centrations which produced 70–80% of maximum response and then washed every 15 min for 60 min. Then the cumulative dose–response curve with norepinephrine (10^{-10}–10^{-7} mol/L) was made. Ascorbic acid (1.13×10^{-5} mol/L) was present during the elaboration of each norepinephrine dose–response curve.

Presynaptic α_2-Adrenergic Activity on Guinea Pig Vas Deferens. Vasa deferentia were removed from guinea pigs weighing 300–500 g. The vasa were prepared according to the method of Drew.[62] They were suspended under 1 g of tension in Tyrode solution, maintained at 36 °C, and bubbled with 5% CO_2 in O_2. The composition of the Tyrode solution was (mmol/L) 137 NaCl, 2.7 KCl, 1.1 $MgCl_2$, 1.8 $CaCl_2$, 0.4 NaH_2PO_4, 11.2 $NaHCO_3$, 5.5 glucose. The bath fluid contained atropine (1.7×10^{-3} mmol/L) to exclude the effects of cholinergic nerve stimulation. Preparations were set up between two platinum ring electrodes (ring diameter 5 mm, distance between rings 32 mm). Field stimulation was carried out at 6 Hz using square-wave pulses of 1-ms duration. Voltage was adjusted to at least 1.5 times the level which gave a maximum contraction of the tissue. The preparation was stimulated for 1 s every 15 s. The agonist and antagonist potencies of the molecules were determined as mentioned above, but the reference agonist molecule was clonidine.

Serotonergic activity (5-HT_2) and dopaminergic activity (D_2) were tested by the ability of our compounds to inhibit [^{3}H]spiperone binding to a rat cortex preparation. Binding experimental conditions were as described by Billard[63] et al.

Evaluation of the Results. 1. Agonist Activity. ED_{50} values (mol/L) were determined with the method of Ariens and Van Rossum[64] (ED_{50}: dose which produces 50% of the maximum effect). Intrinsic activity was expressed as the ratio of the maximum response to each compound to the maximum response to either norepinephrine or clonidine, according to the method of Ariens.[65]

2. Antagonist Activity. The antagonistic activities were expressed in terms of pA_2 value for competitive antagonists according to the method of Arunlakshana and Schild.[66] When the antagonism was not competitive, it was expressed as pAH (–log of the molar concentration of antagonist which inhibit 50% of the maximum effect of the agonist), according to the method of Ariens and Van Rossum.[64]

Acknowledgment. We thank Alcon Laboratories, Inc., for their financial support.

(62) Drew, G. M. *Eur. J. Pharmacol.* **1977**, *42*, 123.
(63) Billard, W.; Ruperto, V.; Crosby, G.; Iorio, L. C.; Barnett, A. *Life Sci.* **1984**, *35*, 1885.
(64) Ariens, E. J.; Van Rossum, J. M. *Arch. Int. Pharmacodyn. Ther.* **1957**, *110*, 275.
(65) Ariens, E. J. *Arch. Int. Pharmacodyn. Ther.* **1954**, *99*, 32.
(66) Arunlakshana, O.; Schild, H. O. *Br. J. Pharmacol.* **1959**, *14*, 48.

(Aminoalkyl)carbamates of Forskolin: Intermediates for the Synthesis of Functionalized Derivatives of Forskolin with Different Specificities for Adenylyl Cyclase and the Glucose Transporter

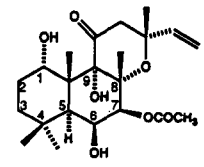
Joan D. Robbins,† Antonio Laurenza,† Raymond W. Kosley, Jr.,‡ Gerard J. O'Malley,‡ Bettina Spahl,‡ and Kenneth B. Seamon*,†

Laboratory of Molecular Pharmacology, Center for Biologics Evaluation and Research, Food and Drug Administration, 8800 Rockville Pike, Bethesda, Maryland 20892, and Hoechst-Roussel Pharmaceuticals, Somerville, New Jersey 08876.
Received December 18, 1990

(Aminoalkyl)carbamates of forskolin were synthesized at the 6- and 7-hydroxyl positions of forskolin with the length of the alkyl chain varying from ethyl to heptyl. Two of these derivatives, 7-[[(2-aminoethyl)amino]carbonyl]-7-desacetylforskolin (**2**) and 6-[[(2-aminoethyl)amino]carbonyl]forskolin (**3**), were used to synthesize iodinated derivatives of forskolin that bind with high affinity to adenylyl cyclase in bovine brain membranes and the glucose transporter in human erythrocyte membranes, respectively. Hydroxyphenyl derivatives of forskolin were prepared from the (aminoalkyl)carbamates and tested for their ability to bind to adenylyl cyclase in bovine brain membranes and the glucose transporter in human erythrocyte membranes. The 6-derivative (**18**) of forskolin had a K_d of 9 nM at adenylyl cyclase and was more potent than either the 7-derivatives or the 6-derivatives of 7-desacetylforskolin. The 7-derivatives were more potent at binding to the glucose transporter than forskolin. In contrast, the 6-derivatives had K_d's > 100 μM at the glucose transporter. Isothiocyanates and *N*-bromoacetyl derivatives were synthesized from **2** and **3** as potential alkylating agents for forskolin binding sites. The alkylating agents produced an irreversible loss of forskolin binding to adenylyl cyclase. In contrast, the alkylating agents bound reversibly to the glucose transporter.

Introduction

Forskolin, a diterpene natural product originally isolated from methanol extracts derived from the roots of *Coleus forskohlii* found in the Indian subcontinent,[1] interacts with a diverse group of membrane proteins including adenylyl cyclase and the glucose transporter.[2] Forskolin produces marked cardiotonic effects due to its ability to activate the enzyme adenylyl cyclase and increase intracellular cyclic AMP.[3] The ability of forskolin to interact directly with the catalytic subunit of adenylyl cyclase is a unique property of this diterpene and has been exploited extensively by biomedical researchers.[4] Forskolin increases cyclic AMP in vivo, which has promoted many investigations into the therapeutic potential of forskolin to treat



Forskolin

* To whom correspondence should be addressed at the Food and Drug Administration, 8800 Rockville Pike, Bethesda, MD 20892.
† Food and Drug Administration.
‡ Hoechst-Roussel Pharmaceuticals.

(1) Bhat, S. V.; Bajwa, B. S.; Dornauer, H.; De Souza, N. J. *Tetrahedron Lett.* **1977**, *19*, 1669.
(2) Laurenza, A.; McHugh-Sutkowski, E.; Seamon, K. B. *Trends Pharmacol. Sci.* **1989**, *10*, 442.
(3) Metzger, H.; Lindner, E. *Arzneim. Forsch.* **1981**, *31*, 1248.
(4) Seamon, K. B.; Daly, J. W. *Adv. Cyclic Nucl. Res.* **1986**, *20*, 1.

0022-2623/91/1834-3204$02.50/0

a number of indications including asthma,[5] glaucoma,[6] and heart disease.[7] There has also been a great interest in developing synthetic strategies for forskolin, and this has resulted in the complete synthesis of the diterpene.[8]

Analogues of forskolin with different acyl groups esterified at the 1α-, 6β-, and 7β-hydroxyl groups[9] as well as water-soluble derivatives of forskolin[10] have been synthesized and tested for their ability to activate adenylyl cyclase. Other derivatives of forskolin have been synthesized primarily for use in biochemical studies on forskolin binding proteins. These include α-haloacetyl derivatives of forskolin such as 7-(bromoacetyl)-7-desacetylforskolin and 7-(chloroacetyl)-7-desacetylforskolin,[11] which irreversibly block the forskolin binding site on adenylyl cyclase. 7-Desacetyl-7-hemisuccinylforskolin has been coupled to solid supports[12] for the isolation and purification of adenylyl cyclase and used as an intermediate for the synthesis of iodinated photoactivatable derivatives of forskolin for covalently labeling adenylyl cyclase and the glucose transporter.[13] However, there are potential problems in using ester analogues for in vivo and in vitro studies due to their susceptibility to hydrolysis and rearrangement under mildly basic conditions.[14,15]

Forskolin interacts with proteins other than adenylyl cyclase; however, the derivatives of forskolin that have been synthesized to date have not been designed to be specific for the different sites of action of forskolin. Most forskolin analogues have been synthesized in order to improve potency of stimulation of adenylyl cyclase. It would be desirable to have analogues of forskolin that would be stable and specific for forskolin binding proteins. The synthesis of such analogues could best be achieved by the use of intermediates that would have the following properties: (1) they would be stable compounds, (2) they would have reactive groups that would be readily modified to produce a number of different derivatives, and (3) the reactive groups would be placed at positions on forskolin such that they would afford derivatives with differing specificities for forskolin binding proteins.

Procedures have been developed for the selective acylation of the 6- or 7-hydroxyl groups via protection of the 1- and 9-hydroxyl groups with dimethylformamide dimethyl acetal.[15] A method has been developed to produce 6- and 7-carbamate derivatives of forskolin containing different groups attached to forskolin through a stable carbamate linkage.[16] 7-Carbamate derivatives were produced by the nucleophilic attack of primary and secondary amines on a 7-acyl imidazole intermediate of forskolin, and 6-carbamates were produced by the regioselective attack of primary and secondary amines on the 6,7-carbonate ester of forskolin. We have utilized these procedures to produce (aminoalkyl)carbamates of forskolin which were envisioned to be excellent compounds to serve as useful intermediates of forskolin. Carbamates are intrinsically more stable to hydrolysis than esters and are resistant to esterases. Primary amino groups react rapidly with activated esters such as succinimido esters to form amides and with isothiocyanates to form stable thioureas. With this rationale in mind, procedures were developed for the synthesis of analogues of forskolin which would be selective for different forskolin binding proteins.

Chemistry

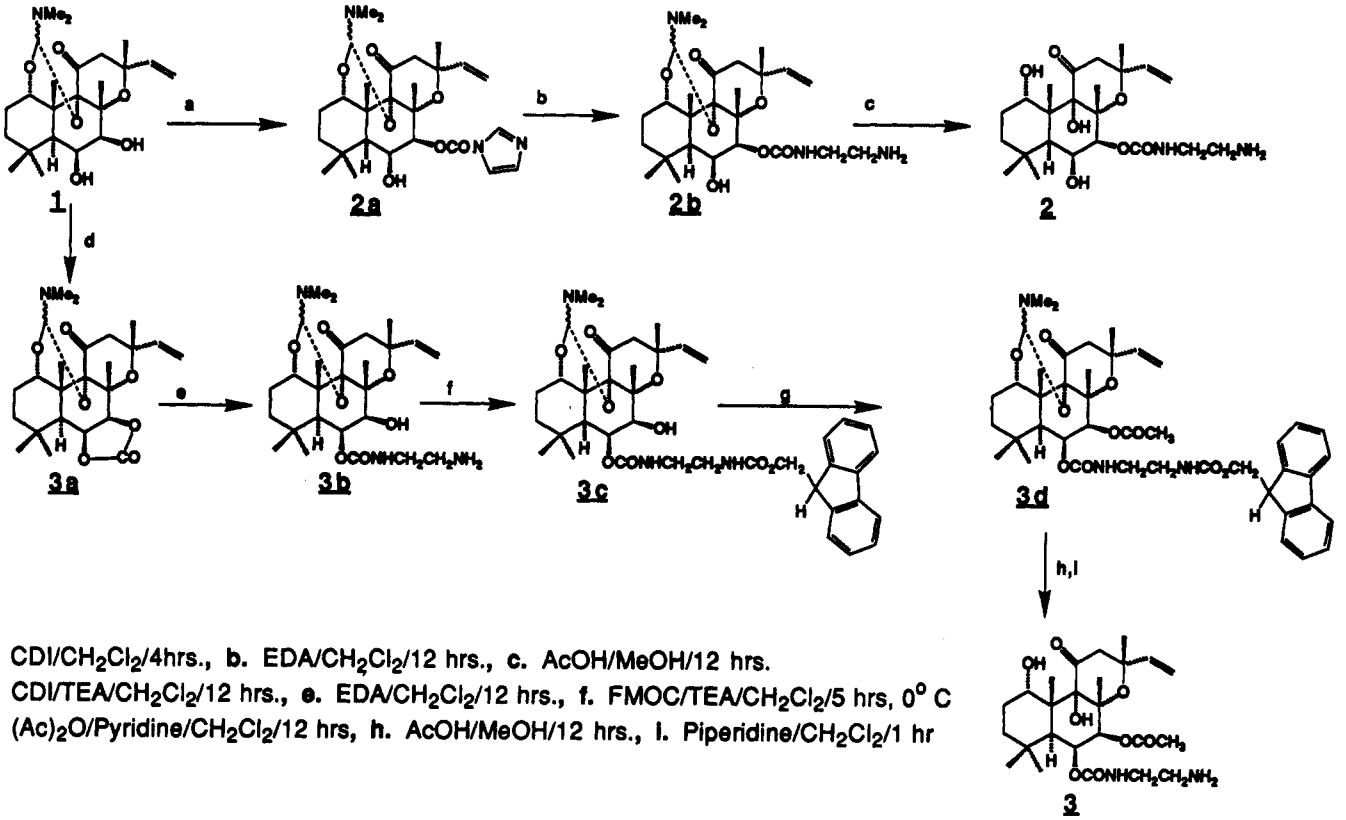
Forskolin contains four groups at which carbamate functionality could be introduced. These are the 1α-, 9α-, and 6β-hydroxyl groups and the 7β-acetoxy moiety. Derivatives of forskolin with the 1- or 9-hydroxyl group modified or absent fail to activate adenylyl cyclase. In contrast, 1,9-dideoxyforskolin inhibits glucose transport in adipocyte vesicles.[17] Previous studies demonstrated that derivatives of forskolin with heterocyclic amino acids esterified at the 6β-hydroxyl group are very potent at activating adenylyl cyclase.[10] Therefore, 6-(aminoalkyl)-carbamates of forskolin were synthesized as intermediates to form forskolin derivatives with specificity for adenylyl cyclase. Forskolin derivatives with lipophilic groups esterified at the 7β-hydroxyl group of forskolin can inhibit glucose transport[17] and are more potent than forskolin at the glucose transporter.[13a] Therefore, 7-(aminoalkyl)carbamates of forskolin were synthesized as intermediates to produce derivatives with enhanced affinity for the glucose transporter.

The (aminoalkyl)carbamates (**2**, **3**) were synthesized with use of the 1,9-dimethylformamide acetal of 7-desacetylforskolin as starting material[15] (Scheme I). The selective reactivity of carbonyldiimidazole (CDI) with the equatorial 7-hydroxyl group of forskolin was exploited to form the 7-acylimidazole intermediate **2a**.[16] Subsequent reaction of **2a** with ethylenediamine (EDA) gave the (aminoethyl)carbamate **2b** in 81% yield. Formation of the migration product, 6-(aminoethyl)carbamate **3b**, was observed if the reaction with CDI was allowed to proceed longer than 8 h prior to the addition of EDA. The formamide acetal group was removed with acetic acid in methanol to give **2**, which was purified by silica gel chromatography. The same procedure was utilized to produce **4**–**7**.

Synthesis of the regioisomeric product **3** was accomplished via the cyclic carbonate **3a** as a key intermediate. Nucleophilic ring opening of the 6,7-carbonate **3a** by primary or secondary amines produced exclusively 6-carbamates.[16] The 6,7-carbonate **3a** was synthesized by reacting the 1,9-dimethylformamide acetal of 7-desacetyl-

(5) Burka, J. *Can. J. Physiol. Pharmacol.* **1983**, *61*, 581.
(6) Caprioli, J.; Sears, M.; Bausher, L.; Gregory, D.; Mead, A. *Invest. Opthalmol. Vis. Sci.* **1984**, *25*, 268.
(7) Briston, M. R.; Ginsburg, R.; Strosberg, A.; Montgomery, W.; Minobe, W. *J. Clin. Invest.* **1984**, *74*, 212.
(8) (a) Jenkins et. al. *J. Chem. Soc., Chem. Commun.* **1984**, 1423. (b) Nicolaou, K. C.; Li, W. S. *J. Chem. Soc., Chem. Commun.* **1985**, 421. (c) Ziegler, F. E.; Jaynes, B. H.; Saindane, M. T. *Tetrahedron Lett.* **1985**, *26*, 3307. (d) Ziegler, F. E.; Jaynes, B. H.; Saindane, M. T. *J. Am. Chem. Soc.* **1987**, *109*, 8115. (e) Hashimoto, S.; Sakata, S.; Sonegawa, M.; Ikegami, S. *J. Am. Chem. Soc.* **1988**, *110*, 3670. (f) Corey, E. J.; Jardine Da Silva, P.; Rohloff, J. C. *J. Am. Chem. Soc.* **1988**, *110*, 3672.
(9) (a) Bhat, S. V.; Dohadwalla, A. N.; Bajwa, B. S.; Dadkar, N. K.; Dornauer, H.; DeSouza, N. J. *J. Med. Chem.* **1983**, *26*, 486. (b) Seamon, K. B.; Daly, J. W.; Metzger, H.; DeSouza, N. J.; Reden, J. *J. Med. Chem.* **1983**, *26*, 436.
(10) (a) Khandelwal, Y.; Rayeshwari, R.; Rajagopalan, R.; Lakshim, S. W.; Dohadwalla, A. N.; DeSouza, N. J.; Rupp, R. H. *J. Med. Chem.* **1988**, *31*, 1872. (b) Laurenza, A.; Khandelwal, Y.; DeSouza, N. J.; Rupp, R. H.; Metzger, H.; Seamon, K. B. *Mol. Pharmacol.* **1987**, *32*, 133.
(11) Laurenza, A.; Morris, D. I.; Seamon, K. B. *Mol. Pharmacol.* **1990**, *37*, 69.
(12) (a) Pfeuffer, E.; Dreher, R. M.; Metzger, H.; Pfeuffer, T. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *83*, 3086. (b) Pfeuffer, E.; Mollner, S.; Pfeuffer, T. *EMBO J.* **1985**, *4*, 3675. (c) Smigel, M. D. *J. Biol. Chem.* **1986**, *201*, 1976.
(13) (a) Wadzinski, B. E.; Shanahan, M. F.; Ruoho, A. E. *J. Biol. Chem.* **1987**, *262*, 17683. (b) Pfeuffer, E.; Pfeuffer, T. *FEBS Lett.* **1989**, *248*, 13.
(14) Selfe, S.; Storm, D. R. *Biochem. Biophys. Res. Commun.* **1985**, *133*, 52.
(15) Kosley, R. W., Jr.; Cherill, R. J. *J. Org. Chem.* **1989**, *54*, 2972.
(16) O'Malley, G. J.; Spahl, B.; Cherill, R. J.; Kosley, R. W., Jr. *J. Org. Chem.* **1990**, *55*, 1102.
(17) Joost, H. G.; Habberfield, A. D.; Simpson, I. A.; Laurenza, A.; Seamon, K. B. *Mol. Pharmacol.* **1988**, *33*, 449.

Scheme I



forskolin with CDI and triethylamine for 16 h. Further reaction of **3a** with EDA gave exclusively the desired intermediate **3b**. The transformation of **3b** to the target carbamate **3** was accomplished in the following sequential steps: conversion of **3b** to the (fluorenylmethoxy)carbonyl (FMOC) derivative **3c**; acylation with acetic anhydride to give **3d**; removal of the formamide acetal group with acetic acid in methanol; FMOC deprotection by treatment with piperidine; and purification by silica gel chromatography yielding the crystalline product **3**. This general procedure was used for the synthesis of compounds 8–10.

Results and Discussion

Binding Assays. The (aminoethyl)carbamate derivatives **2** and **3** were reacted with commercially available [^{125}I]-Bolton-Hunter reagent to produce the radioactive 2-[3-(4-hydroxy-3-[^{125}I]iodophenyl)propanamido] derivatives. These were then used to develop ligand binding assays for adenylyl cyclase in bovine brain membranes and the glucose transporter in human erythrocyte membranes. The filtration binding assays used rapid filtration through glass fiber filters to separate the bound from free ligands.

The iodinated derivative synthesized from **2**, [^{125}I]-2-[3-(4-hydroxy-3-iodophenyl)propanamido]-*N*-ethyl-7-(aminocarbonyl)-7-desacetylforskolin ([^{125}I]-7-IHPP-Fsk), binds with high affinity to the glucose transporter in human erythrocyte membranes and to the glucose transporter in other tissues.[18] The affinity of [^{125}I]-7-IHPP-Fsk to human erythrocyte membranes was determined by incubating tracer amounts of [^{125}I]-7-IHPP-Fsk with increasing amounts of nonradioactive 7-IHPP-Fsk. The data were then analyzed using the Ligand program developed by Munson and Rodbard.[19] Data were analyzed for a one site fit or a two site fit. Combined data from two different experiments analyzed for a one-site fit are shown in Figure 1A. The affinity of [^{125}I]-7-IHPP-Fsk to human erythrocyte membranes was 83 nM ± 20 nM. There was no significant improvement in the analysis by utilizing a

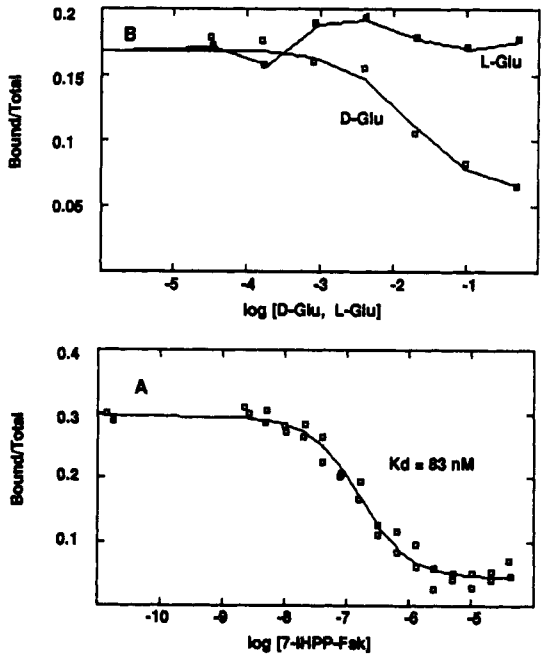
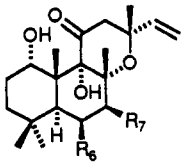


Figure 1. Binding of [^{125}I]-7-IHPP-Fsk to human erythrocyte membranes. (A) Displacement of [^{125}I]-7-IHPP-Fsk from human erythrocyte membranes by nonradioactive 7-IHPP-Fsk. Data from two experiments is shown and was analyzed using the Ligand program. The K_d for [^{125}I]-7-IHPP-Fsk was 83 ± 11 nM. (B) Displacement of [^{125}I]-7-IHPP-Fsk binding to bovine brain membranes by D-glucose (□) and L-glucose (■). Data from two different experiments for each compound are shown and were analyzed by the Ligand program. The K_d for D-glucose was 17 ± 5 mM.

(18) Laurenza, A.; Robbins, J. D.; Simpson, I. A.; Seamon, K. B. Unpublished results.
(19) Munson, P. J.; Rodbard, D. *Anal. Biochem.* **1980**, *107*, 239.

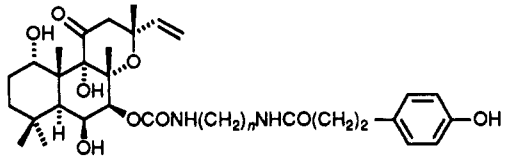
Table I. Affinity of (Aminoalkyl)carbamates of Forskolin at Adenylyl Cyclase and the Glucose Transporter



compd	R_6	R_7	K_d[a] adenylyl cyclase	K_d[a] glucose transporter
forskolin	OH	$OCOCH_3$	29 ± 7 nM	5 ± 2 μM
2	OH	$OCONH(CH_2)_2NH_2$	98 ± 17 nM	28 ± 5 μM
3	$OCONH(CH_2)_2NH_2$	$OCOCH_3$	34 ± 10 nM	>100 μM[b]
7	OH	$OCONH(CH_2)_2C_6H_4OH$	61 ± 20 nM	0.24 ± .04 μM
10	$OCONH(CH_2)_2C_6H_4OH$	OH	236 ± 52 nM	>100 μM[b]

[a] K_d's ± SE were determined from the inhibition of [^{125}I]-6-IHPP-Fsk binding to bovine brain membranes for adenylyl cyclase or the inhibition of [^{125}I]-7-IHPP-Fsk binding to human erythrocyte membranes for the glucose transporter as described in the Experimental Section. [b] There was less than 50% inhibition of control binding at a concentration of 100 μM.

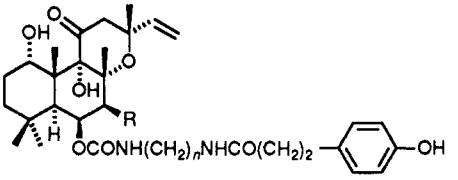
Table II. Effect of Alkyl Chain Length on Affinity of 7-Carbamate Derivatives of Forskolin at Adenylyl Cyclase and the Glucose Transporter



compd	n	K_d[a] adenylyl cyclase	K_d[a] glucose transporter
11	2	13 ± 3 nM	1.1 ± 0.2 μM
12	4	60 ± 14 nM	0.8 ± 0.1 μM
13	6	82 ± 16 nM	0.4 ± 0.1 μM
14	7	53 ± 9 nM	0.1 ± 0.03 μM

[a] K_d's ± SE were determined from the inhibition of [^{125}I]-6-IHPP-Fsk binding to bovine brain membranes for adenylyl cyclase or the inhibition of [^{125}I]-7-IHPP-Fsk binding to human erythrocyte membranes for the glucose transporter as described in the Experimental Section.

Table III. Effect of Alkyl Chain Length and 7-Acetylation on the Affinity of 6-Carbamate Derivatives of Forskolin at Adenylyl Cyclase and the Glucose Transporter



compd	R	n	K_d[a] adenylate cyclase	K_d[a] glucose transporter
15	OH	2	174 ± 70 nM	>100 μM[b]
16	OH	4	80 ± 24 nM	>100 μM[b]
17	OH	6	94 ± 25 nM	>100 μM[b]
18	$OCOCH_3$	2	9 ± 2 nM	2.8 ± 0.7 μM

[a] K_d's ± SE were determined from the inhibition of [^{125}I]-6-IHPP-Fsk binding to bovine brain membranes for adenylyl cyclase or the inhibition of [^{125}I]-7-IHPP-Fsk binding to human erythrocyte membranes for the glucose transporter as described in the Experimental Section. [b] There was less than 50% inhibition of control binding at a concentration of 100 μM.

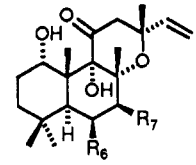
two-site fit of the data. [^{125}I]-7-IHPP-Fsk binding to human erythrocyte membranes was inhibited by D-glucose but not by L-glucose consistent with the binding being associated with the glucose transporter (Figure 1B). The glucose transport inhibitor cytochalasin B, but not its inactive analogue cytochalasin E, inhibited the binding of [^{125}I]-7-IHPP-Fsk to human erythrocyte membranes (data not shown). Forskolin derivatives were tested for their ability to bind to the glucose transporter by measuring their inhibition of [^{125}I]-7-IHPP-Fsk binding to human erythrocyte membranes. Human erythrocyte membranes were incubated with tracer amounts of [^{125}I]-7-IHPP-Fsk (about 30 000 dpm) and varying concentrations of the forskolin derivative. The inhibition data from two experiments were analyzed together using the Ligand program, and the K_d's are given in Tables I–IV.

[^{125}I]-7-IHPP-Fsk has been used to measure the glucose transporter in bovine brain membranes.[18] However, a small percentage, about 10%, of the binding does appear to be associated with adenylyl cyclase. The D-glucose inhibitable binding of [^{125}I]-7-IHPP-Fsk to bovine brain membranes can be defined as that component of [^{125}I]-7-IHPP-Fsk binding associated with the glucose transporter. Therefore, [^{125}I]-7-IHPP-Fsk can be used as a ligand for the glucose transporter; however, this ligand does have some affinity for adenylyl cyclase. It is interesting to note that a photoaffinity label synthesized from **2** identified the glucose transporter in bovine brain membranes but did not label the adenylyl cyclase in the same membranes.[20]

The iodinated derivative of **3**, [^{125}I]-2-[3-(4-hydroxy-3-iodophenyl)propanamido]-*N*-ethyl-6-(aminocarbonyl)forskolin ([^{125}I]-6-IHPP-Fsk), binds with high affinity to adenylyl cyclase in bovine brain membranes. The affinity of [^{125}I]-6-IHPP-Fsk to bovine brain membranes was determined by incubating a tracer amount of [^{125}I]-6-IHPP-Fsk with increasing concentrations of nonradioactive 6-IHPP-Fsk. The binding was then analyzed using the Ligand program. [^{125}I]-6-IHPP-Fsk binds to bovine brain membranes at a single class of sites with a K_d of 8.8 nM ± 3 nM (Figure 2A). There was no significant improvement in the analysis of the data by utilizing a two-site fit. The binding of [^{125}I]-6-IHPP-Fsk to bovine brain membranes is inhibited by forskolin but not by 1,9-dideoxyforskolin consistent with these sites being associated with adenylyl cyclase (Figure 2B). The affinity of forskolin to inhibit [^{125}I]-6-IHPP-Fsk binding, 28 nM, is the same as the affinity for [^{3}H]forskolin binding to bovine brain membranes. The binding of [^{125}I]-6-IHPP-Fsk to bovine brain membranes exhibited the same binding characteristics as that of [^{3}H]forskolin.[18] The binding of forskolin derivatives to adenylyl cyclase was determined from their

(20) Morris, D.; Robbins, J. D.; McHugh-Sutkowski, E.; Ruoho, A.; Seamon, K. B. *J. Biol. Chem.* **1991**, *266*, 13377. Morris, D.; Speicher, L.; Ruoho, A.; Tew, K.; Seamon, K. B. *Biochemistry* **1991**, *30*, 8371.

Table IV. Affinity of *N*-Bromoacetyl and Isothiocyanate Derivatives of Forskolin at Adenylyl Cyclase and the Glucose Transporter



compd	R_6	R_7	adenylate cyclase, K_d(exp),[a] nM	glucose transporter, K_d,[a] μM
19	OH	$OCONH(CH_2)_2NHCOCH_2Br$	10 ± 5	1.3 ± 0.6
20	OH	$OCONH(CH_2)_2NCS$	110 ± 40	0.5 ± 0.2
21	$OCONH(CH_2)_2NHCOCH_2Br$	$OCOCH_3$	10 ± 3	17 ± 3
22	$OCONH(CH_2)_2NCS$	$OCOCH_3$	130 ± 50	0.3 ± 0.1

[a] K_d's ± SE were determined from the inhibition of [^{125}I]-6-IHPP-Fsk binding to bovine brain membranes for adenylyl cyclase or the inhibition of [^{125}I]-7-IHPP-Fsk binding to human erythrocyte membranes for the glucose transporter as described in the Experimental Section. The experiments measuring the binding of [^{125}I]-6-IHPP-Fsk to bovine brain membranes did not account for the irreversible loss of binding sites and therefore an experimentally determined apparent K_d(exp) is reported. There was no irreversible inhibition of [^{125}I]-7-IHPP-Fsk binding to human erythrocyte membranes, and therefore the values for the glucose transporter are given as K_d's.

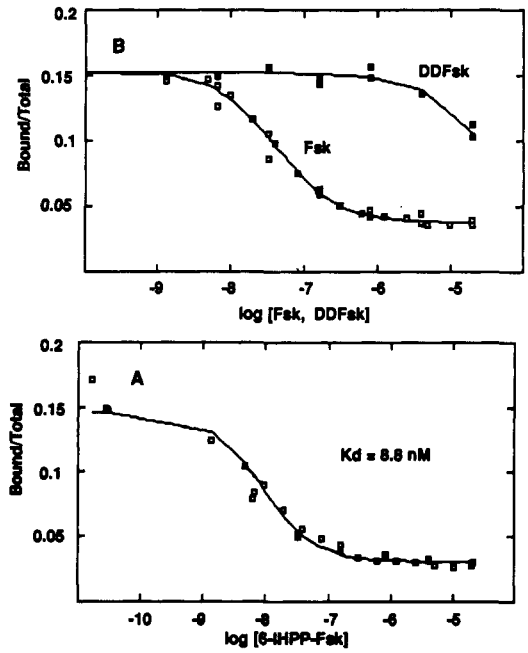


Figure 2. Binding of [^{125}I]-6-IHPP-Fsk to bovine brain membranes. (A) Displacement of [^{125}I]-6-IHPP-Fsk from bovine brain membranes by nonradioactive 6-IHPP-Fsk. Data from two experiments is shown and was analyzed using the Ligand program. The K_d for [^{125}I]-6-IHPP-Fsk was 8.8 nM ± 3 nM. (B) Displacement of [^{125}I]-6-IHPP-Fsk binding to bovine brain membranes by forskolin (□) and 1,9-dideoxyforskolin (■). Data from two different experiments for each compound is shown and were analyzed using the Ligand program. The K_d for forskolin binding was 28 ± 7 nM and the K_d for 1,9-dideoxyforskolin was 30 ± 3 μM.

inhibition of [^{125}I]-6-IHPP-Fsk binding to bovine brain membranes. Bovine brain membranes were incubated with tracer amounts of [^{125}I]-6-IHPP-Fsk, about 30 000 dpm, and the unlabeled derivative. The binding data from two different experiments was analyzed using the Ligand program and the K_d's are given in Tables I–IV.

There was no displaceable binding of [^{125}I]-6-IHPP-Fsk to human erythrocyte membranes using the filtration binding assay as described in the Experimental Section. Therefore, this ligand does not bind to the glucose transporter with an affinity high enough to be measured using a filtration binding assay.

Adenylyl Cyclase. The (aminoalkyl)carbamates of forskolin were tested for their ability to inhibit the high affinity binding of [^{125}I]-6-IHPP-Fsk to bovine brain membranes (Table I). Compound **3**, with a K_d of 34 nM, was equipotent with forskolin while compound **2** was less potent with a K_d of 98 nM.

(Hydroxyphenyl)propanamido derivatives of forskolin were synthesized by reacting the (aminoalkyl)carbamates with *N*-succinimidyl-3-(4-hydroxyphenyl)propionic acid. The 7-alkylcarbamate derivatives **11–14**, were relatively potent with **11** being equipotent with forskolin at adenylyl cyclase with a K_d of 13 nM (Table II). However, derivatives produced from the butyl, hexyl, and heptyl intermediates, **12–14**, were less potent than the ethyl derivative, **11**. The 6-alkylcarbamate derivatives of 7-desacetylforskolin, **15–17**, were less potent than forskolin at adenylyl cyclase with K_d's of about 100 nM (Table III). Interestingly, the 7-[[(4-hydroxyphenyl)ethylamino]carbonyl] derivative of 7-desacetylforskolin, **7**, had a K_d of 61 nM and was more potent than 7-desacetyl-6-[[*N*-[2-(4-hydroxyphenyl)ethyl]amino]carbonyl]forskolin, **10**, which had a K_d of 236 nM. These results are consistent with other data that indicate that 6-acyl derivatives of 7-desacetylforskolin are much less potent than the same 6-acyl derivatives of forskolin.[11] The presence of the acetyl group at the 7-position is important for potency at adenylyl cyclase. Acetylation of the 7-hydroxyl group of derivative **15** to produce **18** is associated with almost a 20-fold increase in potency at adenylyl cyclase.

Attempts to define the optimal chain length for the alkyl group did not produce derivatives that were more potent than **3**. Increasing the alkyl chain from ethyl to hexyl for the 6-derivatives of 7-desacetylforskolin, **15–17**, produced only a moderate increase in potency at adenylyl cyclase.

Glucose Transporter. Forskolin binds to the glucose transporter with an affinity of 5 μM. However, forskolin derivatives that contain lipophilic groups esterified at the 7-position are more potent than forskolin at the glucose transporter.[13a] The 7-(aminoethyl)carbamate **2** had a K_d of 28 μM and was less potent than forskolin (Table I). In contrast, the 6-(aminoethyl)carbamate **3** was not effective at inhibiting the binding and had a K_d that was greater than 100 μM. The [[(4-hydroxyphenyl)ethyl]amino]-carbonyl derivatives **7** and **10** exhibited the same selectivity with the 7-derivative **7** having a K_d of 0.24 μM and the 6-derivative **10** having a K_d greater than 100 μM.

The effect of the length of the alkyl side chain of the 7-(aminoalkyl)carbamate derivatives was examined. Compounds **11–14** were relatively potent at the glucose transporter (Table II). The heptyl derivative, **14**, was the most potent with a K_d of 0.1 μM and the least potent was the ethyl derivative, **11**, with a K_d of 1.1 μM. The iodinated

analogue of **11**, [^{125}I]-7-IHPP-Fsk, binds to the glucose transporter with an affinity of 83 nM. The presence of the iodine increased the potency about 10-fold. It is interesting to note that the iodinated derivative of **14** synthesized by reacting **6** with [^{125}I]-Bolton-Hunter reagent did not exhibit displaceable binding to human erythrocyte membranes. The addition of iodine to **14** may have had no effect or possibly caused a reduction in potency at the glucose transporter.

6-Derivatives of 7-desacetylforskolin, **15–17**, were not potent at binding to the glucose transporter and had K_d's greater than 100 μM (Table III). Compound **18**, synthesized from the 6-(aminoethyl)carbamate to forskolin, had a K_d of 2.8 μM and was more potent than the corresponding derivative of 7-desacetylforskolin, **15**.

Alkylating Derivatives of Forskolin. *N*-Bromoacetyl and isothiocyanate derivatives of **2** and **3** were synthesized as potential affinity ligands for forskolin binding sites. The derivatives were tested for their ability to irreversibly inhibit the binding of [^{125}I]-6-IHPP-Fsk to adenylyl cyclase in bovine brain membranes and [^{125}I]-7-IHPP-Fsk binding to the glucose transporter in human erythrocyte membranes.

The *N*-bromoacetyl derivatives **19** and **21** and the isothiocyanate derivatives **20** and **22** at concentrations of 20 μM irreversibly inhibited over 65% of the specific [^{125}I]-6-IHPP-Fsk binding to bovine brain membranes (Figure 3A). The experimentally determined K_d's, K_d(exp), for the alkylating derivatives to inhibit [^{125}I]-6-IHPP-Fsk binding to bovine brain membranes was determined by using the same protocol as for the other forskolin derivatives. These experiments did not account for the irreversible loss of binding sites, and therefore an experimentally determined apparent K_d(exp) is reported. The *N*-bromoacetyl derivatives **19** and **21** had K_d(exp)'s of 10 nM. In contrast, the isothiocyanate derivatives **20** and **22** were less potent than the *N*-bromoacetyl derivatives and had K_d(exp)'s of about 100 nM.

In contrast to the results with adenylyl cyclase, there was no irreversible inhibition of [^{125}I]-7-IHPP-Fsk binding to human erythrocyte membranes (Figure 3B). The binding of the alkylating derivatives to the glucose transporter was determined as described for the other forskolin derivatives. The 6- and 7-isothiocyanate derivatives **22** and **20** were equipotent at inhibiting [^{125}I]-7-IHPP-Fsk binding with K_d's 0.3 and 0.5 μM, respectively. The 7-*N*-bromoacetyl derivative **19** had a K_d of 1.3 μM and was more potent than the 6-*N*-bromoacetyl derivative **21**, which had a K_d of 17 μM.

Characteristics of Forskolin Binding Sites. A model has been proposed whereby forskolin binds at the glucose transporter in a similar manner as α-D-glucose.[2,17] In this model the 6-hydroxyl group of forskolin and the 8,13-ether oxygen of forskolin bind at similar sites as the 1-hydroxyl group and the 3-hydroxyl group of α-D-glucose, respectively. Lipophilic derivatives of **2** are about 100-fold more potent at the glucose transporter than the same lipophilic derivatives of **3**. Therefore, it seems reasonable that the inability of derivatives of **3** to bind to the glucose transporter is due to the absence of the free hydroxyl group which is present in derivatives of **2**. This would suggest that there is an important hydrogen-bond interaction at the 6-hydroxyl group of forskolin (1-hydroxyl of D-glucose) which is important for binding. Although the 6-hydroxyl of forskolin is in an axial configuration and there is a dimethyl group at C-4 of forskolin, space-filling models indicate that the 6-hydroxyl of forskolin is relatively accessible for hydrogen-bonding interactions. An almost

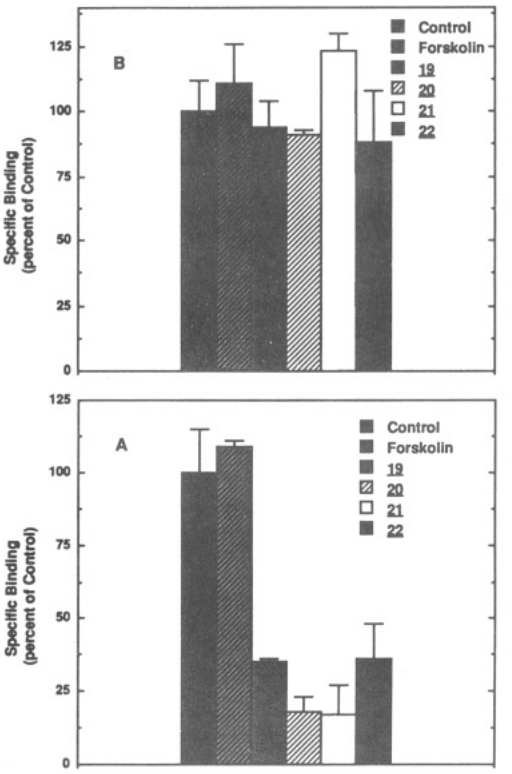


Figure 3. Effect of alkylating derivatives of forskolin on [^{125}I]-6-IHPP-Fsk binding to bovine brain membranes and [^{125}I]-7-IHPP-Fsk binding to human erythrocyte membranes. (A) Bovine brain membranes were incubated with 20 μM of the indicated derivative, washed extensively, and [^{125}I]-6-IHPP-Fsk binding was determined as described in the experimental section. Specific binding was defined as the difference between total binding and nonspecific binding determined in the presence of 100 μM forskolin. (B) Human erythrocyte membranes were incubated with 20 μM of the indicated derivative, washed extensively, and [^{125}I]-7-IHPP-Fsk binding was determined as described in the experimental section. Specific binding was defined as the difference between total binding and nonspecific binding determined in the presence of 100 μM forskolin.

identical configuration can be obtained for the 1-hydroxyl group of the β-anomer of D-glucose. Lipophilic derivatives of forskolin at the 7-position are more potent at the glucose transporter than forskolin. The enhanced affinity of lipophilic derivatives of forskolin at the glucose transporter is consistent with the lipophilic groups occupying a hydrophobic binding domain. We would therefore suggest that forskolin is binding at a substrate binding site of the glucose transporter in a manner analogous to transported hexoses and the lipophilic side chains of compounds such as [^{125}I]-7-IHPP-Fsk are occupying a hydrophobic binding domain that is spatially close to the substrate binding site.

Many of the 6-(aminoethyl)carbamyl derivatives of forskolin are equipotent with forskolin at adenylyl cyclase. However, none of these compounds has a potency that is much greater than that of forskolin. Studies have clearly demonstrated the need for the 1- and 9-hydroxyl groups of forskolin;[9] however, there has not been any demonstration of structural modifications in forskolin that increase the potency at adenylyl cyclase. Although, some of the derivatives described in this paper, **11** and **18**, are slightly more potent than forskolin at adenylyl cyclase, they do not indicate any strong leads for significantly improving the potency of forskolin at adenylyl cyclase. However, it is interesting that forskolin can accommodate both lipophilic groups and hydrophilic groups at the 6-position without a loss of potency at the forskolin binding

site on adenylyl cyclase. This is in contrast to the binding region oriented toward the 7-position which appears to be more restrictive and does not accommodate lipophilic groups but rather can accommodate charged groups.[9,10,17]

Conclusion

The purpose of this work was to synthesize intermediates of forskolin that would be useful in the synthesis of forskolin derivatives with different specificities at forskolin binding proteins. (Aminoalkyl)carbamates of forskolin were synthesized at the 6- and 7-positions on forskolin. The synthetic protocol for making the 7-(aminoalkyl)-carbamate has also been utilized to synthesize 1-(aminoalkyl)carbamates of forskolin. The (aminoalkyl)carbamates are easy to synthesize and provide stable crystalline products that are useful in the synthesis of a variety of forskolin derivatives, for example, fluorescent derivatives, biotinylated derivatives, affinity columns, and radioactive photoaffinity labels.

Derivatives of 6-[(aminoethyl)carbamyl]forskolin bind with high affinity to adenylyl cyclase and do not bind to the glucose transporter in human erythrocyte membranes. Derivatives of 7-(aminoalkyl)carbamates are more potent than forskolin at binding to the glucose transporter and can be used to develop ligand binding assays as well as photoaffinity agents for the glucose transporter. Although, the 7-derivatives are relatively potent at adenylyl cyclase, the selectivity of these ligands for the glucose transporter can be assessed by their sensitivity to agents that bind to the glucose transporter such as D-glucose and cytochalasin B. It is anticipated that the (aminoalkyl)carbamate derivatives of forskolin will be useful for synthesizing ligands whose specificities differ for forskolin binding proteins. For example, iodinated photoaffinity labels have been synthesized with different specificities for adenylyl cyclase, the glucose transporter, and the P-glycoprotein.[20]

Experimental Section

Synthetic Procedures. Analytical thin-layer chromatography was performed on silica gel (silica gel on polyester with indicator, Sigma Chemical Co.). Visualization of the plates was by iodine vapor, UV light, or detection of amines with trinitrobenzenesulfonic acid (TNBS) spray. ^{1}H NMR spectra were recorded on a 300-MHz Bruker spectrometer in $CDCl_3$ unless otherwise indicated. Chemical shifts are reported in parts per million (δ) downfield from tetramethylsilane as internal standard, with peak multiplicities being indicated as follows: s, singlet; d, doublet; t, triplet; m, multiplet, dd, double of doublets. Chemical-ionization mass spectra were recorded on an Extrel ELQ-400-3 spectrometer and samples were introduced on a Vacumetrics DCI probe. The mass spectra revealed $(M + H)^+$ for each compound synthesized. Elemental analysis on compounds **2** and **3** were performed by Atlantic Microlab, Inc. Atlanta, GA, and were within 0.4% of theoretical. Preparative column chromatography was performed on silica gel (Analtech, sorbent silica gel 10 μ). Organic materials were from Aldrich Chemicals and were used without further purification. The starting material, the 1,9-dimethylformamide acetal protected 7-desacetylforskolin, was synthesized by a published procedure.[15]

Forskolin is a naturally occurring diterpene, and it is difficult to obtain large quantities of the compound for semisynthetic studies. The (aminoalkyl)carbamates and derivatives were synthesized in small amounts sufficient for biological testing and for testing by NMR spectroscopy, mass spectroscopy, and TLC in order to conserve starting materials. The chemical shifts of the H-1, H-6, and H-7 protons of forskolin have been extensively studied[1,9a,15,16] and upon modification of their paired hydroxyl group exhibit a unique and reliable indication of the chemical change at each of these positions. Furthermore, the chemical shifts of the methyl groups, the H-12 protons (α to the carbonyl group), and the H-14, H-15 vinyl protons are very sensitive to any modifications of forskolin. Proof of purity therefore depended on single spots by TLC using two different solvent systems as well as the absence of "double sets of resonances" determined by ^{1}H NMR spectroscopy.

Synthesis of 7-[[(2-Aminoethyl)amino]carbonyl]-7-desacetylforskolin (2). The 1,9-dimethylformamide acetal of 7-desacetylforskolin (300 mg, 0.709 mmol) was dissolved in 3 mL of methylene chloride. Carbonyldiimidazole (172 mg, 1.06 mmol) was added and the reaction was stirred at room temperature for 6–8 h. A 5-fold excess of ethylenediamine (213 mg, 3.5 mmol) was added and the reaction was stirred overnight. The reaction was diluted with methylene chloride (3 mL) and washed one time with water (3 mL). The organic layer was dried in vacuo. The acetal protecting group was removed by overnight hydrolysis in acetic acid/methanol 1:1.5 (3 mL). The reaction was brought to pH 10 with saturated sodium carbonate and extracted three times with methylene chloride (5 mL). The crude product was dried in vacuo, dissolved in a minimum volume of chloroform, and purified by flash chromatography on a silica gel column (12 g) equilibrated with chloroform/methanol (9/1) and eluted with chloroform/methanol/triethylamine (9:1:0.1). The column was monitored by TLC using a spray reagent of trinitrobenzenesulfonic acid (TNBS) in acetone to visualize the amine product. Pure **2** (211 mg, 65%) was dried to a white crystalline solid in vacuo: ^{1}H NMR δ 5.95 (dd, 1 H, H14), 5.24 (d, 1 H, H15), 5.20 (d, 1 H, H7), 4.95 (d, 1 H, H15), 4.52 (m, 2 H, H1,6), 3.27 (m, 2 H, $NHCH_2CH_2NH_2$), 3.20 (d, 1 H, H12), 2.84 (t, 2 H, $NHCH_2CH_2NH_2$), 2.42 (d, 1 H, H12), 2.30 (d, 1 H, H5), 1.70 (s, 3 H, CH_3), 1.42 (s, 3 H, CH_3), 1.34 (s, 3 H, CH_3), 1.24 (s, 3 H, CH_3), 1.01 (s, 3 H, CH_3); R_f = 0.34 (EtOAc/HOAc/H_2O 8/1/1), R_f = 0.25 ($CHCl_3$/MeOH/NH_3 9:1:0.1) ($C_{23}H_{38}O_7N_2$) C, H, N.

Synthesis of 7-(Aminoalkyl)carbamates (4–7). 7-(4-Aminobutyl)carbamate **4**, 7-(6-aminohexyl)carbamate **5**, 7-(7-aminoheptyl)carbamate **6**, and 4-[(hydroxyphenyl)ethyl]carbamate **7** were synthesized as described for **2** with 1,4-diaminobutane, 1,6-diaminohexane, 1,7-diaminoheptane, and tyramine, respectively, instead of ethylenediamine. All compounds were purified by chromatography on silica gel in $CHCl_3$/MeOH 9:1.

Synthesis of 6-[[(2-Aminoethyl)amino]carbonyl]forskolin (3). The 1,9-dimethylformamide acetal of 7-desacetylforskolin (300 mg, 0.709 mmol) was dissolved in 3 mL of methylene chloride. Triethylamine (246 μL, 1.77 mmol) and carbonyldiimidazole (172 mg, 1.06 mmol) were added, and the reaction was stirred overnight at room temperature and monitored by TLC for the formation of the 6,7-carbonate **3a** (see Scheme I; R_f = 0.70, methylene chloride/ethyl acetate (1:1)). A 5-fold excess (213 mg, 3.5 mmol) of ethylenediamine was added, and the reaction was stirred overnight. The sample was diluted in methylene chloride (5 mL), washed with water (3 mL), and dried in vacuo. The free amine **3b** was dissolved in 2 mL of methylene chloride and put in an ice bath. Triethylamine (197 μL) and (9-fluorenylmethoxy)-carbonyl chloride (FMOC, 183 mg, 1.4 mmol) were added, the reaction was stirred on ice for 5 h and applied to a 5-g silica gel column eluted with hexane/ethyl acetate (4:6). The FMOC derivative **3c** was dried and dissolved in 2 mL of methylene chloride, and pyridine (52 μL, 1.06 mmol), a crystal of (dimethylamino)-pyridine (ca. 1 mg), and acetic anhydride (203 μL, 3.5 mmol) were added. The reaction was stirred overnight at room temperature to form the 7-acetyl derivative **3d** and then dried in vacuo. The acetal protecting group was removed by overnight hydrolysis in 3 mL of acetic acid/methanol (1:1.5). The sample was diluted in methylene chloride and washed one time with water. The FMOC group was removed in 20% piperidine/methylene chloride (0.4 mL/2 mL) at room temperature for 1 h, then dried, and purified by silica gel (12 g) chromatography eluting with chloroform/methanol 9:1. The TNBS-positive fractions were pooled and dried in vacuo, resulting in a white crystalline product (138 mg, 39%): ^{1}H NMR δ 5.95 (dd, 1 H, H14), 5.68 (t, 1 H, H6), 5.51 (d, 1 H, H7), 5.30 (d, 1 H, H15), 5.00 (d, 1 H, H15), 4.60 (t, 1 H, H1), 3.27 (m, 2 H, $NHCH_2CH_2NH_2$), 3.22 (d, 1 H, H12), 2.84 (m, 2 H, $NHCH_2CH_2NH_2$), 2.40 (d, 1 H, H12), 2.42 (d, 1 H, H5), 2.15 (s, 3 H, $OCOCH_3$), 1.66 (s, 3 H, CH_3), 1.43 (s, 3 H, CH_3), 1.41 (s, 3 H, CH_3), 1.06 (s, 3 H, CH_3) 1.00 (s, 3 H, CH_3); R_f = 0.14 (EtOAC/HOAc/H_2O 8:1:1), R_f = 0.28 ($CHCl_3$/MeOH/NH_3 9:1:0.1) ($C_{25}H_{40}O_8N_2$) C, H, N.

(21) Steck, T. L.; Kant, J. A. *Methods Enzymol.* **1974**, *31*, 172.

Synthesis of 6-Carbamates 8–10. 6-(4-Aminobutyl)carbamate **8**, 6-(6-aminohexyl)carbamate **9**, and 6-[[2-(4-hydroxyphenyl)-ethyl]amino]carbamate **10** were synthesized as described for **3** with 1,4-diaminobutane, 1,6-diaminohexane, and tyramine, respectively, instead of ethylenediamine. These intermediates were not reacylated as described for **3**.

6-[[(4-Aminobutyl)amino]carbonyl]-7-desacetylforskolin (8): ^{1}H NMR δ 6.12 (dd, 1 H, H14), 5.68 (t, 1 H, H6), 5.25 (d, 1 H, H15), 5.02 (d, 1 H, H15), 4.64 (t, 1 H, H1), 4.27 (d, 1 H, H7), 3.25 (t, 2 H, NHCH_2CH$_2$NH$_2$), 3.19 (d, 1 H, H12), 2.73 (t, 2 H, NHCH$_2$CH_2NH$_2$), 2.54 (d, 1 H, H12), 2.31 (d, 1 H, H5), 1.62 (s, 3 H, CH$_3$), 1.43 (s, 3 H, CH$_3$), 1.37 (s, 3 H, CH$_3$), 1.10 (s, 3 H, CH$_3$), 1.03 (s, 3 H, CH$_3$); R_f = 0.20 (EtOAc/HOAc/H$_2$O 8:1:1); recovery 55%.

Synthesis of 3-(4-Hydroxyphenyl)propionamide Derivatives of Forskolin (11–18). The (aminoalkyl)carbamyl intermediate of forskolin (for example, 10 mg of **2**) was dissolved in a small volume (200 μL) of dimethylformamide. A 1.5 molar equiv of *N*-succinimidyl-3-(4-hydroxyphenyl)propionic acid was added to the reaction and the mixture allowed to stir at room temperature for 2 h. The product was purified by flash chromatography on silica gel with ethyl acetate as solvent.

2-[3-(4-Hydroxyphenyl)propanamido]-*N*-ethyl-7-(aminocarbonyl)-7-desacetylforskolin (11): ^{1}H NMR δ 7.04 + 6.76 (dd, 4 H, Ph), 5.97 (dd, 1 H, H14), 5.27 (d, 1 H, H15), 5.17 (d, 1 H, H7), 4.93 (d, 1 H, H15), 4.55 (m, 2 H, H1, H6), 3.20 (m, 2 H, -NHCH_2CH$_2$NH-), 3.19 (d, 1 H, H12), 2.94 (m, 4 H, CH_2C$H_2$4-OH-Ph), 2.83 (t, 2 H, -NHCH$_2$CH_2NH-), 2.44 (d, 1 H, H12), 2.14 (d, 1 H, H5), 1.71 (s, 3 H, CH$_3$), 1.42 (s, 3 H, CH$_3$), 1.34 (s, 3 H, CH$_3$), 1.25 (s, 3 H, CH$_3$), 1.01 (s, 3 H, CH$_3$); R_f = 0.69 (EtOAc), R_f = 0.44 (CHCl$_3$/MeOH 9:1); recovery 85%.

2-[3-(4-Hydroxyphenyl)propanamido]-*N*-ethyl-6-(aminocarbonyl)-7-desacetylforskolin (15): ^{1}H NMR δ 7.04 + 6.74 (dd, 4 H, Ph), 6.12 (dd, 1 H, H14), 5.67 (t, 1 H, H6), 5.20 (d, 1 H, H15), 5.02 (d, 1 H, H15), 4.65 (t, 1 H, H1), 4.27 (d, 1 H, H7), 3.27 (m, 4 H, -NHCH_2CH_2NH-), 3.18 (d, 1 H, H12), 2.87 (t, 2 H, CH$_2$C$H_2$4-OH-Ph), 2.52 (d, 1 H, H12), 2.42 (t, 2 H, CH_2CH$_2$4-OH-Ph), 2.35 (d, 1 H, H5), 1.5 (s, 3 H, CH$_3$), 1.41 (s, 3 H, CH$_3$), 1.35 (s, 3 H, CH$_3$), 1.12 (s, 3 H, CH$_3$), 1.07 (s, 3 H, CH$_3$); R_f = 0.50 (EtOAc), R_f = 0.49 (CHCl$_3$/MeOH 9:1); recovery 82%.

Synthesis of *N*-Bromoacetyl Derivatives (19, 21). A 1.5-fold excess of bromoacetic acid (7 mg, 0.05 mmol) and a 5-fold excess of *N,N'*-dicyclohexylcarbodiimide (34 mg, 0.065 mmol) was added to a solution of **2** (15 mg, 0.03 mmol) dissolved in 0.5 mL of methylene chloride. The reaction was stirred at room temperature for 6 h. The sample was filtered through glass wool, dried in vacuo, and purified by chromatography on silica gel eluting with chloroform to give the pure **19**. The *N*-bromoacetyl derivative **21** was synthesized from **3** by using the same procedure.

7-[[*N*-[2-(Bromoacetyl)ethyl]amino]carbonyl]-7-desacetylforskolin (19): ^{1}H NMR (CDCl$_3$) δ 6.01 (dd, 1 H, H14), 5.29 (d, 1 H, H15), 5.21 (d, 1 H, H7), 5.00 (d, 1 H, H15), 4.57 (m, 2 H, H1, H6), 3.87 (s, 2 H, CH$_2$Br), 3.22 (m, 4 H, NHCH_2CH_2NHCOCH$_2$Br), 3.19 (1 H, H12), 2.48 (d, 1 H, H12), 1.70 (s, 3 H, CH$_3$), 1.43 (s, 3 H, CH$_3$), 1.37 (s, 3 H, CH$_3$), 1.26 (s, 3 H, CH$_3$), 1.05 (s, 3 H, CH$_3$); R_f = 0.77 (CHCl$_3$), R_f = 0.80 (EtOAc); recovery 53%.

6-[[*N*-[2-(Bromoacetyl)ethyl]amino]carbonyl]forskolin (21): ^{1}H NMR δ 6.29 (dd, 1 H, H14), 5.66 (t, 1 H, H6), 5.50 (d, 1 H, H7), 5.28 (d, 1 H, H15), 4.97 (d, 1 H, H15), 3.85 (s, 2 H, CH$_2$Br), 3.45 (m, 4 H, NHCH_2CH_2NHCOCH$_2$Br), 3.21 (d, 1 H, H12), 2.47 (d, 1 H, H12), 2.36 (d, 1 H, H5), 2.04 (s, 3 H, OCOCH$_3$), 1.71 (s, 3 H, CH$_3$), 1.65 (s, 1 H, CH$_3$), 1.26 (s, 3 H, CH$_3$), 1.18 (s, 3 H, CH$_3$), 1.05 (s, 3 H, CH$_3$); R_f = 0.73 (CHCl$_3$), R_f = 0.75 (EtOAc); recovery 21%.

Synthesis of Isothiocyanate Derivatives 20 and 22. **2** (32 mg, 0.07 mmol) was dissolved in 2.5 mL of chloroform, and sodium bicarbonate (30 mg, 0.42 mmol) in 1 mL of water was added. A 1.5 molar excess of thiophosgene (7 μL, 0.09 mmol) was added, and the reaction was allowed to proceed for 1 h with vigorous shaking. The chloroform layer was dried, and the product was purified by flash chromotography on silica gel equilibrated in chloroform/ethyl acetate 1:1 to give pure **20**. The isothiocyanate derivative **22** was synthesized from **3** by using the same procedure.

7-[[*N*-(2-Isothiocyanatoethyl)amino]carbonyl]-7-desacetylforskolin (20): ^{1}H NMR δ 6.04 (dd, 1 H, H14), 5.30 (d, 1 H, H15), 5.23 (d, 1 H, H7), 5.00 (d, 1 H, H15), 4.58 (s, 2 H, H1, H6), 3.68 (m, 2 H, NHCH$_2$CH_2NCS), 3.47 (m, 2 H, NHCH_2CH$_2$NCS), 3.17 (d, 1 H, H12), 2.49 (d, 1 H, H12), 2.18 (d, 1 H, H5), 1.73 (s, 3 H, CH$_3$), 1.43 (s, 3 H, CH$_3$), 1.39 (s, 3 H, CH$_3$), 1.26 (s, 3 H, CH$_3$), 1.05 (s, 3 H, CH$_3$); R_f = 0.51 (CHCl$_3$/EtOAc 1:1), R_f = 0.52 (hexane/EtOAc 4:6); IR peak 2200 cm^{-1}; recovery 52%.

6-[[*N*-(2-Isothiocyanatoethyl)amino]carbonyl]forskolin (22): ^{1}H NMR δ 5.99 (dd, 1 H, H14), 5.69 (t, 1 H, H6), 5.51 (d, 1 H, H7), 5.30 (d, 1 H, H15), 5.00 (d, 1 H, H15), 4.60 (t, 1 H, H1), 3.69 (m, 2 H, NHCH$_2$CH_2NCS), 3.48 (m, 2 H, NHCH_2CH_2NCS), 3.22 (d, 1 H, H12), 2.49 (d, 1 H, H12), 2.38 (d, 1 H, H5), 2.06 (s, 3 H, OCOCH$_3$), 1.66 (s, 3 H, CH$_3$), 1.42 (s, 3 H, CH$_3$), 1.41 (s, 3 H, CH$_3$), 1.06 (s, 3 H, CH$_3$), 1.01 (s, 3 H, CH$_3$); R_f = 0.56 (CHCl$_3$/EtOAc 1:1), R_f = 0.53 (hexane/EtOAc 4:6); IR peak 2200 cm^{-1}; recovery 75%.

Synthesis of Iodinated Derivatives of Forskolin, [^{125}I]-6-IHPP-Fsk and [^{125}I]-7-IHPP-Fsk. One millicurie of [^{125}I]-Bolton-Hunter reagent from NEN was concentrated to dryness under N$_2$. Twenty microliters of a solution of **3** (or **2**) (1 mg/mL in CH$_2$Cl$_2$) was added to the reaction vial and allowed to react at room temperature overnight. It is important to use as little volume as possible for the reaction due to the low concentration of the [^{125}I]-Bolton-Hunter reagent. The reaction was monitored by TLC with ethyl acetate as developing solvent and visualized by autoradiography. The ^{125}I-labeled Bolton-Hunter reagent has an R_f of 0.9 and the iodinated derivatives of forskolin have R_f's of 0.6. **2** and **3** do not migrate on silica under these conditions, and thus it is possible to use an excess of **2** or **3** to improve the yield. The reaction was applied to a small silica column (0.9 cm × 2 cm) equilibrated with ethyl acetate. Fractions (0.3 mL) were collected and monitored by TLC, followed by autoradiography. The fractions corresponding to the iodinated forskolin derivatives were pooled and stored at room temperature in ethanol. The [^{125}I]-6-IHPP-Fsk and [^{125}I]-7-IHPP-Fsk were separated completely from the (aminoalkyl)carbamates **2** and **3** using these chromatographic conditions and therefore the labeled compounds were assumed to be carrier-free with the specific activity of the [^{125}I]-Bolton-Hunter reagent (about 2175 Ci/mmol). The yields for the radioactive products range from 50 to 75% of the starting radioactivity. The purified [^{125}I]-6-IHPP-Fsk and [^{125}I]-7-IHPP-Fsk are stable for at least 2 months when stored in ethanol at room temperature.

Biological Evaluation. Preparation of Membranes. Bovine brains, obtained from a local slaughterhouse, were immersed in ice-cold homogenization buffer and brought to the lab. The homogenization buffer contained Tris·HCl 10 mM pH 7.4, sucrose 0.32 M, EDTA 10 mM, dithiothreitol 1 mM, benzamidine 1 mM, leupeptin 10^{-7} M, pepstatin A 10^{-6} M, and PMSF (phenyl methanesulfonyl fluoride) 0.5 mM. The cortex was dissected and homogenized in a Waring blender using a ratio of about 500 mL of buffer per brain. The homogenate was passed twice through four layers of cheesecloth and then centrifuged at 20000*g* for 30 min. The pellet was resuspended to the original volume with homogenization buffer in a Potter homogenizer and then centrifuged at 20000*g* for 40 min. The last step was repeated, and the resuspended material was divided in 40-mL aliquots, frozen in liquid nitrogen, and stored at −80 °C.

Washed human erythrocytes and membrane ghosts were prepared by the method of Steck and Kant.[21] Ghost membranes in 5 mM sodium phosphate buffer, pH 8, were isolated by centrifugation at 17400*g* for 15 min in a Sorvall SS-34 rotor and washed 3–4 times with the same buffer. Membrane ghosts were frozen at −70 °C until use.

Binding of [^{125}I]-6-IHPP-Fsk to Bovine Brain Membranes. The membrane suspension was prepared as described above, diluted with ice-cold 50 mM Tris·HCl buffer, centrifuged at 20000*g* for 10 min, and resuspended in 50 mM Tris·HCl buffer, pH 7.5, at a protein concentration of 2 mg/mL. The incubations were carried out at room temperature for 60 min in 12 mm × 75 mm glass test tubes in a total volume of 0.4 mL 50 mM Tris·HCl buffer with 5 mM MgCl$_2$, 10 mM NaF, pH 7.4, with a tracer amount of [^{125}I]-6-IHPP-Fsk (about 30 000 dpm) in the presence of derivatives. Forskolin analogues were tested at concentrations ranging from 1.28 nM to 20 μM. Binding equilibrium was reached by 45 min and was constant for at least 2 h (data not shown). The

assay was terminated by rapid filtration over Whatman GF/C filters using a Brandel cell harvester (Gaithersburg, MD). The filters were quickly washed three times with 4 mL of ice-cold buffer and filters counted in a gamma counter. The data were analyzed using the Ligand program.[19] The $K_d \pm$ SE values were determined by analysis of two independent experiments with each data point being determined in triplicate.

Binding of [^{125}I]-7-IHPP-Fsk to Human Red Blood Cell Membranes. The membrane suspension was prepared as described above, diluted with ice-cold 50 mM Tris·HCl buffer, centrifuged at 20000*g* for 10 min and resuspended in 50 mM Tris·HCl buffer, pH 7.5, at a protein concentration of 10 μg/mL. The incubations were carried out at room temperature for 60 min in 12 mm × 75 mm glass test tubes in a total volume of 0.4 mL 50 mM Tris·HCl buffer, 5 mM $MgCl_2$, pH 7.4. The membranes (10 μg/tube) were incubated with a tracer amount of [^{125}I]-7-IHPP-Fsk, about 30000 dpm, and forskolin derivatives at concentrations ranging from 1.28 nM to 20 μM. In some experiments the derivatives were tested at concentrations up to 100 μM. The assay was terminated by rapid filtration over Whatman GF/C filters using a Brandel cell harvester (Gaithersburg, MD). The filters were quickly washed three times with 4 mL of ice-cold buffer and filters counted in a gamma counter. The data were analyzed using the Ligand program. The $K_d \pm$ SE values were determined by analysis of two independent experiments with each data point being determined in triplicate.

Irreversible Loss of Binding Sites. The irreversible effects of the alkylating derivatives **19–22** were tested on [^{125}I]-6-IHPP-Fsk binding to bovine brain membranes or [^{125}I]-7-IHPP-Fsk binding to human erythrocyte membranes as described previously.[11] Membranes were incubated with either no additions (control) or 20 μM of forskolin or the indicated alkylating derivatives in 1 mL of buffer used for binding experiments for 30 min at room temperature. The membranes were then washed seven times with 50 mL of buffer. Membranes were resuspended in buffer, and the binding was determined as described above with a tracer amount of label, about 30000 dpm/assay. Specific binding was determined as the difference between total binding and nonspecific binding determined in the presence of 100 μM forskolin.

Acknowledgment. We would like to acknowledge many helpful discussions with Dr. Kenner Rice, NIDDK, NIH, Bethesda, MD, and Dr. Radhakrishnan Iyer, Division of Biochemistry and Biophysics, FDA, Bethesda, MD. We would also like to thank Dr. Lawrence Philips, Division of Biochemistry and Biophysics, FDA, Bethesda, MD, for ^{1}H NMR spectra and Noel Whitaker, NIDDK, NIH, Bethesda, MD, for mass spectral data. We would also like to thank Dr. Ian Simpson, NIDDK, NIH, Bethesda, MD, for kindly supplying us with the human erythrocyte membranes.

Registry No. 1 (isomer 1), 136378-26-2; 1 (isomer 2), 136378-27-3; 2, 135159-49-8; 3, 135159-50-1; 4, 136327-79-2; 5, 136327-80-5; 6, 136327-81-6; 7, 136327-82-7; 8, 136327-83-8; 9, 136327-84-9; 10, 136327-85-0; 11, 132523-83-2; 11-^{125}I, 135159-45-4; 12, 136327-86-1; 13, 136327-87-2; 14, 136327-88-3; 15, 136327-89-4; 15-^{125}I, 136327-96-3; 16, 136327-90-7; 17, 136327-91-8; 18, 136327-92-9; 19, 136327-93-0; 20, 136327-94-1; 21, 136357-51-2; 22, 136327-95-2; adenylyl cyclase, 9012-42-4.

Supplementary Material Available: NMR data for compounds 4–7, 9, 10, 12–14, and 16–18 (3 pages). Ordering information is given on any current masthead page.

Synthesis and Selective Class III Antiarrhythmic Activity of Novel *N*-Heteroaralkyl-Substituted 1-(Aryloxy)-2-propanolamine and Related Propylamine Derivatives

John A. Butera,*,† Walter Spinelli,§ Viji Anantharaman,†,‖ Nicholas Marcopulos,† Roderick W. Parsons,§ Issam F. Moubarak,§ Catherine Cullinan,‡,§ and Jehan F. Bagli†

Wyeth-Ayerst Research, Division of Exploratory Chemistry and Cardiovascular Pharmacology, CN 8000, Princeton, New Jersey 08534-8000. *Received May 20, 1991*

The synthesis and biological evaluation of a series of novel 1-(aryloxy)-2-propanolamines and several related deshydroxy analogues are described. Compounds **4–29** were prepared and investigated for their class III electrophysiological activity in isolated canine Purkinje fibers and in anesthetized open-chest dogs. None of these compounds showed any class I activity. On the basis of the in vitro data, structure–activity relationships for the series are discussed. Two compounds, *N*-[4-[2-hydroxy-3-[methyl(2-quinolinylmethyl)amino]propoxy]phenyl]methanesulfonamide (**12**, WAY-123,223) and *N*-[2-[[methyl[3-[4-[(methylsulfonyl)amino]phenoxy]propyl]amino]methyl]-6-quinolinyl]-methanesulfonamide (**24**, WAY-125,971) were identified and characterized as potent and specific class III antiarrhythmic agents in vitro and in vivo. Compound **12** was found to be orally bioavailable, to produce large increases of ventricular fibrillation threshold (VFT), and, in some instances, to restore sinus rhythm from ventricular fibrillation in anesthetized open-chest dogs at a dose of 5 mg/kg (iv). The enantiomers of **12** (i.e., **13** and **14**) were synthesized and were found to exhibit similar electrophysiological effects in the Purkinje fiber screen. Compound **24**, a propylamine analogue with potency and efficacy comparable to those of UK-68798 (**2**) and E-4031 (**3**), was studied in voltage-clamp experiments (isolated cat myocytes) and was found to be a potent and specific blocker of the delayed rectifier potassium current (I_K).

Cardiovascular diseases are responsible for the deaths of over 1 million people annually in the United States.[1] Statistics indicate that sudden cardiac death (SCD) resulting from ventricular tachycardia (VT) and/or ventricular fibrillation (VF) plays a major role in 40–60% of these deaths.[2,3] Most of these life-threatening ventricular

† Department of Chemistry.

‡ Present address: Merck Sharp & Dohme Research Laboratories, Rahway, NJ 07065.

§ Department of Cardiovascular and Metabolic Disorders.

‖ Present address: University of Illinois at Urbana-Champaign, Urbana, IL 61801.

(1) Kaplan, H. R. Advances in Antiarrhythmic Drug Therapy: Changing Concepts. *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **1986**, *45*, 2184–2185.

0022-2623/91/1834-3212$02.50/0