these compounds many important aspects of vitamin D metabolism, its regulation, and the mechanism of action had been investigated under physiological conditions. One of the major metabolites of vitamin D is (24*R*)-24,25-dihydroxyvitamin $D_3$ [(24*R*)-24,25-$(OH)_2D_3$][1] (Suda et al., 1970a,b; Holick et al., 1972). Though a number of important roles have been suggested for this metabolite, its function is still not understood. An important tool in such an investigation is radiolabeled (24*R*)-24,25-$(OH)_2D_3$ of high specific activity. Several 24,25-$(OH)_2D_3$ syntheses have been reported, including some very elegant stereoselective methods; however, none of these appeared suitable for the synthesis of the title compound, mainly because the 26,27-dimethyl groups were introduced early in the synthesis, making radioactive labeling impractical (Lam et al., 1973; Seki et al., 1973; Eyley et al., 976; Partridge et al., 1976; Takayama et al., 1980; N. Koizumi, M. Ishiguro, M. Yasuda, and N. Ikekawa, unpublished results). It is the purpose of this paper to report the chemical synthesis of (24*R*)-24,25-$(OH)_2$[26,27-$^3$H]$D_3$ and

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[1] Abbreviations: 24,25-$(OH)_2D_3$, 24,25-dihydroxyvitamin $D_3$; 24,25-$(OH)_2$[26,27-$^3$H]$D_3$, 24,25-dihydroxy[26,27-$^3$H]vitamin $D_3$; (+)-MTPA, (+)-$\alpha$-methoxy-$\alpha$-(trifluoromethyl)phenylacetate or the corresponding acetyl radical; UV, ultraviolet; NMR, nuclear magnetic resonance; HPLC, high-performance liquid chromatography.

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