Forskolin Carbamates: Binding and Activation Studies with Type I Adenylyl Cyclase

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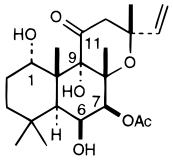
Three series of analogs were regioselectively prepared from a protected forskolin precursor to afford 7-carbamoyl-7-desacetylforskolins (series 1), 6-carbamoyl-7-desacetylforskolins (series 2), and 6-carbamoylforskolins (series 3). The analogs were pharmacologically evaluated for binding (IC_{50}) to and activation (EC_{50}) of type I adenylyl cyclase in membranes from stably transfected Sf9 cell lines expressing a single adenylate cyclase subtype. The following ranges were determined for the IC_{50}'s and EC_{50}'s of each individual series: series 1, IC_{50} = 43−1600 nM, EC_{50} = 0.5−9.6 μM; series 2, IC_{50} = 65−680 nM, EC_{50} = 0.63−6.5 μM; series 3, IC_{50} = 21−271 nM, EC_{50} = 0.5−8.1 μM (forskolin IC_{50} = 41 nM and EC_{50} = 0.5 μM). Activation paralleled binding; however, some analogs exhibited poor binding and good activation whereas others demonstrated good binding but poor activation. Steric bulk tended to diminish binding and activation when at the 6- or 7-position, although bulk was accommodated at the 6-position if the 7-site was reacetylated. Acylation of the 7-position by the carbamoyl linker or acetyl was important for obtaining good binding and activation; however, the effect was more pronounced with binding. For both binding and activation, small, linear, lipophilic substituents (propyl, allyl, isopropyl) are well tolerated at the 7-position but less so in the 6-position, even when the 7-site is reacetylated. Planar aromatic moieties (phenyl and 2-pyridinyl) demonstrated moderate to good potency for binding and activation when located at either the 6- or 7-positions. There is an overall trend toward increasing potency for both binding and activation with polar substituents.

Introduction

Forskolin **(1)**, a naturally occurring diterpene isolated from *Coleus forskholii*, directly activates adenylyl cyclase (AC) through its catalytic subunit to increase intracellular levels of cyclic adenosine monophosphate (cAMP).[1] The physiological consequences of cAMP elevation are varied and include cardiac inotropy, hypotension, bronchodilation, and reduction of intraocular pressure.[2]



Forskolin (1)

Although forskolin is widely used as a biochemical probe for characterizing AC-coupled biological responses, it has not found clinical application due to its poor water solubility, synthetic complexity, and broad spectrum of nonspecific pharmacological activities. Forskolin analogs have been prepared with improved water solubility,[3] and several total (albeit lengthy) syntheses[4] have been published. However, forskolin indiscriminantly activates all AC subtypes and binds to other membrane proteins.[5] Clearly, more information is needed regarding forskolin binding to and regulation of the different AC subtypes in order for a forskolin derivative to become therapeutically useful as a selective agent.

Eight mammalian isoforms of AC with specific regulatory requirements have been reported.[6] They are widely distributed, having been found in heart, brain, kidney, lung, liver, and intestine. All eight have been localized in the brain. Advances characterizing the tissue distribution, regulation, and homology between these isoforms have been the subject of recent reviews.[7] Each of the AC isoforms have been expressed through stably transfected Sf9 cell lines by using the baculovirus expression system.[8] The availability of membranes from these cloned cells permits the evaluation of forskolin analogs for specific AC isoform binding and activation requirements. No reports of an AC isoform specific forskolin derivative have yet appeared.

Three series of forskolin carbamate derivatives with an array of hydrophilic and hydrophobic functionalities were designed to probe the physicochemical determinants of forskolin binding to, and activation of, AC isoforms. The preparation and biological evaluation of the analogs in type I AC is described in the following sections. The work presented is part of a larger investigation into the binding and activation of all subtypes of AC.

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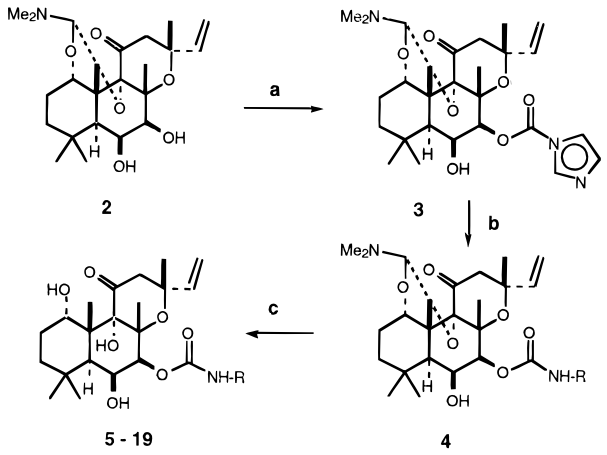
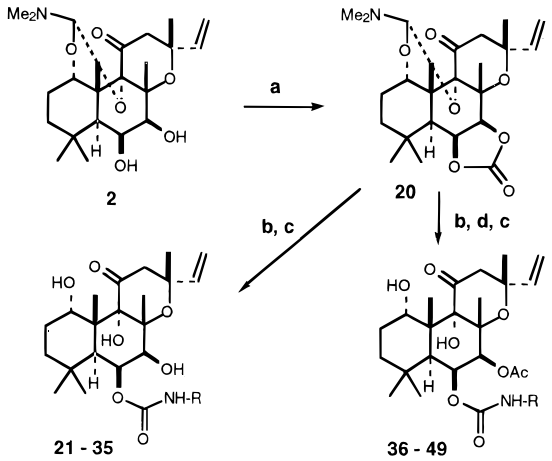
Scheme 1. 7-Carbamoyl-7-desacetylforskolin Derivatives[a]



[a] (a) Carbonyldiimidazole/CH_2Cl_2/room temperature/4−6 h; (b) R-NH_2; (c) AcOH/MeOH.

Scheme 2. 6-Carbamoyl-7-desacetylforskolin and 6-Carbamoylforskolin Derivatives[a]



[a] (a) Carbonyldiimidazole/triethylamine/room temperature/overnight; (b) R-NH_2; (c) AcOH/MeOH; (d) Ac_2O/pyridine.

Chemistry

The 6- and 7-hydroxyls of forskolin are relatively insensitive sites on the molecule for derivatization, and a flexible strategy had been previously developed to regioselectively prepare both 6- and 7-carbamate analogs.[9,10] The carbamate group was chosen as the linker based on prior successes with the chemistry involved and the hydrolytic and metabolic stability of a carbamate. The amine-containing reactants for subsequent carbamate formation were selected due to their polar or hydrophobic characteristics and a two-carbon span between the reactive amine and the desired terminal moiety in the final derivative. The three general methodologies provided either 6-carbamoyl-7-desacetylforskolins, 7-carbamoyl-7-desacetylforskolins, or 6-carbamoylforskolins. The general methodologies have been described in related work.[11]

7-Carbamoyl-7-desacetylforskolin Derivatives. The preparation of this series is outlined in Scheme 1. The acetal protected precursor **2** was prepared as previously described[9] and then treated with carbonyldiimidazole (CDI) in CH_2Cl_2 for 4−6 h at room temperature. The intermediate imidazolide **3** was not isolated, but immediately treated with an excess of the desired amine to form the 1,9-protected-7-carbamate **4**. The acetal was subsequently removed using glacial acetic acid in methanol to yield the general 7-carbamoyl-substituted derivatives **5**−**19**.

6-Carbamoyl-7-desacetylforskolin Derivatives. This series of analogs was prepared as shown in Scheme 2. The acetal-protected cyclic carbonate **20** was prepared as previously described[10] followed by addition of the desired amine resulting in regioselective formation of the 6-carbamate derivatives. Deprotection of the 1,9-hydroxyls was again effected by glacial acetic acid/methanol to yield the general compounds **21**−**35**.

6-Carbamoylforskolin Derivatives. This series of compounds follows the steps outlined in the previous section except that the acetal protected, 6-carbamates were treated with acetic anhydride in pyridine to effect acetylation as shown in Scheme 2. Removal of the acetal was again achieved by glacial acetic acid/methanol to afford the general structures **36**−**49**. Compounds **42** and **46** required additional protection of the terminal amine before acetylation by treatment with 9-fluorenylmethyl chloroformate (FMOC) as has been previously described.[11] Analog **47** was prepared from the acetal-protected 6-ethanolamine carbamate where the ethanolamine hydroxyl was subsequently masked with a 4,4′-dimethoxytrityl protecting group. The 7-hydroxyl was acetylated using Ac_2O/pyridine, and both protecting groups were removed by treatment with glacial acetic acid/methanol.

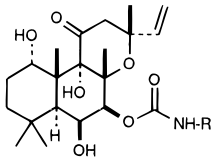
Results and Discussion

Biological Data: (1) 7-Carbamoyl-7-desacetylforskolin Series (Table 1). (A) Binding. Derivatives **5**−**9** exhibited decreasing affinity for type I AC as the side chain became bulkier and more hydrophobic. The phenyl moiety, derivative **10**, caused a 6-fold decrease in affinity over forskolin but was 6-fold greater than the cyclohexyl ligand **(9)**. This observation could be due to a $\pi-\pi$ interaction at the binding site or the fact that the planar phenyl ring occupies less space than the cyclohexyl derivative. Addition of a 4-hydroxy **(11)** or 4-amino **(16)** group to the phenyl ligand increased its affinity 2-fold, demonstrating again that the phenyl group is well tolerated at the 7-position of forskolin and a more polar phenyl ligand is preferred. The primary **(12)** and tertiary **(13)** amines and the piperidinyl **(14)** and pyridyl ligands **(15)** bound 3−6 times less than forskolin. The hydroxy ligand **(17)** was equal to forskolin, but the ether **(18)** and ester **(19)** ligands were 4-fold less potent.

(B) Activation. Generally, activation (EC_{50}) paralleled binding (IC_{50}) in this series. As the groups became more hydrophobic and bulkier, the less active they were. However, bulk or hydrophobicity alone were not sufficient to decrease activation since the compounds containing either a phenyl **(10)** or piperdinyl **(14)** group were still active (though 2−4-fold less active than forskolin). The fold stimulation of AC by each derivative in this series was similar to the value for the fold binding affinity. All but three compounds, **8**, **9**, and **19**, were between 2- and 5-fold less active than forskolin. None were more active.

(2) 6-Carbamoyl-7-desacetylforskolin Series (Table 2). (A) Binding. The affinities of derivatives **21** and **23**−**25** were 10−16 times less than forskolin with **22** being 7-fold less. The phenyl derivative **(26)**

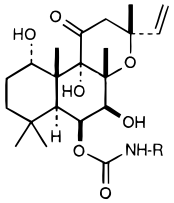
Table 1. Binding and Activation of 7-Carbamoyl-7-desacetylforskolins



compd	R	binding[a] IC_{50} (nM)	activation[b] EC_{50} (μM)	% maximal activation[c]
forskolin (**1**)	see text	41 ± 5	0.5 ± 0.05	100
5	$CH_2CH_2CH_3$	89 ± 13	1.3 ± 0.3	101
6	$CH_2CH{=}CH_2$	157 ± 14	2.4 ± 0.8	102
7	$CH_2CH(CH_3)_2$	112 ± 8	2.7 ± 0.2	72
8	$CH_2CH_2C(CH_3)_3$	608 ± 79	9.6 ± 3.3	58
9	CH_2-c-C_6H_{11}	1600 ± 100	6.4 ± 0.4	44
10	$CH_2CH_2C_6H_5$	251 ± 32	2.1 ± 0.5	75
11[d]	$CH_2CH_2C_6H_4$-4-OH	95 ± 8	1.4 ± 0.3	45
12[d]	$CH_2CH_2NH_2$	128 ±17	0.7 ± 0.4	85
13	$CH_2CH_2N(CH_3)_2$	228 ± 16	2.2 ± 0.4	89
14	CH_2CH_2-1-piperidinyl	184 ± 16	1.2 ± 0.03	81
15	CH_2CH_2-2-pyridyl	143 ± 11	1.1 ± 0.1	91
16	$CH_2CH_2C_6H_4$-4-NH_2	64 ± 5	1.1 ± 0.4	68
17	CH_2CH_2OH	43 ± 6	1.4 ± 0.8	119
18	$CH_2CH_2OCH_3$	174 ± 12	1.6 ± 0.3	97
19	$CH_2CH_2CO_2Et$	185 ± 18	3.6 ± 0.5	127

[a] IC_{50}'s ± SE were determined from the inhibition of [^{125}I]-6-IHPP-forskolin binding to type I AC membranes as described in the Experimental Section. [b] EC_{50}'s ± SE were determined by assaying for the conversion of [^{32}P]ATP to [^{32}P]cAMP as described in the Experimental Section. [c] Maximal stimulation was determined by dividing the fitted maximum stimulation obtained for the analog by the maximum stimulation obtained by treatment of the same membranes with 100 μM forskolin. [d] Reference 11.

Table 2. Binding and Activation of 6-Carbamoyl-7-desacetylforskolins



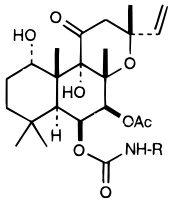
compd	R	binding[a] IC_{50} (nM)	activation[b] EC_{50} (μM)	% maximal activation[c]
21	$CH_2CH_2CH_3$	555 ± 66	4.9 ± 2.5	61
22	$CH_2CH{=}CH_2$	306 ± 37	3.5 ± 3.3	94
23	$CH_2CH(CH_3)_2$	593 ± 142	2.5 ± 0.7	88
24	$CH_2CH_2C(CH_3)_3$	680 ± 68	2.1 ± 0.1	63
25	CH_2-c-C_6H_{11}	433 ± 35	1.0 ± 0.1	51
26	$CH_2CH_2C_6H_5$	65 ± 8	1.1 ± 0.1	72
27[d]	$CH_2CH_2C_6H_4$-4-OH	236 ± 28	0.63 ± 0.64	86
28	$CH_2CH_2NH_2$	192 ± 31	ind[e]	ind[e]
29	$CH_2CH_2N(CH_3)_2$	247 ± 54	4.9 ± 1.4	131
30	CH_2CH_2-1-piperidinyl	123 ± 20	1.7 ± 0.5	80
31	CH_2CH_2-2-pyridyl	117 ± 15	1.8 ± 0.1	59
32	$CH_2CH_2C_6H_4$-4-NH_2	162 ± 23	5.1 ± 0.6	83
33[f]	CH_2CH_2OH	230 ± 30	3.6 ± 1.2	85
34	$CH_2CH_2OCH_3$	318 ± 60	3.1 ± 1.4	84
35	$CH_2CH_2CO_2Et$	171 ± 58	6.5 ± 1.5	75

[a] IC_{50}'s ± SE were determined from the inhibition of [^{125}I]-6-IHPP-forskolin binding to type I AC membranes as described in the Experimental Section. [b] EC_{50}'s ± SE were determined by assaying for the conversion of [^{32}P]ATP to [^{32}P]cAMP as described in the Experimental Section. [c] Maximal stimulation was determined by dividing the fitted maximum stimulation obtained for the analog by the maximum stimulation obtained by treatment of the same membranes with 100 μM forskolin. [d] Reference 11. [e] Indeterminate, the percent of maximal activation was approximately 10%. [f] Reference 10.

bound almost as well as forskolin, while adding either a 4-hydroxy **(27)** or 4-amino **(32)** group to the phenyl decreased affinity at least 2−4-fold. The amino functionalities **28**−**31** were comparable with the analogous 7-carbamoyl-7-desacetyl series, i.e. being 3−6-fold less potent than forskolin. The hydroxy **(33)** and ether **(34)** ligands were 6−8-fold less potent than forskolin, while the ester **(35)** was the same as the comparable 7-carbamoyl-7-desacetyl series, being 4-fold less potent.

(B) Activation. This series showed more diversity, and the derivatives were generally less active than the comparable ligands in the 7-carbamoyl-7-desacetyl series. An interesting point to note is that the first five derivatives, compounds **21**−**25** become increasingly active with bulk in contrast to the 7-carbamoyl-7-desacetyl series. Polar and amine functionalities conferred poor activation properties, causing these derivatives to be 6−13-fold less active than forskolin.

Table 3. Binding and Activation of 6-Carbamoylforskolins



compd	R	binding[a] IC_{50} (nM)	activation[b] EC_{50} (μM)	% maximal activation[c]
36	$CH_2CH_2CH_3$	76 ± 8	4.3 ± 1.2	80
37	$CH_2CH{=}CH_2$	76 ± 20	4.4 ± 1.1	80
38	$CH_2CH(CH_3)_2$	167 ± 27	4.1 ± 0.9	93
39	$CH_2CH_2C(CH_3)_3$	271 ± 35	8.1 ± 1.6	84
40	CH_2-c-C_6H_{11}	117 ± 19	6.6 ± 1.7	74
41	$CH_2CH_2C_6H_5$	36 ± 5	1.5 ± 0.3	65
42[d]	$CH_2CH_2NH_2$	31 ± 3	0.6 ± 0.1	125
43	$CH_2CH_2N(CH_3)_2$	73 ± 15	2.0 ± 0.05	100
44	CH_2CH_2-1-piperidinyl	82 ± 23	0.5 ± 0.07	131
45	CH_2CH_2-2-pyridyl	21 ± 3	0.6 ± 0.3	97
46	$CH_2CH_2C_6H_4$-4-NH_2	39 ± 14	0.5 ± 0.02	55
47	CH_2CH_2OH	142 ± 17	0.5 ± 0.1	57
48	$CH_2CH_2OCH_3$	32 ± 5	0.7 ± 0.2	71
49	$CH_2CH_2CO_2Et$	44 ± 11	3.5 ± 1.4	157

[a] IC_{50}'s ± SE were determined from the inhibition of [^{125}I]-6-IHPP-forskolin binding to type IAC membranes as described in the Experimental Section. [b] EC_{50}'s ± SE were determined by assaying for the conversion of [^{32}P]ATP to [^{32}P]cAMP as described in the Experimental Section. [c] Maximal stimulation was determined by dividing the fitted maximum stimulation obtained for the analog by the maximum stimulation obtained by treatment of the same membranes with 100 μM forskolin. [d] Reference 11.

(3) 6-Carbamoylforskolin Series (Table 3). (A) Binding. The presence of the 7-acetyl group conferred greater affinity to this series of derivatives in every case over the analogous compounds of the 6-carbamoyl-7-desacetyl series. Compounds **36**−**38** and **40** were 2−4-fold less potent than forskolin with **39** being 7-fold less, showing that hydrophobicity is tolerated, though potency decreases with increasing bulk. The phenyl derivative **(41)** is comparable to forskolin. The amine-containing moieties bind well with the primary amine **(42)**, the phenylamino **(46)** and the pyridinyl **(45)** groups being equal to or more potent than forskolin and the tertiary amino **(43)** and piperidinyl **(44)** derivatives being 2-fold less potent than forskolin. The ether **(48)** and ester **(49)** compounds are equal to forskolin. The hydroxy analog **(47)**, being a polar derivative, was expected to demonstrate good binding; however, it exhibited only moderate affinity.

(B) Activation. This group was characterized by relatively poor activation from the hydrophobic compounds **36**−**40** yet robust activation by the polar amino derivatives **42**−**46** (in contrast to the 6-carbamoyl-7-desacetyl series). The ester **(49)** is anomolous by being polar yet showing poor activation, and the phenyl derivative **(41)** is inconsistent by being hydrophobic yet demonstrating moderate activation, perhaps due to its planar aromatic structure. Compounds **47** and **48** are equiactive to forskolin.

Conclusions

The availability of membranes from stably transfected Sf9 cell lines expressing a single AC subtype allows investigation into the specific binding and activation requirements of forskolin analogs for each individual AC subtype. The purpose of the current work was to use established synthetic and pharmacologic methodology to selectively modify forskolin in a rational way so that structure−activity relationships could be generated for each of the known AC subtypes.

Table 4. Comparison of Binding and Activation

(+ , +)[a]		(+ , −)	(− , +)	(− , −)	
forskolin	**31**	**7**	**14**	**6**	**24**
5	**41**	**36**	**25**	**8**	**29**
11	**42**	**37**	**27**	**9**	**32**
12	**43**	**40**		**10**	**33**
15	**44**	**49**		**13**	**34**
16	**45**			**18**	**35**
17	**46**			**19**	**38**
26	**47**			**21**	**39**
30	**48**			**22**	**40**
				23	

[a] (Binding, activation), **(+)** binding if $IC_{50} \leq 150$ nM, **(−)** binding if $IC_{50} > 150$ nM; **(+)** activation if $EC_{50} \leq 2.0$ μM, **(−)** activation if $EC_{50} > 2.0$ μM.

By referring to Table 4 and Figure 1, several trends can be noted regarding the binding and activation of forskolin analogs to type I AC. In general, as depicted in Table 4 (based on the division of "good" and "poor" binding and activation by the median values for binding (150 nM) and activation (2.0 μM) of all compounds), activation tends to parallel binding. Several interesting exceptions to the binding versus activation trend are important. Compounds **14**, **25**, and **27** all exhibited poor binding but good activation whereas compounds **7**, **36**, **37**, **40**, and **49** all demonstrated good binding but poor activation. No common structural feature is apparent in the first subset (poor binders/good activators), but a bulky, hydrophobic group is shared by many of the compounds in the second subset (good binders/poor activators). The first subset of analogs (poor binders/good activators) may be compounds which elevate cAMP by an AC-independent mechanism. The second subset of compounds may represent partial forskolin antagonists. Both subsets may also be indicative of binding and activation occurring at two distinct yet closely associated sites.

By analyzing the binding and activation data (Figure 1) for each individual moiety across all three substitu-

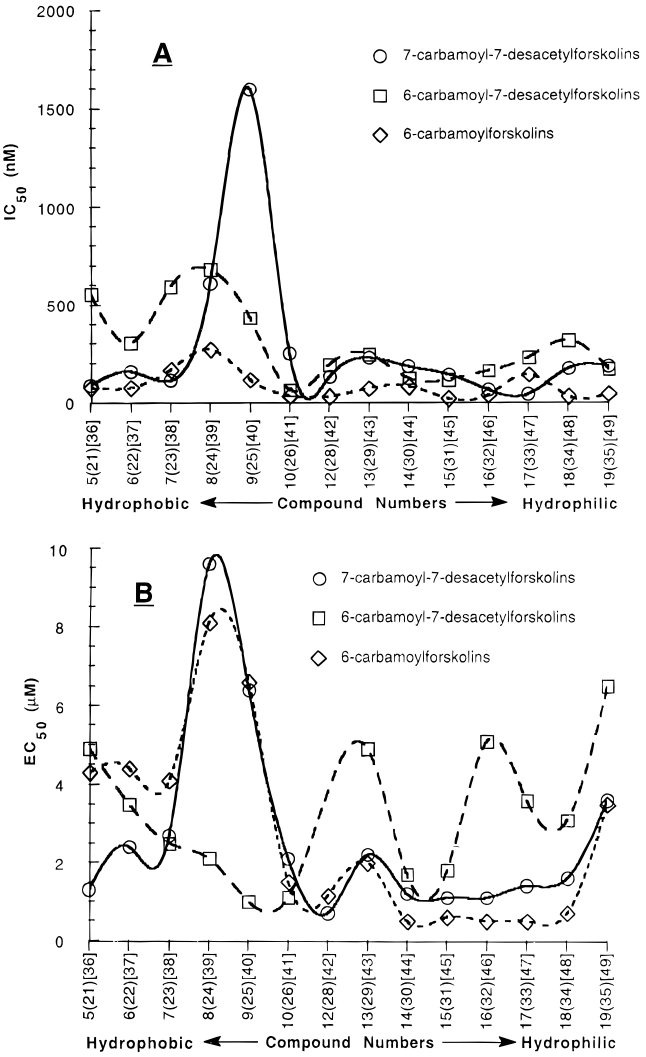


Figure 1. Comparison of the individual moieties at each site of substitution with (A) binding affinity (IC_{50}) and (B) activation (EC_{50}).

tion sites, the following generalizations may be made: (1) Bulky (*tert*-butyl and cyclohexyl) groups adversely influence binding when in the 6-position; however, if the 7-position is reacetylated, this influence is lessened. In the 7-substituted series, bulk is intolerable, giving derivatives (**8** and **9**) with low affinities. For activation, steric bulk is better tolerated in the 6-position than in the 7-position. Additionally, steric bulk tended to diminish the maximal degree of activation except in the 6-carbamoyl-7-desacetylforskolin series. This could be the result of a steric interaction at the receptor site or lessened solubility of the analog. (2) For both binding and activation, small, linear, lipophilic substituents (propyl, allyl, isopropyl) are well tolerated at the 7-position but less so in the 6-position, even when the 7-site is reacetylated. (3) Planar aromatic moieties (phenyl and 2-pyridinyl) demonstrated moderate to good potency for binding and activation when located at either the 6- or 7-position. Addition of a hydroxyl **(11)** or amine **(16)** to the phenyl group resulted in enhanced binding and activation in the 7-carbamoyl-7-desacetyl series, however, in the 6-carbamoyl-7-desacetyl series; addition of the amine **(32)** resulted in diminished binding and activation whereas addition of a hydroxyl **(27)** reduced binding yet enhanced activation. (4) There is an overall trend toward increasing potency for both binding and activation with polar substituents. However, polarity has much less influence on activation if present at the 6-position (as was also seen with the phenyl moiety). Remarkably, no compound demonstrating poor binding contained a polar group, whereas several poor activators (across all three groups) contained a polar group. (5) The most active series for binding was the 6-substituted forskolins, whereas for activation, the 6-substituted forskolins and the 7-substituted-7-desacetylforskolins were approximately equiactive. Reattachment of the 7-acetyl group in the 6-substituted series enhances both the binding and activation of the 6-substituted-7-desacetyl derivatives, but has a larger effect on binding. Importantly, the 7-substituted-7-desacetyl and 6-substituted forskolins tended to trend together, perhaps pointing to a common requirement for 7-substitution in order to obtain full expression of AC stimulation. This finding is in accordance with earlier work highlighting the contribution of the 7-acetyl group.[11]

In summary, the physicochemical requirements at the 6- and 7-position of forskolin, sufficient for expression of type I AC binding and activation, have been determined for a series of analogs. These determinants will be compared against the requirements for the other AC subtypes with the hope of ultimately developing AC subtype specific ligands. Additionally, the current work may provide direction for the design of forskolin antagonists and perhaps indicate novel interactions between forskolin and AC.

Table 5. Characteristic ^{1}H-NMR Shifts for the Analog Series

	series		
signals (ppm)	7-carb-7-desac[a]	6-carb-7-desac[b]	6-carb[c]
CH_3 (I)	1.02 ± 0.01	0.99 ± 0.03	0.97 ± 0.03
CH_3 (II)	1.24 ± 0.01	1.07 ± 0.01	1.04 ± 0.01
CH_3 (III)	1.34 ± 0.02	1.34 ± 0.03	1.33 ± 0.01
CH_3 (IV)	1.41 ± 0.01	1.40 ± 0.01	1.37 ± 0.02
CH_3 (V)	1.68 ± 0.02	1.59 ± 0.02	1.63 ± 0.02
1β	4.53 ± 0.02	4.61 ± 0.05	4.57 ± 0.01
6α	4.51 ± 0.03	5.66 ± 0.03	5.47 ± 0.02
7α	5.25 ± 0.06	4.25 ± 0.01	5.65 ± 0.02
7-OAc			2.03 ± 0.02
12-eq	3.18 ± 0.02	3.17 ± 0.01	3.20 ± 0.01
12-ax	2.46 ± 0.03	2.51 ± 0.01	2.46 ± 0.01
14	5.95 ± 0.03	6.10 ± 0.02	5.93 ± 0.02
15-trans	5.34 ± 0.04	5.19 ± 0.01	5.26 ± 0.01
15-cis	4.98 ± 0.02	4.98 ± 0.02	4.97 ± 0.01

[a] 7-Carbamoyl-7-desacetylforskolin analogs. Average ± SD of 16 analogs. [b] 6-Carbamoyl-7-desacetylforskolin analogs. Average ± SD of 15 analogs. [c] 6-Carbamoylforskolin analogs. Average ± SD of 14 analogs.

Experimental Section

Chemistry. ^{1}H-NMR spectra were obtained on a 300-MHz Varian Gemini spectrometer in $CDCl_3$ unless otherwise noted. Chemical shifts are reported in parts per million (δ) downfield from tetramethylsilane as internal standard with the following peak multiplicities: s, singlet; d, doublet; t, triplet; m, multiplet; dd, double of doublets. A combination of characteristic and specific signals in the ^{1}H-NMR spectra were used for the structural assignments. The characteristic signals (Table 5) are invariant within 0.06 ppm for a particular series of analogs and are a reliable indication for the site of derivatization. The specific signals are unique to a particular compound and are reported under the appropriate heading. 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. Preparative chromatography was performed on silica gel (Analtech, sorbent silica gel 10 μm). Organic

materials were from Aldrich Chemicals and were used without further purification. The starting material, the 1,9-dimethylformamide acetal protected 7-desacetylforskolin **(2)**, was synthesized by a published procedure.[9] The 1,9-dimethylformamide acetal-protected 7-desacetylforskolin-6,7-carbonate **(20)** was also prepared by a published procedure.[10]

Thin-layer chromatography was performed on silica gel (silica gel on polyester with indicator, Sigma Chemical Co.) in two eluents. Visualization of the plates was by iodine vapor or UV light. The following solvents and proportions were used in determining the R_f values for the analogs:

$CHCl_3$/EtOAc	9:1	A
$CHCl_3$/EtOAc	1:1	B
$CHCl_3$/EtOAc	7:3	C
hexane/EtOAc	4:6	D
hexane/EtOAc	6:4	E
$CHCl_3$/MeOH	7:3	F
$CHCl_3$/MeOH	8:2	G
$CHCl_3$/MeOH	9:1	H
$CHCl_3$/MeOH/NH_3	9:1:0.1	I
EtOAc	1	J
EtOAc/HOAc/H_2O	8:1:1	K

In reporting the R_f values, the following convention is used unless otherwise noted: solvent 1(R_f)/solvent 2(R_f).

Forskolin is an expensive and scarce natural product which precludes large-scale synthesis of derivatives. Additionally, the synthetic preparation of the derivatives is a multi-step process and overall yields are low. The typical recovery by weight from the synthetic procedures starting with 10−15 mg of acetal-protected forskolin ranged from 3 to 6 mg. Proof of purity, as carried out previously for this type of reporting,[11] depended on single spots by TLC in two different eluents and characteristic and specific signals by ^{1}H-NMR spectroscopy, as well as the absence of "double resonances".

Synthesis of 7-Carbamoyl-7-desacetylforskolins (5−19). To 20 mg (47 μM) of **2** contained in 200 μL of anhydrous CH_2Cl_2 was added 15.2 mg (94 μM) of carbonyldiimidazole. This was allowed to stir for 4−6 h at room temperature, and then a 5 molar excess (235 μM) of the desired amine was added all at once. The mixture was allowed to react for 20 h, then diluted in CH_2Cl_2, and washed with 2 mL of water. The organic layer was separated and solvent removed by rotary evaporation. The residue was taken up in 360 μL of MeOH, and 240 μL of glacial acetic acid was added all at once to cleave the acetal protecting group. This was permitted to stand overnight, whereupon a saturated aqueous solution of Na_2CO_3 was added to neutralize the acetic acid. The mixture was extracted three times with CH_2Cl_2; the organic fractions were combined, dried over Na_2SO_4, filtered, and evaporated to dryness by rotary evaporation; and the residue was applied to a silica gel column for purification.

7-[(Propylamino)carbonyl]-7-desacetylforskolin (5): ^{1}H-NMR δ 3.16 (m, 2H, NHCH_2CH$_2$CH$_3$), 0.91 (t, 3H, NHCH$_2$-CH$_2$CH_3); R_f = D(0.83)/B(0.64).

7-[(Allylamino)carbonyl]-7-desacetylforskolin (6): ^{1}H-NMR δ 5.87 (m, 1H, NHCH$_2$CH=CH$_2$), 5.16 (t, 2H, NHCH$_2$-CH=CH_2) 3.83 (m, 2H, NHCH_2CH=CH$_2$); R_f = B(0.76)/D(0.83).

7-[[(2-Methylpropyl)amino]carbonyl]-7-desacetylforskolin (7): ^{1}H-NMR δ 3.07, 2.98 (m, m, 1H, 1H, NHCH_2CH-(CH$_3$)$_2$), 0.91 (d, 6H, NHCH$_2$CH(CH_3)$_2$); R_f = C(0.60)/D(0.85).

7-[[(3,3-Dimethylbutyl)amino]carbonyl]-7-desacetylforskolin (8): ^{1}H-NMR δ 3.20 (m, 2H, NHCH_2CH$_2$C(CH$_3$)$_3$), 0.90 (s, 9H, NHCH$_2$CH$_2$C(CH_3)$_3$); R_f = B(0.80)/D(0.88).

7-[[(Cyclohexylmethyl)amino]carbonyl]-7-desacetylforskolin (9): ^{1}H-NMR δ 3.08, 3.01 (m, m, 1H, 1H, NHCH_2-c-C$_6$H$_{11}$); R_f = D(0.87)/B(0.76).

7-[[(2-Phenylethyl)amino]carbonyl]-7-desacetylforskolin (10): ^{1}H-NMR δ 7.21−7.34 (m, 5H, NHCH$_2$CH$_2$C$_6$H$_5$), 3.53, 3.42 (m, m, 1H, 1H, NHCH_2CH$_2$C$_6$H$_5$), 2.85 (t, 2H, NHCH$_2$C-H_2C$_6$H$_5$); R_f = D(0.83)/B(0.51).

7-[[2-(4-Hydroxyphenyl)ethyl]amino]carbonyl]-7-desacetylforskolin (11):[11] ^{1}H-NMR δ 7.07 (d, 2H, NHCH$_2$-CH$_2$C$_6$H$_4$-4-OH), 6.78 (d, 2H, NHCH$_2$CH$_2$C$_6$H$_4$-4-OH), 3.49, 3.37 (m, m, 1H, 1H, NHCH_2CH$_2$C$_6$H$_4$-4-OH), 2.76 (t, 2H, CH$_2$CH_2C$_6$H$_4$-4-OH); R_f = B(0.52)/D(0.46).

7-[[(2-Aminoethyl)amino]carbonyl]-7-desacetylforskolin (12):[11] ^{1}H-NMR δ 3.25 (m, 2H, NHCH_2CH$_2$NH$_2$) 2.67 (m, 2H, NHCH$_2$CH_2NH$_2$); R_f = K(0.34)/I(0.25).

7-[[[2-(Dimethylamino)ethyl]amino]carbonyl]-7-desacetylforskolin (13): ^{1}H-NMR δ 3.23 (m, 2H, NHCH_2CH$_2$N-(CH$_3$)$_2$), 2.41 (m, 2H, NHCH$_2$CH_2N(CH$_3$)$_2$), 2.21 (s, 6H, NHCH$_2$-CH$_2$N(CH_3)$_2$); R_f = H(0.07)/I(0.37).

7-[[[2-(1-Piperidinyl)ethyl]amino]carbonyl]-7-desacetylforskolin (14): ^{1}H-NMR δ 3.29 (m, 2H, NHCH_2CH$_2$-1-piperidinyl), 2.38 (m, 2H, NHCH$_2$CH_2-1-piperidinyl); R_f = I(0.45)/H(0.14).

7-[[[2-(2-Pyridyl)ethyl]amino]carbonyl]-7-desacetylforskolin (15): ^{1}H-NMR δ 8.49 (d, 1H, NHCH$_2$CH$_2$-2-*pyridyl*), 7.61 (dd, 1H, NHCH$_2$CH$_2$-2-*pyridyl*), 7.13−7.19 (m, 2H, NHCH$_2$-CH$_2$-2-*pyridyl*), 3.58 (m, 2H, NHCH_2CH$_2$-2-pyridyl), 2.94−3.02 (m, 2H, NHCH$_2$CH_2-2-pyridyl). R_f = H(0.74)/B(0.20).

7-[[[2-(4-Aminophenyl)ethyl]amino]carbonyl]-7-desacetylforskolin (16): ^{1}H-NMR δ 7.0 (d, 2H, NHCH$_2$CH$_2$C$_6$H$_4$-4-NH$_2$), 6.7 (d, 2H, NHCH$_2$CH$_2$C$_6$H$_4$-4-NH$_2$), 3.47, 3.35 (m,m, 1H, 1H, NHCH_2CH$_2$C$_6$H$_4$-4-NH$_2$), 2.73 (t, 2H, NHCH$_2$CH_2C$_6$H$_4$-4-NH$_2$); R_f = B(0.45)/H(0.74).

7-[[(2-Hydroxyethyl)amino]carbonyl]-7-desacetylforskolin (17): ^{1}H-NMR δ 3.77 (t, 2H, NHCH_2CH$_2$OH), 3.39 (t, 2H, NHCH$_2$CH_2OH); R_f = I(0.65)/D(0.45).

7-[[(2-Methoxyethyl)amino]carbonyl]-7-desacetylforskolin (18): ^{1}H-NMR δ 3.40 (t, 2H, NHCH$_2$CH_2OCH$_3$) 3.37 (s, 3H, NHCH$_2$CH$_2$OCH_3), 3.15 (NHCH_2CH$_2$OCH$_3$); R_f = B(0.60)/D(0.60).

7-[[(2-Carbethoxyethyl)amino]carbonyl]-7-desacetylforskolin (19): ^{1}H-NMR δ 4.14 (q, 2H, NHCH$_2$CH$_2$CO$_2$CH_2-CH$_3$) 3.46 (m, 2H, NHCH_2CH$_2$CO$_2$Et), 2.55 (t, 2H, NHCH$_2$CH_2-CO$_2$Et), 0.83 (t, 3H, NHCH$_2$CH$_2$CO$_2$CH$_2$CH_3); R_f = B(0.70)/D(0.78).

Synthesis of 6-Carbamoyl-7-desacetylforskolins (21−35). To 20 mg (44 μM) of previously prepared **20** contained in 200 μL of anhydrous CH_2Cl_2 was added a 5 molar excess of the desired amine all at once. The mixture was allowed to react overnight, diluted with CH_2Cl_2 and then washed with 2 mL of water. The organic layer was separated and solvent removed by rotary evaporation. The residue was taken up in 360 μL of MeOH, and 240 μL of glacial acetic acid was added all at once. This was permitted to stand overnight, whereupon a saturated aqueous solution of Na_2CO_3 was added to neutralize the acetic acid. The mixture was extracted three times with CH_2Cl_2; the organic fractions were combined, dried over Na_2SO_4, filtered, and evaporated to dryness by rotary evaporation; and the residue was applied to a silica gel column for purification.

6-[(Propylamino)carbonyl]-7-desacetylforskolin (21): ^{1}H-NMR δ 3.16 (m, 2H, NHCH_2CH$_2$CH$_3$), 0.90 (t, 3H, NHCH$_2$-CH$_2$CH_3); R_f = C(0.56)/D(0.31).

6-[(Allylamino)carbonyl]-7-desacetylforskolin (22): ^{1}H-NMR δ 5.84 (m, 1H, NHCH$_2$CH=CH$_2$), 5.14 (t, 2H, NHCH$_2$-CH=CH_2) 3.87 (m, 2H, NHCH_2CH=CH$_2$); R_f = D(0.31)/C(0.33).

6-[[(2-Methylpropyl)amino]carbonyl]-7-desacetylforskolin (23): ^{1}H-NMR δ 3.03 (dd, 2H, NHCH_2CH(CH$_3$)$_2$), 0.89 (d, 6H, NHCH$_2$CH(CH_3)$_2$); R_f = C(0.60)/D(0.40).

6-[[(3,3-Dimethylbutyl)amino]carbonyl]-7-desacetylforskolin (24): ^{1}H-NMR δ 3.20 (m, 2H, NHCH_2CH$_2$C(CH$_3$)$_3$), 0.91 (s, 9H, NHCH$_2$CH$_2$C(CH_3)$_3$); R_f = A(0.45)/D(0.44).

6-[[(Cyclohexylmethyl)amino]carbonyl]-7-desacetylforskolin (25): ^{1}H-NMR δ 3.05 (m, 2H, NHCH_2-c-C$_6$H$_{11}$); R_f = D(0.37)/C(0.53).

6-[[(2-Phenylethyl)amino]carbonyl]-7-desacetylforskolin (26): ^{1}H-NMR δ 7.20−7.32 (m, 5H, NHCH$_2$CH$_2$C$_6$H$_5$), 3.46 (m, 2H, NHCH_2CH$_2$C$_6$H$_5$), 2.83 (m, 2H, NHCH$_2$CH_2C$_6$H$_5$); R_f = C(0.47)/D(0.37).

6-[[[2-(4-Hydroxyphenyl)ethyl]amino]carbonyl]-7-desacetylforskolin (27):[11] ^{1}H-NMR δ 7.03 (d, 2H, NHCH$_2$-CH$_2$C$_6$H$_4$-4-OH), 6.74 (d, 2H, NHCH$_2$CH$_2$C$_6$H$_4$-4-OH), 3.39 (m, 2H, NHCH_2CH$_2$C$_6$H$_4$-4-OH), 2.77 (dd, 2H, CH$_2$CH_2C$_6$H$_4$-4-OH); R_f = B(0.65)/D(0.83).

6-[[(2-Aminoethyl)amino]carbonyl]-7-desacetylforskolin (28): ^{1}H-NMR δ 3.28 (m, 2H, NHC*H*$_2$CH$_2$NH$_2$) 2.71 (m, 2H, NHCH$_2$C*H*$_2$NH$_2$); R_f = K(0.51)/F(0.50).

6-[[[2-(Dimethylamino)ethyl]amino]carbonyl]-7-desacetylforskolin (29): ^{1}H-NMR δ 3.29 (m, 2H, NHC*H*$_2$CH$_2$N(CH$_3$)$_2$), 2.33 (m, 2H, NHCH$_2$C*H*$_2$N(CH$_3$)$_2$), 2.21 (s, 6H, NHCH$_2$CH$_2$N(C*H*$_3$)$_2$); R_f = F(0.06)/I(0.37).

6-[[[2-(1-Piperidinyl)ethyl]amino]carbonyl]-7-desacetylforskolin (30): ^{1}H-NMR (CD$_3$OD) δ (m, 2H, NHC*H*$_2$CH$_2$-1-piperidinyl), (m, 2H, NHCH$_2$C*H*$_2$-1-piperidinyl); R_f = F(0.11)/I(0.60).

6-[[[2-(2-Pyridyl)ethyl]amino]carbonyl]-7-desacetylforskolin (31): ^{1}H-NMR δ 8.47 (d, 1H, NHCH$_2$CH$_2$-2-*pyridyl*), 7.57 (dd, 1H, NHCH$_2$CH$_2$-2-*pyridyl*), 7.10−7.18 (m, 2H, NHCH$_2$CH$_2$-2-*pyridyl*), 3.61 (m, 2H, NHC*H*$_2$CH$_2$-2-pyridyl), 2.99 (t, 2H, NHCH$_2$C*H*$_2$-2-pyridyl); R_f = H(0.61)/J(0.27).

6-[[2-(4-Aminophenyl)ethyl]amino]carbonyl]-7-desacetylforskolin (32): ^{1}H-NMR δ 6.96 (d, 2H, NHCH$_2$CH$_2$C$_6$*H*$_4$-4-NH$_2$), 6.60 (d, 2H, NHCH$_2$CH$_2$C$_6$*H*$_4$-4-NH$_2$), 3.37 (m, 2H, NHC*H*$_2$CH$_2$C$_6$H$_4$-4-NH$_2$), 2.67 (m, 2H, NHCH$_2$C*H*$_2$C$_6$H$_4$-4-NH$_2$); R_f = H(0.56)/CHCl$_3$:EtOAc (2:8)(0.35).

6-[[(2-Hydroxyethyl)amino]carbonyl]-7-desacetylforskolin (33):[10] ^{1}H-NMR δ 3.72 (t, 2H, NHC*H*$_2$CH$_2$OH), 3.37 (t, 2H, NHCH$_2$C*H*$_2$OH); R_f = H(0.32)/I(0.10).

6-[[(2-Methoxyethyl)amino]carbonyl]-7-desacetylforskolin (34): ^{1}H-NMR δ 3.35 (m, 2H, NHCH$_2$C*H*$_2$OCH$_3$) 3.35 (s, 3H, NHCH$_2$CH$_2$OC*H*$_3$), 3.34 (NHC*H*$_2$CH$_2$OCH$_3$); R_f = A(0.48)/H(0.68).

6-[[(2-Carbethoxyethyl)amino]carbonyl]-7-desacetylforskolin (35): ^{1}H-NMR δ 4.11 (q, 2H, NHCH$_2$CH$_2$CO$_2$C*H*$_2$CH$_3$) 3.45 (m, 2H, NHC*H*$_2$CH$_2$CO$_2$Et), 2.53 (t, 2H, NHCH$_2$C*H*$_2$CO$_2$Et), 1.24 (t, 3H, NHCH$_2$CH$_2$CO$_2$CH$_2$C*H*$_3$); R_f = B(0.42)/H(0.82).

Synthesis of 6-Carbamoylforskolins (36−47). To 20 mg (44 μM) of previously prepared **20** contained in 200 μL of anhydrous CH$_2$Cl$_2$ was added a 5 molar excess of the desired amine all at once. The mixture was allowed to react overnight, diluted with CH$_2$Cl$_2$, and then washed with 2 mL of water. The organic layer was separated and solvent removed by rotary evaporation. To the residue was added 200 μL of pyridine and 200 μL of acetic anhydride and the mixture allowed to react overnight. To the reaction mixture was added 2 mL of water followed by extraction with two 2 mL portions of CH$_2$Cl$_2$. The fractions were combined, solvent was removed by rotary evaporation, and then residual pyridine was removed by high vacuum. The residue was taken up in 360 μL of MeOH, and 240 μL of glacial acetic acid was added all at once. This was permitted to stand overnight, whereupon 500 μL of a saturated aqueous solution of Na$_2$CO$_3$ was added to neutralize the acetic acid. The mixture was extracted three times with CH$_2$Cl$_2$; the organic fractions were combined, dried over Na$_2$SO$_4$, and evaporated to dryness by rotary evaporation; and the residue was applied to a silica gel column for purification.

Additionally Protected Derivatives (42, 46, and 47). The FMOC derivatives leading to **42** and **46** were prepared as previously described.[11] Compound **47** was synthesized as follows:

To 20 mg (47 μM) of **2** contained in 300 μL of anhydrous CH$_2$Cl$_2$ were added 15.2 mg (94 μM) of carbonyldiimidazole and 19.4 μL (5-fold excess) of triethylamine. This was allowed to stand for 24 h, and then 14.4 mg (235 μM) of ethanolamine was added all at once. This was permitted to stand for 24 h and then washed with water, and solvent was removed. The residue was dissolved in 100 μL of pyridine, and 32 mg (94 μM) of 4,4′-dimethoxytrityl chloride was added and reacted for 24 h at room temperature. This was washed with water, the solvent removed, and the residue purified by flash chromatography. The purified, tritylated material was dissolved in 200 μL of pyridine and then reacted with 22 μL (235 μM) of acetic anhydride, and the mixture was allowed to stand for 24 h. The mixture was washed with water, pyridine was removed by rotary evaporation, and then the acetal and trityl group were removed by treatment with 240 μL of glacial acetic acid and 360 μL of methanol. This mixture was diluted with CH$_2$Cl$_2$ and washed with water and solvent removed. The residue was purified by flash chromatography to yield compound **47**.

6-[(Propylamino)carbonyl]forskolin (36): ^{1}H-NMR δ 3.16 (m, 2H, NHC*H*$_2$CH$_2$CH$_3$), 0.91 (t, 3H, NHCH$_2$CH$_2$C*H*$_3$); R_f = D(0.80)/B(0.56).

6-[(Allylamino)carbonyl]forskolin (37): ^{1}H-NMR δ 5.86 (m, 1H, NHCH$_2$C*H*=CH$_2$), 5.16 (t, 2H, NHCH$_2$CH=C*H*$_2$), 3.84 (m, 2H, NHC*H*$_2$CH=CH$_2$); R_f = D(0.80)/C(0.51).

6-[[(2-Methylpropyl)amino]carbonyl]forskolin (38): ^{1}H-NMR δ 3.02 (m, 2H, NHC*H*$_2$CH(CH$_3$)$_2$), 0.89 (d, 6H, NHCH$_2$CH(C*H*$_3$)$_2$); R_f = C(0.60)/D(0.40).

6-[[(3,3-Dimethylbutyl)amino]carbonyl]forskolin (39): ^{1}H-NMR δ 3.19 (m, 2H, NHC*H*$_2$CH$_2$C(CH$_3$)$_3$), 0.91 (s, 9H, NHCH$_2$CH$_2$C(C*H*$_3$)$_3$); R_f = D(0.86)/C(0.54).

6-[[(Cyclohexylmethyl)amino]carbonyl]forskolin (40): ^{1}H-NMR δ 3.06 (m, 2H, NHC*H*$_2$-c-C$_6$H$_{11}$); R_f = D(0.84)/C(0.53).

6-[[(2-Phenylethyl)amino]carbonyl]forskolin (41): ^{1}H-NMR δ 7.16−7.32 (m, 5H, NHCH$_2$CH$_2$C$_6$*H*$_5$), 3.38 (m, 2H, NHC*H*$_2$CH$_2$C$_6$H$_5$), 2.80 (m, 2H, NHCH$_2$C*H*$_2$C$_6$H$_5$); R_f = D(0.81)/C(0.53).

6-[[(2-Aminoethyl)amino]carbonyl]forskolin (42):[11] ^{1}H-NMR δ 3.28 (m, 2H, NHC*H*$_2$CH$_2$NH$_2$), 2.84 (m, 2H, NHCH$_2$C*H*$_2$NH$_2$); R_f = I(0.28)/F(0.54).

6-[[[2-(Dimethylamino)ethyl]amino]carbonyl]forskolin (43): ^{1}H-NMR δ 3.29 (m, 2H, NHC*H*$_2$CH$_2$N(CH$_3$)$_2$), 2.33 (m, 2H, NHCH$_2$C*H*$_2$N(CH$_3$)$_2$), 2.21 (s, 6H, NHCH$_2$CH$_2$N(C*H*$_3$)$_2$); R_f = H(0.38)/I(0.72).

6-[[[2-(1-Piperidinyl)ethyl]amino]carbonyl]forskolin (44): ^{1}H-NMR δ 3.26 (m, 2H, NHC*H*$_2$CH$_2$-1-piperidinyl), 2.37 (m, 2H, NHCH$_2$C*H*$_2$-1-piperidinyl); R_f = G(0.65)/K(0.49).

6-[[[2-(2-Pyridyl)ethyl]amino]carbonyl]forskolin (45): ^{1}H-NMR δ 8.48 (d, 1H, NHCH$_2$CH$_2$-2-*pyridyl*), 7.59 (dd, 1H, NHCH$_2$CH$_2$-2-*pyridyl*), 7.11−7.16 (m, 2H, NHCH$_2$CH$_2$-2-*pyridyl*), 3.59 (dd, 2H, NHC*H*$_2$CH$_2$-2-pyridyl), 2.99 (t, 2H, NHCH$_2$C*H*$_2$-2-pyridyl); R_f = I(0.50)/H(0.44).

6-[[[2-(4-Aminophenyl)ethyl]amino]carbonyl]forskolin (46): ^{1}H-NMR δ 7.00 (d, 2H, phenyl), 6.63 (d, 2H, phenyl), 3.3−3.5 (2m, 2H, NHC*H*$_2$CH$_2$-phenyl) 2.70 (t, 2H, NHCH$_2$C*H*$_2$-phenyl); R_f = B(0.36)/D(0.66).

6-[[(2-Hydroxyethyl)amino]carbonyl]forskolin (47): ^{1}H-NMR δ 3.22 (m, 2H, NHC*H*$_2$CH$_2$OH), 3.06 (t, 2H, NHCH$_2$C*H*$_2$OH); R_f = H(0.36)/J(0.72).

6-[[(2-Methoxyethyl)amino]carbonyl]forskolin (48): ^{1}H-NMR δ 3.43 (m, 2H, NHCH$_2$C*H*$_2$OCH$_3$), 3.34 (s, 3H, NHCH$_2$CH$_2$OC*H*$_3$), 3.37 (NHC*H*$_2$CH$_2$OCH$_3$); R_f = D(0.66)/B(0.52).

6-[[(2-Carbethoxyethyl)amino]carbonyl]forskolin (49): ^{1}H-NMR δ 4.13 (q, 2H, NHCH$_2$CH$_2$CO$_2$C*H*$_2$CH$_3$), 3.44 (dd, 2H, NHC*H*$_2$CH$_2$CO$_2$Et), 2.53 (t, 2H, NHCH$_2$C*H*$_2$CO$_2$Et), 1.24 (t, 3H, NHCH$_2$CH$_2$CO$_2$CH$_2$C*H*$_3$); R_f = B(0.42)/H(0.78).

Biological Evaluation. Preparation of Membranes. The AC is expressed in Sf9 cells using the recombinant baculovirus expression system as described previously.[8] The AC activity in the cells is approximately 20-fold higher than that of the control. Cells were lysed and membranes washed and resuspended in 20 mM Na HEPES (pH 8.0), 2 mM dithiothreitol, 1 mM EDTA, and 200 mM sucrose plus protease inhibitors: 22 mg/L each of L-1-(tosylamino)-2-phenylethyl chloromethyl ketone, 1-chloro-3-(tosylamino)-7-amino-2-heptanone and phenylmethanesulfonyl fluoride, plus 3.2 mg/L each of leupeptin and lima bean trypsin inhibitor. The protein concentration of the membranes was determined by dye binding using bovine serum albumin as the standard.

Binding of Analogs to Type I Adenylyl Cyclase Membranes. Approximately 40 000 cpm of [^{125}I]-2-[3-(4-hydroxy-3-iodophenyl)propanamido]-*N*-ethyl-6-(aminocarbonyl)forskolin (6-IHPP), 40 μg of membranes and increasing concentrations (0.06 nM to 10 μM) of the selected forskolin analog in a total volume of 0.4 mL of 50 mM Tris-HCl (pH 7.5), and 5 mM of MgCl$_2$ were incubated for 60 min at ambient temperature. The assays were terminated by rapid filtration through glass fiber filters (Whatman GF/C) using a Brandel cell harvester. The filters were washed three times with 4 mL of cold 50 mM Tris-HCl (pH 7.5) and then counted. The binding data was analyzed using the Ligand program.[13] The IC$_{50}$ ± SE were determined by analysis of two independent experiments with

each data point being determined in triplicate. The average SD was less than 10%.

Activation of Type I Adenylyl Cyclase by Analogs. Activation of Type I AC was performed with each derivative using a one-column AC assay system.[12] The membranes were variable in the AC activity observed, therefore activation was expressed as a percent of activation by 100 μM forskolin, which was included in each assay as a positive control. The percent activation by increasing concentrations of each compound were then plotted together along with their standard deviation using the Kaleidagraph program with a four-parameter logistic regression model. The EC_{50}'s generated for each compound were the result of at least two determinations done in triplicate. Some derivatives did not reach the 100% stimulation level of forskolin. Most frequently this was the case with forskolin analogs containing hydrophobic groups or those that were poor stimulators and had not attained a maximum inflection point. This assay is dependent on the formation of [^{32}P]cAMP from [^{32}P]-αATP in membranes. All assays were performed for 10 min at 30 °C with increasing concentrations (0.32−100 μM) of the selected forskolin analog in a final volume of 100 μL in the presence of 10 mM of $MgCl_2$. Following the incubation period, the [^{32}P]-αATP is precipitated by Zn^{2+} and [^{32}P]cAMP is isolated by chromatography over alumina. The raw data were converted into specific activity then transformed to a percent of maximal attained by control (100 μM forskolin). The average SD was less than 10%.

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