Purine-Based Inhibitors of Inositol-1,4,5-trisphosphate-3-kinase

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Inositol 1,4,5-trisphosphate 3-kinase (IP3K) catalyzes the adenosine triphosphate (ATP) dependent phosphorylation of inositol 1,4,5-trisphosphate (IP3) to inositol 1,3,4,5-tetrakisphosphate (IP4)[1] and plays an important role in the regulation of intracellular calcium concentration. IP3 is a well-known second messenger that stimulates intracellular calcium mobilization, which is in turn responsible for numerous intracellular responses related to fertilization, cell growth, and secretion.[2] Production of IP3 is triggered by cell surface receptor activation by extracellular ligands that act downstream of both the receptor tyrosine kinases and the guanosine triphosphate binding protein (G-protein) coupled receptors.[3] In addition, it has been suggested that IP4, the product of the IP3K-catalyzed reaction, also serves as a second messenger that potentiates IP3 as a calcium oscillation regulator through inhibition of hydrolysis of IP3[4][5] (Figure 1) and other mechanisms.[6] Consequently, a specific inhibitor for IP3K will be a useful tool to study the role of this enzyme in transmembrane signal transduction and intracellular calcium metabolism in vitro and in vivo.

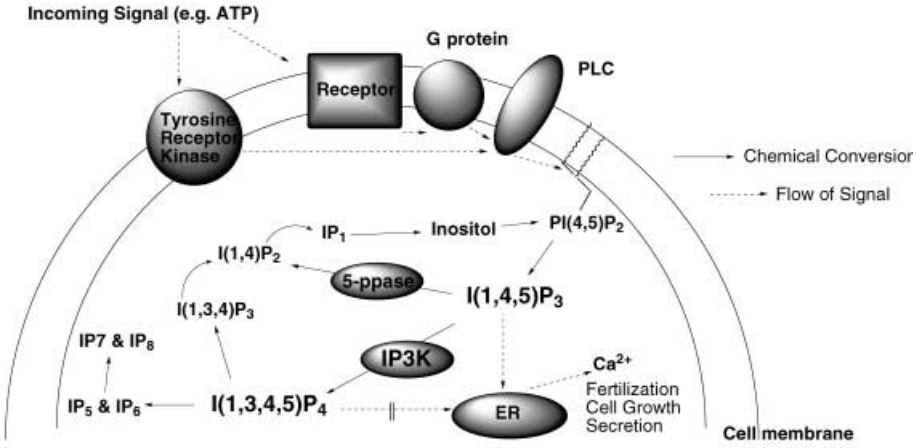

Figure 1. *Inositol phosphate signaling pathway and metabolism. ER = endoplasmic reticulum; 5-ppase = 5-phosphatase; PLC = phospholipase C.*

IP3K most likely has both an ATP binding pocket and an IP3 binding pocket for the catalytic phosphate transfer from ATP to IP3 (Figure 2). We previously synthesized all the possible inositol phosphates (IP1 – IP5) and tested them as competitive inhibitors for the IP3 binding site. Among the 38 inositol phosphate regioisomers, only I(1,4,6)P_3 and three IP4 isomers exhibited weak inhibitory effects on IP3K activity (IC_{50} values in the range 10 – 60 μM) while the natural substrate, IP3, had an IC_{50} value of 3.6 μM.[7] Many other IP3 analogues have also been synthesized and screened against IP3K. IP3K exhibits a remarkable stereo- and regioselectivity that allows recognition of the IP3 binding site.[8] The anthracyclin anticancer drug doxorubucin (adriamycin) has been used as an IP3K inhibitor[9] but it has shown stronger inhibition activity towards topoisomerase II. Herein, we report the first purine-based inhibitors of IP3K that bind to the ATP binding site and their effects on intracellular calcium regulation.

A purine library with structural diversity at the 2, 6, and 9-positions was recently developed to target cyclin-dependent kinases (CDK).[10] The druglike properties of these molecules (molecular weight < 500, computed partition coefficient < 5, etc.) and the fact that more than 10% of the proteins encoded by the genome depend on a purine-containing ligand for their function[11] suggested the possibility of versatile application of purine libraries to many different biological activities. Indeed, novel activities distinct from CDK inhibition, such as microtubule depolymerization[12] and sulfotransferase inhibition,[13] have

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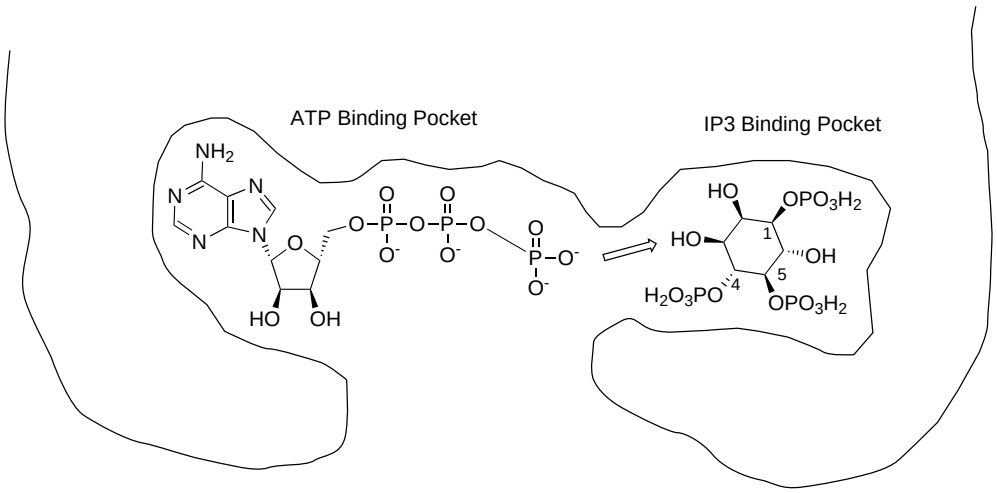
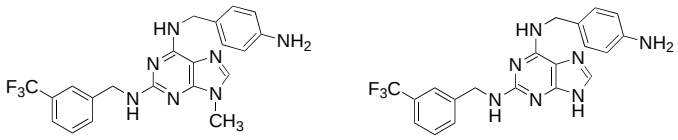


Figure 2. *The two binding sites of IP3K for ATP and IP3.*

recently been demonstrated for compounds in this library, which proves the usefulness of the library as a rich source of lead compound generation. We screened the representative purine library against recombinant IP3K[14] and discovered two promising lead inhibitors of IP3K, **1** and **2** (Figure 3), among hundreds of purines. The active compounds from other screens, such as CDK and compounds capable of microtubule depolymerization, did not show any inhibitory activity against IP3K.



1 IC_{50} = 57.1 μM

2 IC_{50} = 13.5 μM

Figure 3. *The lead compounds selected from purine library screening.*

A sublibrary of these lead structures was synthesized and rescreened against IP3K. The results are listed in Table 1. Several observations stand out with regard to structure – activity relationships (SARs). First, any substitution at the 9-position significantly reduced the inhibitory activity. Second, several *p*-substituted benzyl groups at the 6-position exhibited comparable activities, whereas aniline-substituted purines were much less active (**10**, **12**). Finally, at the 2-position, a substituted benzyl with an electron withdrawing group inhibited the IP3K activity more strongly than a positively charged derivative (**14**).

We derivatized the 2-position with various benzyl groups in order to further enhance the IP3K inhibition activity and to explore the 2-position systematically. The 6-position was fixed as a *p*-nitrobenzyl group (the best substituent in the previous SAR study) (**5**). The results of the IP3K inhibition study of these 2-position derivatives are summarized in Table 2. While different electron withdrawing groups (CF_3, Cl, F) at the 2, 3, or 4-position of 2-benzylamine gave moderate changes in activity, none of the new derivatives in this series were more active than the lead compound **5**. Repeated screening with different concentrations of ATP clearly showed that **5** binds to the ATP binding site of IP3K with an inhibition constant K_i of 4.3 μM (Michaelis constant K_m for ATP = 36.8 μM). It is noteworthy that compound **5** is a significantly weaker inhibitor of CDK1 ($IC_{50} > 100$ μM).

Compound **5** was added to HL60 cells and the intracellular calcium kinetics were measured by real-time imaging (Figure 4) to test the effect of IP3K inhibition in cells. Upon treatment with compound **5**, the intracellular calcium level increased over 1 – 2 mins in a dose-dependent manner and any subsequent ATP-induced calcium release was almost completely absent at a 20 μM concentration of **5**. One explanation is that the inhibition of IP3K caused an accumulation of IP3, which was originally maintained at a constant level by the dynamic inositol recycling machinery, and the elevated IP3 levels caused the release of calcium from the endoplasmic reticulum. The actual change in IP3 level after treatment with **5** was measured by use of radioactive IP3 and specific binding protein; the increase in IP3 concentration was two- to threefold compared to nontreated

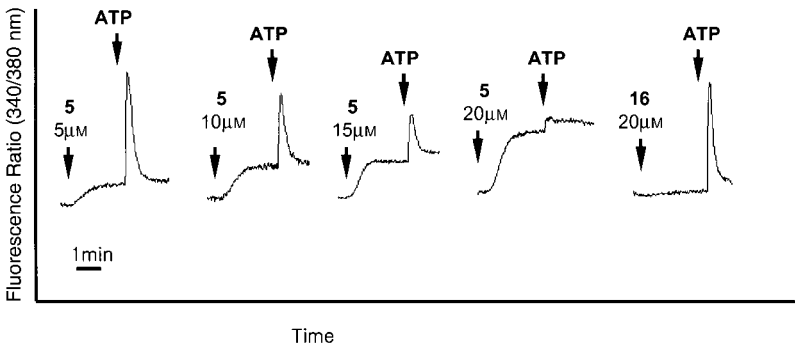


Figure 4. *Intracellular calcium signaling kinetics after the addition of the IP3K inhibitor **5** and ATP. The inactive compound **16** was used as a control.*

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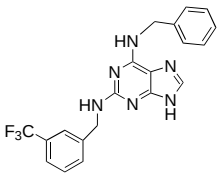
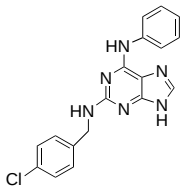
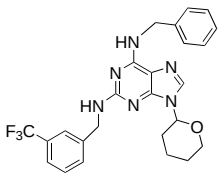
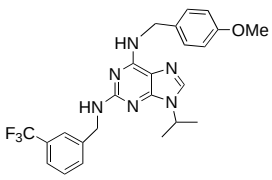
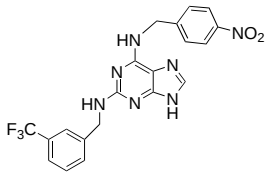
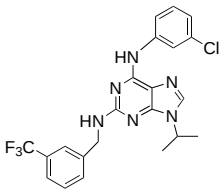
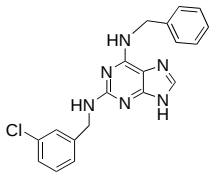
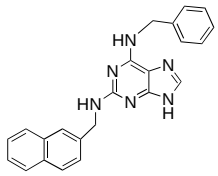
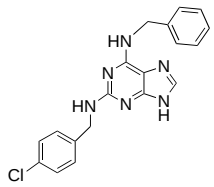
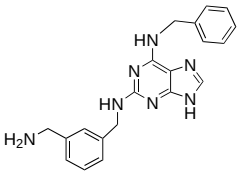
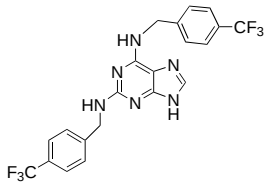
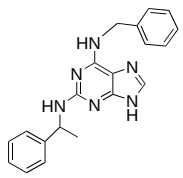
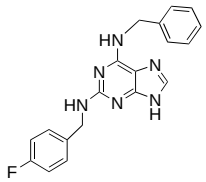
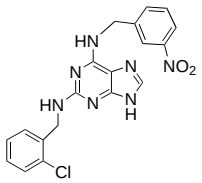
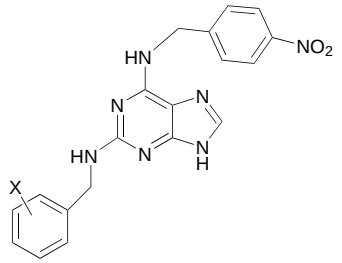
Table 1. *Inhibition of IP3K by the initial sublibrary.*

Compound	Structure	IC_{50} [μM]	Compound	Structure	IC_{50} [μM]
3		13.7	**10**		150
4		43.4	**11**		> 200
5		10.2	**12**		> 200
6		150	**13**		63
7		138	**14**		> 200
8		102	**15**		117
9		220	**16**		> 200

cells. The inactive control compound **16** showed no effect on the calcium signals in cells and subsequent treatment with ATP induced a full calcium-release response. Compound **16** therfore did not induce IP3 accumulation. One of the best CDK1 and CDK2 inhibitors, aminopurvalanol,[15] and the tubulin depolymerizing purine myoseverin,[12] were tested and did not trigger any calcium signal increase. These compounds gave similar responses to that of the control compound **16**, which indicates that the calcium effect of compound **5** is IP3K specific. Interestingly, the previously reported IP3K inhibitor doxorubucin did not show any

Table 2. *IP3K inhibition by 2-substituted derivatives of 6-(4-nitrobenzyl)-purines.*



Derivatives	IC_{50} [μM]
5: X = 3-CF_3	10.2
17: X = 2-CF_3	18.2
18: X = 4-CF_3	67.6
19: X = 2-F	23.4
20: X = 3-F	49.0
21: X = 4-F	28.8
22: X = 2-Cl	28.0
23: X = 3-Cl	20.0
24: X = 4-Cl	12.7

Ca^{2+} ion effect in either our experiment with HL60 cells or other experiments that used Jurkat cells.[9]

Alternatively, the Ca^{2+} ion increase and IP3 accumulation might arise either from an inhibition of IP3 degradation by IP3 5-ppase or from stimulation of IP3 production by PLC (Figure 1). Compound **5**, however, did not exhibit any significant effect on the enzymatic activity of either 5-ppase or PLC at 200 μM.

In conclusion, we have identified here the first purine based IP3K inhibitors to target the ATP binding site and have demonstrated their in vivo calcium regulation effect. Unlike the inositol phosphate analogue inhibitors, which are highly negatively-charged, these purine inhibitors will easily penetrate the cell membrane and will thus be of great use in the study of the role of IP3K in vivo.

Experimental Section

2**IP3K inhibition assay**: IP3K reactions were carried out in a solution (200 μL) that contained tris(hydroxymethyl)aminomethane (Tris) – Cl (50 mM, pH 8.0), KCl (100 mM), ethylene glycol tetraacetic acid (EGTA; 2 mM), ATP (50 μM), dithiothreitol (DTT; 1 mM), 2,3-diphosphoglycerate (1 mM), D-I(1,4,5)P_3 (20 μM), [^{3}H]-I(1,4,5)P_3 (20,000 dpm), and various amounts of synthetic purine analogues. The reaction was initiated by addition of IP3K that had been overexpressed in *E. coli* and purified to near homogeneity and the mixture was incubated for 10 min at 37 °C. The reaction was terminated by addition of H_3PO_4 (1 N, 50 μL). The reaction mixture was then neutralized by the addition of KOH (1 N, 50 μL) and diluted with water (500 μL). Each sample (500 μL) was immediately applied to an anion exchange column that contained Dowex AG1-X8 (formate form, 100 – 200 mesh) and then subsequently washed ten times with ammonium formate (0.55 M, 2 mL) in formic acid (0.1 M) to remove unreacted D-I(1,4,5)P_3. The product of IP3K activity, [^{3}H]-I(1,3,4,5)P_4, was eluted with ammonium formate (1.5 M, 1.5 mL) in formic acid (0.1 M) and then mixed with a complete counting cocktail solution (Budget-Solve, Research Product International Corp.; 10 mL) and its radioactivity was counted by a liquid scintillation counter. The inhibitory efficiency of the purine analogue against IP3K was represented by an IC_{50} value of 50%, which was estimated by interpolation of concentration – inhibition curves.

Intracellular calcium measurement: HL60 cells were cultured in a RPMI 1640 medium (Life Technologies, Inc.) supplemented with 20% bovine calf serum (Hyclone Laboratories, Inc.) in a humidified atmosphere of 95% air and 5% CO_2. The concentration of Ca^{2+} ions within the cells was determined by Grynkiewicz's method by using fura-2/AM dye. Briefly, cultured cells were incubated with fura-2/AM (3 μM) at 37 °C for 50 min in fresh serum-free RPMI 1640 medium with continuous stirring. A 2×10^6-cell aliquot was used for each assay in Ca^{2+}-free Locke's solution (NaCl (154 mM), KCl (5.6 mM), $MgCl_2$ (5.6 mM), 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (5 mM, pH 7.3), glucose (10 mM), and EGTA (0.2 mM). Fluorescence changes at two excitation wavelengths (340 nm and 380 nm) and an emission wavelength of 500 nm were measured.

Quantification of inositol 1,4,5-trisphosphate: I(1,4,5)P_3 concentration in the cells was determined by competition assay with [^{3}H]-I(1,4,5)P_3 as described previously.[16] In brief, HL60 cells were treated with compounds **5** and **16** for 10 min. The reaction was terminated by addition of 15% (w/v) ice-cold trichloroacetic acid that contained EGTA (0.2 mM). The cells were left on ice for 30 min to extract the water-soluble inositol phosphates. Trichloroacetic acid was then removed by extraction with diethyl ether. The final preparation was neutralized with Trizma base (0.2 mM), and its pH value was adjusted to 7.5. Assay buffer (Tris buffer (0.2 mM) containing ethylenediaminetetraacetate (EDTA; 4 mM) and bovine serum albumin (4 mg mL^{-1})), [^{3}H]-I(1,4,5)P_3 (0.1 μCi mL^{-1}), and IP3-binding protein were added to the cell extract. The mixture was incubated for 15 min on ice and then centrifuged at 2000 × g for 10 min. Water and the scintillation mixture were added to the pellet and radioactivity was measured. The I(1,4,5)P_3 concentration in the sample was determined based on a standard curve and expressed as pmol (mg protein)$^{-1}$ in the cell extract. The IP3-binding protein was prepared from bovine adrenal cortex according to the method of Challiss et al.[17] The amounts of IP3 determined are as follows (pmol (mg protein))$^{-1}$: control (120 ± 30), compound **5** (310 ± 50), and compound **16** (135 ± 42).

Phospholipase C and I(1,4,5)P_3 5-phosphatase assays: PLC and 5-phosphatase assays were performed as described previously, with slight modifications.[18] The whole rat brain was homogenized at 4 °C in a buffer that contained Tris – HCl (50 mM, pH 7.5), sucrose (250 mM), EDTA (0.5 mM), EGTA (1 mM), DTT (1 mM), leupeptin (2 μg mL^{-1}), aprotinin (2 μg mL^{-1}), and phenylmethanesulfonyl fluoride (0.5 mM). The homogenate was centrifuged at 2000 × g for 10 min and the resulting supernatant was used as a source of I(1,4,5)P_3 5-phosphatase. The reaction was initiated by addition of an appropriate amount of the 5-phosphatase to a solution containing 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES; 50 mM, pH 7.0), $MgCl_2$ (1 mM), DTT (1 mM), [^{3}H]-I(1,4,5)P_3 (20 000 cpm), and I(1,4,5)P_3 (2 μM) in a final volume of 200 μL. The reaction mixture was incubated at 37 °C for 5 min, terminated, and neutralized by the same method as described for the IP3K assay. The diluted reaction mixture was analyzed directly on a Partisil SAX 10 HPLC column to measure the amount of I(1,4)P_2 produced from the reaction.

The enzyme activity of PLC was measured in the assay mixture (200 μL), which contained phosphatidyl inositol diphosphate ([^{3}H]PIP_2 20,000 cpm; 10 μM), EGTA (1 mM), $CaCl_2$ (10 mM), 0.1% sodium deoxycholate, bovine serum albumin (1 mg mL^{-1}) and HEPES (50 mM, pH 6.8). The reaction mixture was incubated with the appropriate amount of purified PLC at 37 °C for 10 min and terminated by the addition of chroloform/methanol/HCl (1 mL; 50:50:0.3; v/v). The reaction product, [^{3}H]-I(1,4,5)P_3, was extracted

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with HCl (1 N, 0.3 mL) that contained EGTA (5 mM). After centrifugation, an aliquot (0.5 mL) of the upper aqueous phase was removed and the radioactivity was measured with scintillation counting.

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[1] R. F. Irvine, A. J. Letcher, J. P. Heslop, M. J. Berridge, *Nature* **1986**, *320*, 631 – 634.
[2] V. Sorrentino, R. Rizzuto, *Trends Pharmacol. Sci.* **2001**, *22*, 459 – 464.
[3] M. J. Berridge, *Nature* **1993**, *361*, 315 – 325.
[4] M. C. Hermosura, H. Takeuchi, A. Fleig, A. M. Riley, B. V. L. Potter, M. Hirata, R. Penner, *Nature* **2000**, *408*, 735 – 740.
[5] D. M. Zhu, E. Tekle, C. Y. Huang, P. B. Chock, *J. Biol. Chem.* **2000**, *275*, 6063 – 6066.
[6] R. Irvine, *Curr. Biol.* **2001**, *11*, R172 – R174.
[7] G. Choi, Y. T. Chang, S. K. Chung, K. Y. Choi, *Bioorg. Med. Chem. Lett.* **1997**, *7*, 2709 – 2714.
[8] B. V. L. Potter, D. Lampe, *Angew. Chem.* **1995**, *107*, 2085 – 2125; *Angew. Chem. Int. Ed. Engl.* **1995**, *34*, 1933 – 1972.
[9] C. P. Da Silva, F. Emmrich, A. H. Guse, *J. Biol. Chem.* **1994**, *269*, 12521 – 12526.
[10] a) Y. T. Chang, N. Gray, G. R. Rosania, D. P. Sutherlin, S. Kwon, T. Norman, R. Sarohia, M. Leost, L. Meijer, P. G. Schultz, *Chem. Biol.* **1999**, *6*, 361 – 375; b) N. S. Gray, S. Kwon, P. G. Schultz, *Tetrahedron Lett.* **1997**, *38*, 1161 – 1164; c) N. S. Gray, L. Wodicka, A. M. Thunnissen, T. C. Norman, S. Kwon, F. H. Espinoza, D. O. Morgan, G. Barnes, S. LeClerc, L. Meijer, S. H. Kim, P. G. Schultz, *Science* **1998**, *281*, 533 – 538; d) T. C. Norman, N. S. Gray, J. T. Koh, P. G. Schultz, *J. Am. Chem. Soc.* **1996**, *118*, 7430 – 7431.
[11] Information from a search of a yeast genome database: http://www.quest7.proteome.com
[12] a) G. R. Rosania, Y. T. Chang, O. Perez, D. Sutherlin, H. Dong, D. J. Lockhart, P. G. Schultz, *Nat. Biotechnol.* **2000**, *18*, 304 – 308; b) Y. T. Chang, S. M. Wignall, G. R. Rosania, N. S. Gray, S. R. Hanson, A. I. Su, J. Merlie, Jr., H. S. Moon, S. Sangankar, S. B., O. Perez, R. Heald, P. G. Schultz, *J. Med. Chem.* **2001**, *44*, 4497 – 4500; c) O. D. Perez, Y. T. Chang, G. Rosania, D. Sutherlin, P. G. Schultz, *Chem. Biol.* **2002**, *9*, 475 – 483.
[13] a) J. I. Armstrong, A. R. Portley, Y. T. Chang, D. M. Nierengarten, B. N. Cook, K. G. Bowman, A. Bishop, N. S. Gray, K. M. Shokat, P. G. Schultz, C. R. Bertozzi, *Angew. Chem.* **2000**, *112*, 1359 – 1362; *Angew. Chem. Int. Ed.* **2000**, *39*, 1303 – 1306; b) D. E. Verdugo, M. T. Cancilla, X. Ge, N. S. Gray, Y. T. Chang, P. G. Schultz, M. Negishi, J. A. Leary, C. R. Bertozzi, *J. Med. Chem.* **2001**, *44*, 2683 – 2686.
[14] Recombinant plasmids for the rat brain IP3K were constructed and overexpressed in *E. Coli*: K. Y. Choi, H. Y. Kim, S. Y. Lee, K. H. Moon, S. S. Sim, J. W. Kim, H. K. Chung, S. G. Rhee, *Science* **1990**, *248*, 64 – 66. The enzyme was purified to near homogeneity and screened with ATP (50 μM) and IP3 (10 μM): S. Y. Lee, S. S. Sim, J. W. Kim, K. H. Moon, J. H. Kim, S. G. Rhee, *J. Biol. Chem.* **1990**, *265*, 9434 – 9440.
[15] G. R. Rosania, J. Merlie, Jr., N. Gray, Y. T. Chang, P. G. Schultz, R. Heald, *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 4797 – 4802.
[16] T. J. Park, S. K. Song, K. T. Kim, *J. Neurochem.* **1997**, *68*, 2177 – 2185.
[17] R. A. Challiss, E. R. Chilvers, A. L. Willcocks, S. R. Nahorski, *Biochem. J.* **1990**, *265*, 421 – 427.
[18] K. H. Moon, S. Y. Lee, S. G. Rhee, *Biochem. Biophys. Res. Commun.* **1989**, *164*, 370 – 374.

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Solution- and Solid-Phase Synthesis of the Polybasic Lipid-Modified C Termini of Rho A and K-Ras 4B

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Lipid-modified peptides and semisynthetic lipid-modified proteins have proven to be invaluable chemical tools for the study of biological processes like membrane localization of, and signal transduction through, lipidated proteins.[1] Paramount to the success of this chemical – biological approach is the availability of efficient methods for the synthesis of such peptide conjugates that include the attachment of suitable reporter groups for application in subsequent biological or biophysical experiments.[1, 2–4]

The techniques developed so far have given access to different acid- and base-labile lipidated peptides. However, peptide conjugates that include a farnesyl or geranylgeranyl thioether and a stretch of basic amino acids, in particular a polybasic oligolysine sequence, proved to be, and still are, major challenges to the synthetic chemist. Currently, efficient methods for the synthesis of such conjugates are not available. Combinations of an *S*-lipidated cysteine with polybasic amino acid sequences frequently occur in signal-transducing proteins that belong to the Ras super family, such as the Rho proteins and K-Ras 4B. This structural motif is thought to mediate, for instance, selective intracellular localization and trafficking of the proteins (in particular in the case of K-Ras 4B[5]) and to play a decisive role in protein activation/deactivation (both the Ras and the Rho proteins may be targets of farnesyltransferase inhibitors[6] currently under clinical investigation.)

The problems encountered in the synthesis of polybasic lipidated peptides are highlighted by the synthesis of the C terminus of the K-Ras 4B protein (**2**) by Gelb et al.[7] In this

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