Synthesis and Activity of 6-Substituted Purine Linker Amino Acid Immunostimulants†

Boulos Zacharie, Lyne Gagnon, Giorgio Attardo, Timothy P. Connolly, Yves St-Denis, and Christopher L. Penney*

Department of Medicinal Chemistry, BioChem Therapeutic Inc., 275 Armand-Frappier Boulevard, Laval, Quebec, H7V 4A7

Received December 12, 1996⊗

A series of 6-substituted purinyl alkoxycarbonyl amino acids were synthesized and evaluated for their ability to stimulate cytotoxic T lymphocytes (CTLs) and the mixed lymphocyte reaction (MLR). A few of these compounds, in particular [[5-[6-(*N*,*N*-dimethylamino)purin-9-yl]pentoxy]-carbonyl]D-arginine (BCH-1393, **4a**), displayed an *in vitro* stimulation of CTLs comparable to interleukin 2 (IL 2). BCH-1393 increased the CTL response between 10^{-9} M and 10^{-5} M. Further, this potent *in vitro* activity was reflected as a significant increase in CTL cell number *in vivo*. However, immunophenotyping of some of the other equipotent compounds did not reveal a parallel relative increase in CTLs *in vivo*. It was difficult to formulate a rigorous structure−activity relationship based on *in vitro* CTL activity. Nevertheless, the activity was dependent upon the nature of the 6-substituent on the purine, the type and stereochemistry of the amino acid, and the distance and spatial freedom between the purine and amino acid as defined by the length and rigidity of the linker. These compounds were generally nontoxic, as exemplified by BCH-1393. BCH-1393 is a promising immunostimulant which may be targeted for those disease states which require an increased CTL or TH1 type response.

Introduction

Pharmaceutically induced stimulation of the immune system offers an important approach to the control of disease. In addition to the toxicity associated with the use of chemotherapeutics, there is increasing concern regarding the development of resistance to antibacterial, antiviral, and antitumor agents. Yet, research into this important area of immunology has lagged behind the more traditional pharmaceutical sector. Few well defined synthetic immunostimulants have been approved for use in North America. One example is the substituted thiazole, levamisole, which was approved for Duke's class C colorectal cancer in combination with 5-fluorouracil. Levamisole has the ability to act primarily on the T-cell lineage in a manner similar to thymic hormones and so is classified as a "thymomimetic" immunostimulant.[1] More recently, another T-cell stimulant, tucaresol, has been described[2] and is now in clinical trials.

Among the classes of molecules which can nonspecifically stimulate the immune system, nucleosides have displayed promising activity. In particular, a group of substituted guanosines, exemplified by loxoribine, cause significant B and NK-cell stimulation.[3] This is primarily attributed to the ability of these compounds to induce interferon. However, another group of nucleosides with important thymomimetic properties comprise analogues of inosine or the base portion, hypoxanthine. The immunological importance of inosine is demonstrated by the severe immunodeficiency which occurs in the congenital absence of adenosine deaminase.[4] While inosine may be expected to function as an immunostimulant *in vivo*, the high activity of purine nucleoside phosphorylase in lymphoid cells rapidly metabolizes it. A slow-release formulation of inosine, isoprinosine, has weak immunostimulant activity *in vivo* and is licensed in many countries as an antiviral agent.[5] As such, isoprinosine has served as an important standard for the hypoxanthine class of thymomimetic immunostimulants. Indeed, numerous hypoxanthine derivatives have been described such as NPT 15392, NPT 16416, and more recently methyl inosine monophosphate (MIMP).[6] Although these compounds are nontoxic, they are generally not very active, especially as regards the ability to stimulate CTLs.

In an effort to enhance the immunostimulant activity of hypoxanthine derivatives, a compound was synthesized which contained hypoxanthine and the amino acid L-arginine connected by a pentamethylene linker. L-Arginine was selected because it plays a role in immune activation.[7] The biology of this immunostimulant, hypoxanthine pentoxycarbonyl L-arginine (ST 789, **1b**),[7] has been thoroughly described, but the data reported does not indicate that it is significantly superior. Interestingly, a number of analogues have been reported in the patent and scientific literature in which the L-arginine portion of ST 789 has been replaced with small peptides. For example, introduction of the bombesin carboxy terminal dipeptide, leucyl methionine, gave a compound which was reported to modulate the proliferation of hematopoietic cells.[8] However, little has been stated as regards replacement of hypoxanthine with other purine moieties. One report[9] cites work with other naturally occurring bases (adenine, guanine), but no details were provided regarding immunological activity. In summary, the literature suggests that thymomimetic activity arises from the presence of hypoxanthine, and any improved or altered immunological activity requires the presence of L-arginine or naturally occurring peptide sequences covalently linked to hypoxanthine.

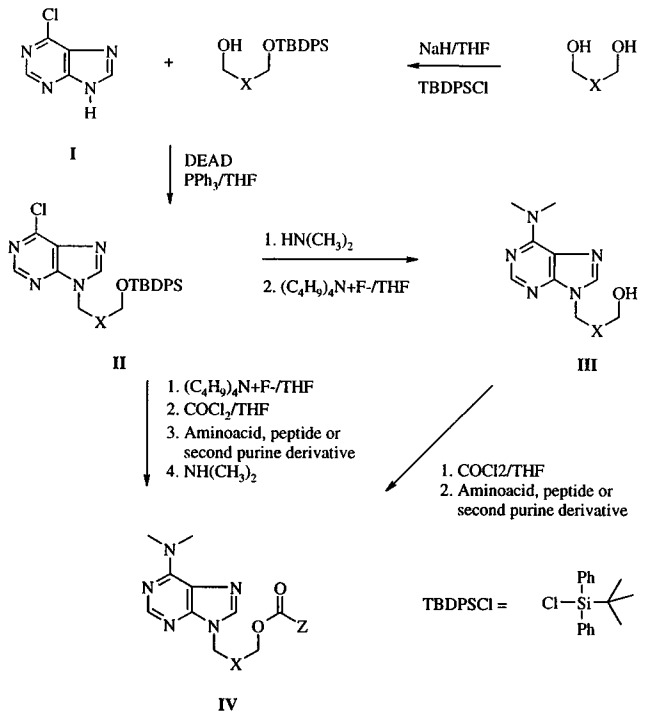
In this paper, we report on the immunological activity of compounds which possess a structural motif resem-

† Part of this work was presented at the 87th Annual Meeting of the American Association for Cancer Research, Washington D.C., April 20−24, 1996.

⊗ Abstract published in *Advance ACS Abstracts,* August 1, 1997.

S0022-2623(96)00844-8 CCC: $14.00

Scheme 1[a]



[a] X = acyclic or cyclic alkyl, or aryl group, with or without heteroatoms; Z = amino acid, peptide, or second alkylpurine unit. For BCH 1393, X = $(CH_2)_3$, Z = D-ARG.

bling ST 789: arginine covalently attached to a purine via a pentamethylene linker. However, these compounds are significantly different from ST 789. The purine is not hypoxanthine or another naturally occurring base, and the amino acid is the unnatural D-enantiomer. Further, immunological activity does not require the presence of an amino acid, and the natural L-enantiomer can be antagonistic.

Chemistry

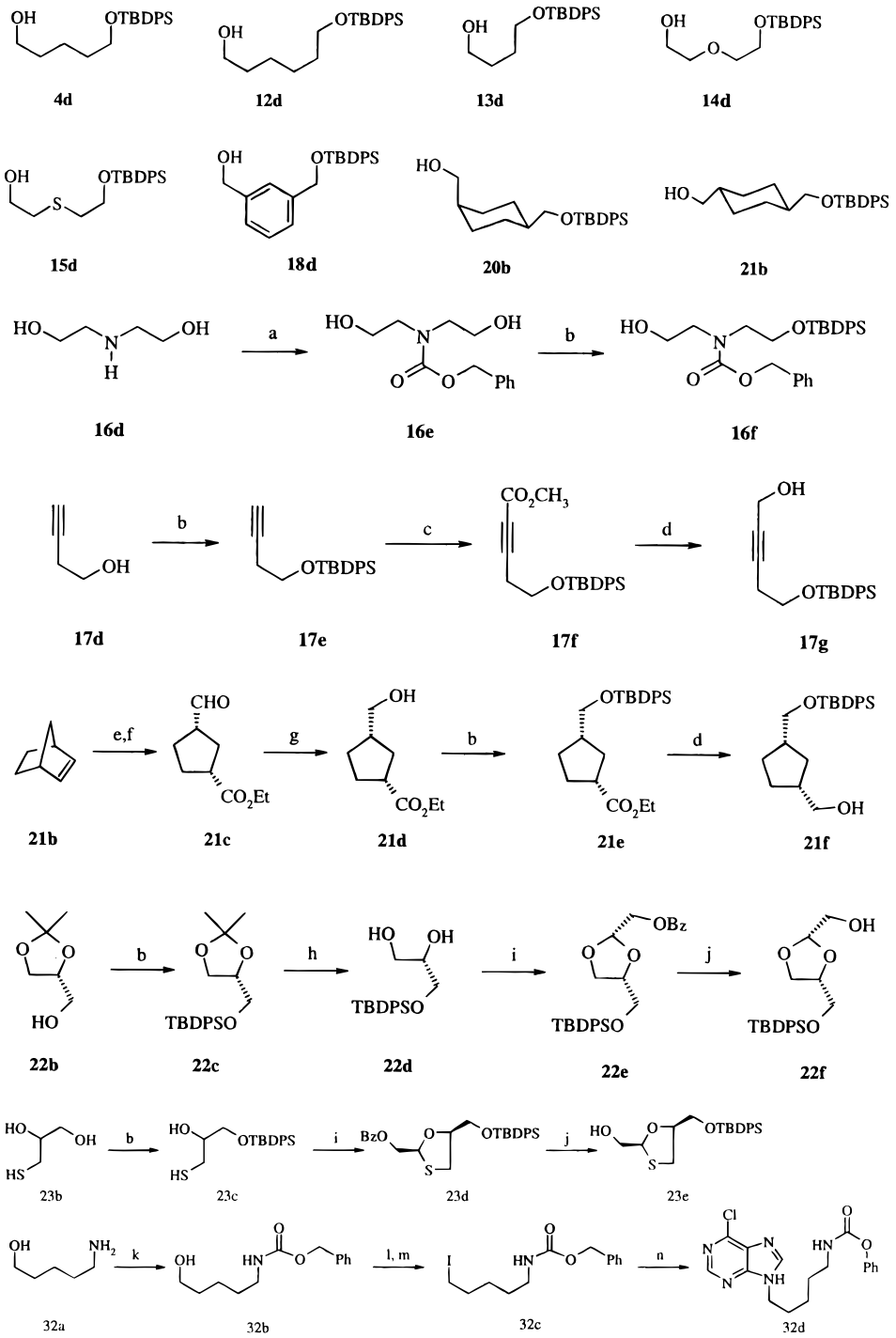
Our first approach to the synthesis of substituted purine compounds followed that described for ST 789 and analogues.[9] That is, the purine ring was built from 5-amino-4,6-dichloropyrimidine while incorporating the linker portion at the same time. However, this route was long and the yield of product poor. Therefore, our goal was to develop a simplified synthesis starting from commercially available substituted purine. An approach was selected in which the linker was attached to 6-chloropurine **I** by means of a Mitsunobu reaction with monoprotected diol (Scheme 1).

The diols were protected by reaction of *tert*-butylchlorodiphenylsilane (TBDPSCl) with acyclic or cyclic alkyl or aralkyl diols, in the presence of base. These precursor diols are commercially available or conveniently prepared according to Scheme 2. For example, protected butynol **17e** was treated with *n*-butyllithium and methyl chloroformate to give ester **17f** which was reduced to the corresponding alcohol **17g**. We also developed a simple procedure to prepare ester **21e**. Ozonolysis of norbornylene in methanol[10] gave aldehyde **21c** which was reduced with sodium borohydride to alcohol **21d**. The latter was protected and converted to the monoprotected alcohol **21f** by reduction of ester **21e** with DIBAL in dichloromethane. The preparation of other cyclic diols, 1,3-dioxolane- and 1,3-oxathiolane-2,5-dimethanol, was also undertaken. Protection of 1,3-dioxolane methanol **22b** followed by selective hydrolysis of the silylated acetonide **22c** with trifluoroacetic acid (TFA) afforded the monoprotected triol **22d**. Treatment of this compound or thiol **23c** with (benzoyloxy)acetaldehyde gave benzoate **22e** and **23d**, respectively. Alkaline hydrolysis gave the corresponding monoprotected alcohol **22f** or **23e**. The Mitsunobu approach was also successfully applied to amino alcohols. In this case, the amino function of compounds **16d** and **32a** was protected as the carbobenzyloxy (Cbz) derivative, and the remaining diol of **16e** was monoprotected as a TBDPS ether using the procedure described above.

The Mitsunobu reaction proceeded in moderate yield (50−60%) and is general for different diols and amino alcohols. In addition, the desired N-9 adduct was formed in high yield relative to the N-7 adduct. However, this procedure is limited by the difficult removal of the triphenylphosphine oxide byproduct, which makes this approach awkward for large scale synthesis. Therefore, it was decided to explore a more practical approach for the preparation of chloropurine **II**. Recently, we demonstrated that coupling between 4-substituted 1*H*-pyrazolo[3,4-*d*]pyrimidine bases and protected or unprotected iodoalkanols proceeded efficiently and in reasonable yield.[11] We therefore investigated the reaction between 6-chloropurine and iodoalkanols. These compounds were prepared by treatment of the corresponding mesylate with sodium iodide in refluxing acetone.[11] Other routes were also examined for the preparation of iodoalkanols. For example, treatment of protected diol with triphenylphosphine, imidazole, and iodine[12] gave the expected iodoalkanol in good yield. Specific linkers of four or five carbon atoms length were prepared by reaction of tetrahydrofuran or tetrahydropyran with iodotrimethylsilane (TMSI) to give ring opening of the cyclic ether and the corresponding silyl protected iodoalkanol.[13a−c] This latter approach is practical and amenable to scale-up. Therefore, preparation of purine derivative **II** was achieved in a single step by preparation of iodoalkanol *in situ*, followed by reaction with chloropurine and cesium carbonate (Scheme 3). Similar results were obtained with protected iodoalkylamine. For example, treatment of protected iodoamine **32c** with 6-chloropurine and cesium carbonate in DMF at 0 °C afforded **32d** in high yield.

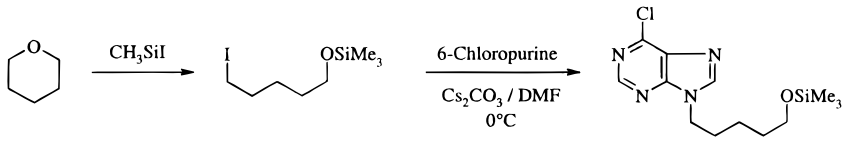
Removal of the silyl group of purine **II** was accomplished by treatment with fluoride, followed by neutralization with dilute HCl or acetic acid, to give the corresponding alcohol. These compounds were then linked to amino acids, peptides, or a second purine derivative by a carbamate or ester function. The latter was prepared in good yield by activation of the acid with carbodiimide, followed by treatment with the alcohol. The carbamate group was introduced by carbonylation of alcohol **III** followed by treatment of the resulting chloroformate with different amines (Scheme 1). A number of procedures were examined for the preparation of the carbamates. These included 1,1′-carbonyldiimidazole and triphosgene, of which neither gave satisfactory results. However, phosgene in toluene gave the desired purine derivatives **IV**. In the case of amino acids, the carboxylic function was converted *in situ* to the sodium salt, thus requiring no protection. The free amine of these salts was then treated with the chloroformate in aprotic solvents such as THF or DMF. The reactions proceeded slowly and product yield was low.

Scheme 2[a]



[a] Key: (a) Benzyl chloroformate, K_2CO_3, CH_3CN; (b) imidazole, TBDPSCl, CH_2Cl_2; (c) n-BuLi, methyl chloroformate, hexane, −78 °C; (d) DIBAL-H, CH_2Cl_2, −78 °C; (e) Ozone, CH_2Cl_2, MeOH, −78 °C; (f) Ac_2O, Et_3N, CH_2Cl_2, 0 °C to rt; (g) $NaBH_4$, MeOH, 0 °C; (h) CF_3COOH, THF/H_2O; (i) $BzOCH_2CHO$, PPTS, toluene; (j) MeONa/MeOH; (k) benzyl chloroformate, aq. Na_2CO_3; (l) MsCl, TEA, CH_2Cl_2; (m) NaI, acetone; (n) 6-chloropurine, Cs_2CO_3, DMF.

Scheme 3



This was due presumably to the lower solubility of the anion in those solvents. Homogeneous reaction conditions were examined in an attempt to improve product yields. In a mixture of THF:H_2O (1:1), the amine reacts efficiently with the chloroformate to give compound **IV** in high yield. This procedure is applicable to all purine derivatives and has been employed to supply gram quantities of BCH-1393. It offers the advantage of a practical and general route for the preparation of a variety of analogues.

In order to prepare *N*,*N*-dimethylpurine derivatives **III** and **IV**, two approaches were considered (Scheme

1). The first route was the deprotection of the silyl group of **II** followed by introduction of the carbamate function to give **IV**. The second approach was the conversion of the 6-chloropurine of **II** to *N*,*N*-dimethyl group, followed by removal of the protecting group, to afford alcohol **III**. The latter was converted to the final product **IV** as described above. Both approaches gave high yield of *N*,*N*-dimethyl derivative **IV** and were appropriate for large-scale synthesis.

In Vitro Analysis: SAR Studies

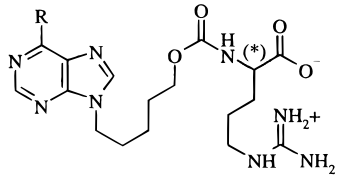
The two commonly used *in vitro* models for T-cell mediated responses are the cytotoxic T lymphocyte (CTL) reaction and the mixed lymphocyte reaction (MLR). A unidirectional CTL/MLR assay was undertaken by incubation of murine splenocytes, along with irradiated allogeneic splenocytes, for five days in the presence or absence of candidate immunostimulant. Early in the program, a sample of ST 789, *N*-[(hypoxanthin-9-ylpentoxy)carbonyl]-L-arginine (**1b**), was synthesized, along with the D-enantiomer (**1a**), for evaluation of *in vitro* CTL activity. The adenine (6-aminopurine) analogues, with L- and D-arginine, were also made, along with the (monomethylamino)- and (dimethylamino)purine equivalents. Measurement of the CTL activity induced by each of these eight compounds revealed some interesting points, as shown in Table 1. ST 789, which was predicated upon the importance of L-arginine, possesses the same CTL activity as the D-enantiomer. However, amongst the L-arginine compounds, the adenine analogue **2b** is the most active. Indeed, in the case of the monomethylamino and dimethylamino analogues **3b** and **4b**, L-arginine appears to function as an antagonist. The most active compound in the group is the (dimethylamino)purine analogue BCH-1393 (**4a**) which contains D-arginine. Clearly, the substituent at the 6-position of the purine base, along with the stereochemistry of arginine, determines the extent of CTL activity. The base does not have to be restricted to hypoxanthine. Other 6-position substituents were therefore examined, as shown in Table 1. None yielded a compound with greater CTL activity than BCH-1393. However, the hydrazino analogues **5a** and **5b** possess good activity. Whereas the activity of BCH-1393 is sensitive to arginine stereochemistry, the activity of the hydrazino compounds is hardly influenced by stereochemistry.

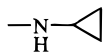
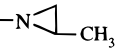
On the basis of the above results, compound **4a** (BCH-1393) was selected as a lead upon which to further define a structure−activity relationship for 6-(*N*,*N*-dimethylamino)purine−linker−amino acid. As shown in Table 2, analogues were synthesized in which the linker length and type were varied while maintaining 6-(dimethylamino)purine and arginine components. The BCH-1393 analogue with a shorter (tetramethylene) linker, **13a**, retains potent CTL activity. However, the potency is reduced with a longer (hexamethylene) linker, as seen with **12a**. The corresponding analogues with L-arginine, **12b** and **13b**, demonstrate a consistent trend. Regardless of the length of the linker, four to six carbons, there is no CTL activity when L-arginine is linked to 6-(*N*,*N*-dimethylamino)purine. Therefore, a series of BCH-1393 analogues were synthesized with five-atom linkers wherein the middle carbon is replaced with a heteroatom. Replacement with oxygen, **14a**, or nitrogen, **16a**, is well tolerated but activity declines with introduction of a larger sulfur atom, **15a**. CTL activity is abrogated upon introduction of a more rigid (π electron) five-carbon alkyne, **17a**. Interestingly, no CTL activity is observed when these five-atom linkers are used to connect L-arginine with 6-(*N*,*N*-dimethylamino)-purine, unless nitrogen is present in the linker, as seen in **16b**. Both **16a** and **16b** display good CTL activity.

It was next decided to introduce rigidity into the linker to examine if a defined geometry or spatial arrangement could improve the CTL activity of BCH-1393. Replacement of three of the five carbons of the linker in BCH-1393 with rigid five-membered rings, **21** to **23**, abolished CTL activity (Table 3). However, replacement with a six-membered cyclohexyl ring, **19** and **20**, preserved some weak activity especially if the purine base and arginine amino acid were in a trans-relationship. Nonetheless, these initial results were not encouraging, and so work was not pursued with L-arginine analogues or other constrained linkers.

Attention was next given to the role of the amino acid arginine. Therefore, analogues of BCH-1393 were made in which D-arginine was replaced with glycine, **24**;

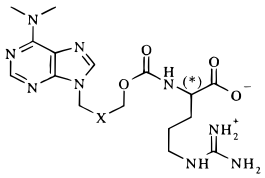
Table 1. *In Vitro* Cytotoxic T-Lymphocyte Activity of 6-Substituted Purine Pentoxycarbonyl Arginine Relative to Interleukin 2



		CTL Activity[b]	
		(*) = D-ARG	(*) = L-ARG
Compound[a]	R	a	b
1	-OH (**b** = ST 789)	+ + (10^{-7}M)	+ + (10^{-7}M)
2	$-NH_2$	+ (10^{-7}M)	+ + + (10^{-7}M)
3	$-NHCH_3$	+ (10^{-5}M)	0
4	$-N(CH_3)_2$ (**a** = BCH-1393)	+ + + + (10^{-7}M)	0
5	$-NHNH_2$	+ + + (10^{-7}M)	+ + + + (10^{-7}M)
6	-Cl	0	0
7	-SH	+ + + (10^{-7}M)	+ (10^{-7}M)
8	-H	+ + (10^{-5}M)	0
9		+ (10^{-7}M)	0
10		+ (10^{-7}M)	0
11		+ + (10^{-7}M)	*c*

[a] All compounds, dissolved in distilled water, were tested at concentrations of 10^{-5}, 10^{-7} and 10^{-9} M. Reported concentrations represent the peak activity. Each active compound was tested 2-4 × except **1b** (ST 789, n = 7) and **4a** (BCH-1393, n = 35). Inactive compounds were tested once. [b] Interleukin 2 represents maximum stimulation or the positive control. Therefore: 0 = 0 - 20% of interleukin 2; + = 21 - 40% of interleukin 2; + + = 41 - 60% of interleukin 2; + + + = 61 - 80% of interleukin 2; + + + + = 81 - 100% of interleukin 2. The reported score represents the mean of the experiment for n > 1. [c] Not tested; crude product difficult to handle/purify.

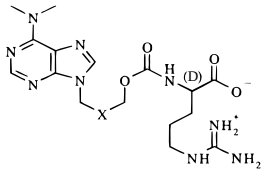
Table 2. *In Vitro* Cytotoxic T-Lymphocyte Activity of Analogues of the Lead Compound **4a**, BCH-1393, with Different Length/Type Linkers

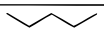
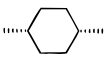
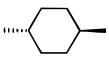


Compound[a]	X	CTL Activity[b] (*) = D-ARG **a**	(*) = L-ARG **b**
4	$-CH_2CH_2CH_2-$	+ + + + (10^{-7} M)	0
12	$-CH_2CH_2CH_2CH_2-$	+ + (10^{-7} M)	0
13	$-CH_2CH_2-$	+ + (10^{-9} M)	0
14	$-CH_2OCH_2-$	+ + + (10^{-7} M)	0
15	$-CH_2SCH_2-$	+ + (10^{-7} M)	0
16	$-CH_2NHCH_2-$	+ + + (10^{-9} M)	+ + + (10^{-9} M)
17	$-C\equiv CCH_2-$	0	0

[a,b] See Table 1 footnotes.

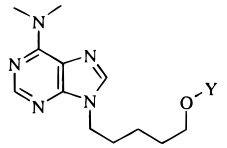
Table 3. *In Vitro* Cytotoxic T-Lymphocyte Activity of Analogues of the Lead Compound **4a**, BCH-1393, with Different Rigid Linkers

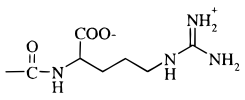
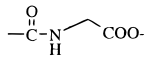
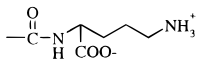
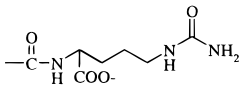
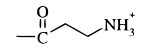
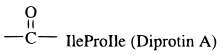
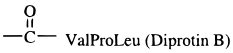
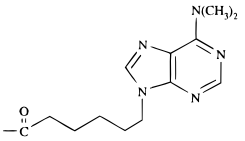
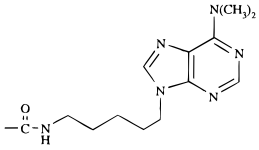
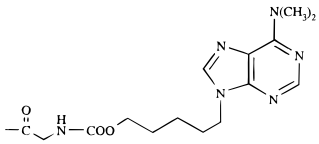


Compound[a]	X	CTL Activity[b]
4a		+ + + + (10^{-7}M)
18		0
19		+ (10^{-5}M)
20		+ + (10^{-7}M)
21		0
22		0
23		0

[a,b] See Table 1 footnotes.

Table 4. *In Vitro* Cytotoxic T-Lymphocyte Activity of Analogues of the Lead Compound **4a**, BCH-1393, with Different Amino Acids/Peptides



Compound[a]	Y	CTL Activity[b]
4		+ + + + (10^{-7}M)
24		+ (10^{-7}M)
25		0
26		+ (10^{-5}M)
27		+ + (10^{-7}M)
28	—H	+ + (10^{-7}M)
29	 IleProIle (Diprotin A)	0
30	 ValProLeu (Diprotin B)	0
31		+ + + (10^{-7}M)
32		+ + (10^{-7}M)
33		0

[a,b] See Table 1 footnotes.

D-ornithine, **25**; and D-citrulline, **26**. Replacement of the guanidino function of arginine with an amino, **25**, or urea function, **26**, severely impaired CTL activity (Table 4). Complete removal of the D-amino acid side chain is better tolerated, as is observed with the glycine compound, **24**. However, among these compounds, the best CTL activity is seen when β-alanine was esterified to the linker, compound **27**. Although **27** is not as active as BCH-1393, it possesses CTL activity which is similar to the desarginine analogue of BCH-1393, compound **28**. Interestingly, the desarginine analogue of **1b** (not shown in Table 4) displayed no CTL activity. It was noted in the introduction that small peptides have been linked to hypoxanthine to provide corresponding analogues of ST 789. Therefore, two analogues of BCH-1393, **29** and **30**, were made wherein arginine was replaced with the tripeptides diprotin A and B. These tripeptides inhibit the enzyme dipeptidyl peptidase IV (CD 26).[14] This enzyme is present on the cell surface of T-lymphocytes. However, **29** and **30** do not activate CTLs. Finally, three analogues of BCH-1393, **31**−**33**, were made wherein arginine was replaced with a second unit of 6-(*N*,*N*-dimethylamino)purine. Essentially, these compounds are dimers of desarginine BCH-1393, **28**. However, one of these dimers, **31**, has improved CTL activity, compared to **28**. Compound **32** has the same activity, while **33**, which is extended by a glycine linker

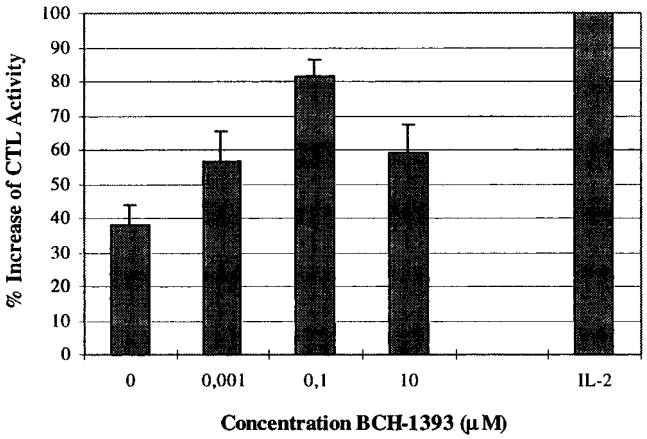


Figure 1. Dose response profile of the *in vitro* cytotoxic T-lymphocyte activity of BCH-1393 in comparison to interleukin-2.

connecting the two units, has no CTL activity. Nonetheless, these dimers are not as active as BCH-1393.

All of the compounds assayed for CTL activity were also examined for their ability to enhance MLR. The majority of compounds had no effect, while a few displayed a modest enhancement of the MLR; approximately 1.5 times at 10^{-7} M. The latter included BCH-1393, analogues of BCH-1393 where the linear linker contained a heteroatom, and dimers of desarginine BCH-1393.

In Vitro Profile of BCH-1393 (4a)

Since the structure−activity study did not reveal any compounds with activity superior to BCH-1393, with regard to the ability to stimulate CTLs, it was decided to examine the reproducibility of this stimulation. This is necessary in view of the variable nature of cell based proliferation assays, variation in cell populations between experiments, and sensitivity to environmental influences. Nonetheless, it was observed that as long as the IL 2 positive control was able to stimulate a significant (maximal) increase in specific cell lysis, BCH-1393 displayed a robust CTL stimulation. The dose−response profile for BCH-1393, as a mean of 13 experiments, is shown in Figure 1. The stimulation of CTLs achieved with 10^{-7} M BCH-1393 is comparable to IL 2. It was also decided to compare the CTL stimulation induced by BCH-1393 with the aromatic aldehyde tucaresol, MIMP, and formylphenoxyacetic acid. T-cell stimulation was reported to be a general property of aromatic aldehydes,[2] and the latter compound is a metabolite of the tuberculosis drug aconiazide.[15] As can be seen in Figure 2, only BCH-1393 at 10^{-7} M induces a significant stimulation of CTLs ($P < 0.001$, $n = 5$) relative to the negative control.

In addition to stimulation of CTL activity and a modest effect on the mixed lymphocyte reaction, BCH-1393 displays weak T-cell proliferative activity (data not shown) in the presence of concanavalin A mitogen. However, BCH-1393 alone does not stimulate NK and B-cell populations (data not shown).

In Vivo Profile of BCH-1393 (4a)

In view of the significant *in vitro* CTL activity of BCH-1393, it was of interest to determine if this activity would translate into an *in vivo* effect. Therefore, immunophenotyping experiments were undertaken with normal immune status mice, in the presence or absence

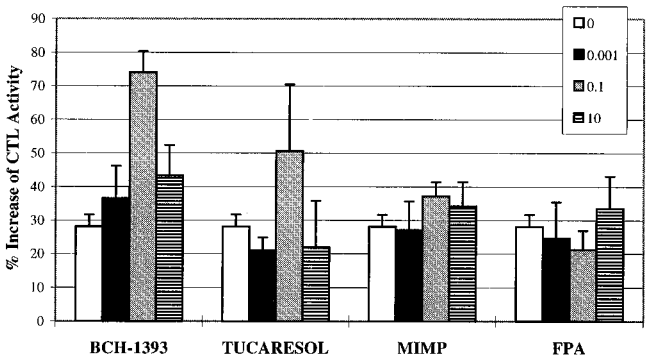


Figure 2. Comparison of the *in vitro* cytotoxic T-lymphocyte activity of BCH-1393 with tucaresol, MIMP, and FPA. Concentration is expressed as μM.

Table 5. Summary of Immunophenotyping Data for Mice Administered One Dose of BCH-1393

cell subsets		0 mg/kg	25 mg/kg	50 mg/kg	100 mg/kg
Blood Immunophenotyping BCH-1393[a]					
CD4−CD8−	mean	65.9	70.7	74.7	82.1
	STD	11.1	3.97	4.92	8.59
	P value		0.187	0.072	0.002
CD4+	mean	20.9	21.9	19.8	14.0
	STD	1.76	3.00	3.89	6.87
	P value		0.289	0.274	0.026
CD8+	mean	8.10	6.30	5.10	3.53
	STD	3.54	0.54	1.14	1.59
	P value		0.131	0.047	0.020
Spleen Immunophenotyping BCH-1393					
CD8+CD45+	mean	7.81	7.46	8.92	9.13
	STD	1.06	0.75	2.36	1.24
	P value		0.300	0.185	0.035

[a] Groups of seven C57BL/6 mice were injected ip for one day with BCH-1393.

of BCH-1393, to determine the increase or decrease of immune cell subset populations, *relative to each other*. Mice were injected intraperitoneally for one, four, or nine consecutive days with BCH-1393 at different concentrations. At the end of the experiment, blood and spleens were collected for determination by flow cytometry of the effect of BCH-1393 on the immune cell subsets within these tissues. The results of the analysis are presented in Tables 5−7.

In blood, one dose of the highest concentration of BCH-1393, 100 mg/kg, significantly increases the relative percentage of non-B- non-T-cells (30%). While this subset was not identified, the probable candidate is a neutrophil population, based on relative occurrence. However, four doses (50 mg/kg) of BCH-1393 shifted the cell subset distribution so that significant ($P \leq 0.041$) increases (23% to 70%) occur in the CTL (CD8+ CD45+), NK (CD3±), and monocytes (CD11b+) populations. Nine doses (50 mg/kg) of BCH-1393 significantly ($P \leq 0.004$) increases (26%) T-helper (CD4+ CD45+) cells. In spleen, one dose of the highest concentration of BCH-1393 induces a significant ($P \leq 0.035$) increase (17%) in CTLs. This correlates with the observed increases in the blood upon administration of multiple doses of BCH-1393. Although not apparent after four doses, it is noteworthy that after nine doses of BCH-1393, the significant ($P \leq 0.035$) increase (44%) in CTLs persists. There is no indication of paradoxical effects[16] or immunoparalysis. Gross pathological examination of the mice at the end of the experiment revealed no abnormalities, including splenomegaly.

Although none of the analogues of BCH-1393 possess

Table 6. Summary of Immunophenotyping Data for Mice Administered Four Doses of BCH-1393

cell subsets		0 mg/kg	25 mg/kg	50 mg/kg
Blood Immunophenotyping BCH-1393[a]				
CD8+CD45+	mean	8.25	11.7	10.1
	STD	1.59	5.54	2.16
	P value		0.108	0.041
NK+CD3−	mean	6.01	5.90	8.14
	STD	0.98	1.39	1.35
	P Value		0.500	0.005
NK+CD3+	mean	3.43	5.84	3.25
	STD	0.76	2.08	0.57
	P value		0.021	0.289
CD4−CD11b+	mean	9.60	13.7	9.68
	STD	2.79	2.68	3.59
	P value		0.015	0.404
Spleen Immunophenotyping BCH-1393				
TCR	mean	38.9	39.8	45.1
	STD	3.83	7.61	7.34
	P value		0.421	0.035
Ly5	mean	55.5	54.1	50.4
	STD	3.44	7.30	6.72
	P value		0.367	0.034

[a] Groups of ten C57BL/6 mice were injected ip for four consecutive days with BCH-1393.

Table 7. Summary of Immunophenotyping Data for Mice Administered Nine Doses of BCH-1393

cell subsets		0 mg/kg	25 mg/kg	50 mg/kg	100 mg/kg
Blood Immunophenotyping BCH-1393[a]					
CD4+CD45+	mean	17.5	18.5	22.1	20.6
	STD	3.26	2.01	2.01	8.85
	P value		0.347	0.004	0.228
Spleen Immunophenotyping BCH-1393					
CD8+CD45+	mean	6.90	8.39	9.06	9.96
	STD	1.36	1.91	2.82	2.28
	P value		0.061	0.066	0.035

[a] Groups of seven C57BL/6 mice were injected ip for nine consecutive days with BCH-1393.

superior *in vitro* CTL activity, six of them (**5a**, **5b**, **13a**, **14a**, **16a**, and **16b**) exhibited similar activity. Therefore, two of these compounds, **5a** and **16a**, were selected for preliminary immunophenotyping to determine if their *in vivo* activity was similar to BCH-1393. This work was undertaken with four consecutive doses of compound and a smaller sample size ($n = 4$; data not shown). Nonetheless, the results clearly demonstrate that unlike BCH-1393, *in vitro* CTL activity does not translate *in vivo* into a corresponding increase in CTLs. In blood, **5a** induces a significant increase in non-T-cells (CD4- CD8-) relative to all other immune cell populations. No significant increases are observed in immune cell subsets in the spleen. In blood, **16a** also induces a significant increase in non-T-cells (CD4- CD8-) relative to all other immune cell populations except monocytes. This significant increase in non-T-cells is also seen in the spleen, along with a significant increase in macrophages.

In view of the unique ability of BCH-1393 to increase T-cell populations after four and nine doses, it was of interest to determine if the desarginine analogue **28** could induce a similar effect. Compound **28** displays good *in vitro* CTL activity, although less than BCH-1393. However, it is reasonable to expect that **28** represents a metabolite of BCH-1393, and so the question of *in vivo* activity becomes more important. Therefore, another preliminary immunophenotyping experiment was undertaken with four consecutive doses of compound and a smaller sample size ($n = 4$; data not shown). Interestingly, the results obtained with nine doses of BCH-1393 paralleled those observed with four doses of desarginine BCH-1393 (**28**). That is, in blood there is a significant increase in T-helper cells, while in spleen there is a significant increase in CTLs. However, a significant decrease in blood monocytes was observed which was not seen at all with BCH-1393.

Throughout these studies, it was observed that BCH-1393 and most of the analogues were well tolerated and devoid of any apparent toxicity. As such, a preliminary chronic toxicity study was undertaken in rats whereby they were administered BCH-1393 by intravenous injection for five consecutive days. Lethality occurs at 1 g/kg, but BCH-1393 is well tolerated at doses up to 500 mg/kg.

Discussion

In this study, the *in vitro* CTL activity of more than 50 6-substituted purine-linker amino acid hybrids and related structures was determined and compared to known T-cell stimulants. On the basis of these results, a lead molecule, BCH-1393 (**4a**), was selected for *in vivo* immunological evaluation. While it was difficult to formulate a rigorous structure−activity relationship as pertains to CTL activity, a few trends emerged which are as follows:

(1) Activity is dependent upon the nature of the substituent at the 6-position of the purine ring. While no substituent, compound **8a**, results in minimal activity, the 6-chloro compounds, **6a** and **6b**, are inactive. Indeed, the nature of the 6-substituent appears to determine if arginine will improve, maintain, or abolish immunological activity.

(2) Activity is dependent upon the type and stereochemistry of the amino acid covalently linked to the purine base. D-Arginine is the most effective amino acid, as exemplified by compounds **4a**, **5a**, **13a, 14a**, and **16a**. L-Arginine can be as effective as the respective D-enantiomer, for example compounds **1b**, **2b**, **5b** and **16b**, but more often it abrogates CTL activity. Other amino acid substituents such as glycine or isosteric citrulline result in reduced activity and the absence of an amino acid can result in the same or better relative activity. This is illustrated by comparison of compounds **24**, **25**, and **26** with **28** or desarginine BCH-1393.

(3) Activity is dependent upon the distance and relative degrees of freedom between the purine and amino acid as defined by the length and rigidity of the linker. A distance of four to five (carbon) atoms appears to represent an optimum, as illustrated by compounds **4a** and **13a**. Introduction of rigidity, for example compounds **18**−**23**, significantly diminishes activity relative to the acyclic linker in **4a** or BCH-1393.

Activity was not improved, relative to BCH-1393, by linking two 6-*N*,*N*-dimethylaminopurine units together as shown by compounds **31**, **32**, and **33**. It is interesting to observe that activity decreases as the distance between the two purine units increases which suggests a dimeric binding site or receptor. This somewhat parallels the relationship noted above between activity and distance between 6-*N*,*N*-dimethylaminopurine and D-arginine. However, this may be coincidence and the possibility cannot be ignored that **31** is, for example, a prodrug form of **28** or desarginine BCH-1393.

The impetus for the design of BCH-1393 (**4a**) came from the compound ST 789; hypoxanthine pentoxycar-

bonyl L-arginine (**1b**). Therefore, a sample of ST 789 was made early in the program and evaluated for *in vitro* CTL activity. The literature[7,17] indicates that ST 789 is able to enhance NK-cell and phagocytic populations and production of IL 6. However, there is no report of CTL activation or induction of IL 2. Therefore, the observation of *in vitro* CTL activity in our assay was unexpected. This was not pursued further since ST 789 is not under development as a T-cell stimulant. Instead, the CTL activity of BCH-1393 was compared with the two well documented T-cell stimulants, Tucaresol and MIMP.[6] While the results in Figure 2 indicate that only BCH-1393 has significant CTL activity, both Tucaresol and MIMP did display greater activity than the control. Furthermore, this is murine data which does not necessarily reflect the results that would be obtained with human CTLs. Nonetheless, these results, along with the extensive *in vitro* analysis of BCH-1393, as exemplified in Figure 1, justified an *in vivo* evaluation of BCH-1393.

Immunophenotyping experiments with BCH-1393 revealed a significant increase in the spleen and blood CTL populations. However, this increase was accompanied by significant increases in other immune cell subsets, and BCH-1393 appeared less specific *in vivo* relative to the *in vitro* profile. Nonetheless, it is difficult to delineate which cell populations are enhanced directly by BCH-1393 as opposed to indirect activation by means of cellular communication. One dose of BCH-1393 did not result in the immediate increase in CTLs in the blood. However, the increase in non-B- non-T-cells (neutrophils?) followed by T-cell, NK, and monocyte populations with repeated doses of BCH-1393 suggests a potential immunorestorative function. On the other hand, the persistent increase in CTLs after nine consecutive doses of BCH-1393 without immunoparalysis or tolerance suggests a potential role in chronic (e.g.: cancer, virus) treatment regimens. Indeed, recent efforts to enhance antitumor CTLs by means of therapeutic vaccination with a peptide representing a specific CTL epitope resulted in tolerance and subsequent increased tumor growth.[18]

The specificity of BCH-1393 observed *in vitro* for stimulation of CTLs and the corresponding lack of stimulation of T-cell proliferation is unusual and does not adhere to a strict classification of BCH-1393 as a thymomimetic drug. It appears unlikely that BCH-1393 shares a mechanistic pathway of immunological activation in common with hypoxanthine-containing thymomimetics. For example, unlike BCH-1393, MIMP increased the mitogenic responses to concanavalin A and phytohemagglutin.[6] Indeed, whereas hypoxanthine compounds may interact with an inosine receptor,[4] BCH-1393 may interact with an adenosine receptor. Perhaps more likely is a transient inhibition by BCH-1393 of adenosine deaminase or purine nucleoside phosphorylase, as has also been suggested for hypoxanthine thymomimetics.[4] Adenosine deaminase, present on the cell surface of T-lymphocytes, is ligated to CD26.[19] In preliminary work,[20] in addition to inducing a significant stimulation of human CTLs, BCH-1393 was shown to increase the specific enzyme activity of CD26 in IL 2/PHA-stimulated human blood T-cells (PBMLs). However, CD26 is preferentially restricted to T-helper (memory) populations.[19] This is contrary to the stimulation of CTLs induced by BCH-1393. The importance of CD26 is therefore uncertain with regard to the mechanism by which BCH-1393 stimulates CTLs. However, an important role for BCH-1393 which merits further investigation may be the potential to influence or alter T-cell apoptosis, in particular the survival of CTLs. Recently, it was reported that tumor necrosis factor (TNF) mediates the death of most CD8+ T-cells (CTLs) while Fas ligand (FasL) mediates the death of most CD4+ T-cells (T-helper).[21] Further, CD26 influences the expression of the receptor for FasL, CD95 (Fas/Apo-1).[19] The effect of BCH-1393 on the *in vivo* expansion of T-helper cells could be accounted for by its effect on the activity of CD26. More importantly, BCH-1393 may inhibit the production of TNF and thereby permit an *in vivo* expansion of CTLs. This inhibition may in turn result from interaction with adenosine deaminase (adenosine sparing effect) or the adenosine receptor. Adenosine has been reported to inhibit the production of TNF.[22] With regard to the structure of BCH-1393, interaction with adenosine deaminase appears more likely and would account for the expansion of *both* T-cell populations. Regardless of the mechanism by which BCH-1393 stimulates CTLs and subsequently expands T-cell populations, the effect on CTLs offers significant potential for the treatment of those diseases, cancer, viral and parasitic infections, where CTLs play an important role.

Experimental Section

Chemistry. Melting points were taken on a Fisher-Johns melting point apparatus and are uncorrected. Merck silica gel (Kieselgel 60) was used for TLC and flash column chromatography (230−400 mesh), with solvent systems (A) MeOH, (B) CH_3COONH_4, 0.01M pH 6, (C) 20% EtOAc/toluene, (D) 5% EtOAc/hexane, (E) 10% EtOAc/hexane, (F) 20% EtOAc/hexane, (G) 25% EtOAc/hexane, (H) 30% EtOAc/hexane, (I) 50% EtOAc/hexane, (J) EtOAc, (K) 10% MeOH/EtOAc, (L) 15% MeOH/EtOAc, (M) 20% MeOH/EtOAc, (N) 30% MeOH/EtOAc, (O) 40% MeOH/EtOAc, (P) 80% MeOH/EtOAc, (Q) BuOH/AcOH/H_2O, 5:1:2, (R) 10% NH_4OH/MeOH, (S) CH_3CN, 0.01% TFA, (T) H_2O, 0.01% TFA pH 2.9. NMR spectra were recorded on a Bruker DRX-400 or a Varian VXR-300 spectrometer. Chemical shifts (δ) are expressed in ppm. Mass spectra were recorded on a Kratos MS-50 TA instrument. Analytical HPLC results were obtained using a Waters instrument with a YMC C-4 (5 μm) 120 Å reverse phase column with a gradient of 20% to 50% (A)/(B), flow rate of 1 mL/min, or with a YMC ODS-A (5 μm) 120 Å reverse phase column with a graident of 0% to 25% (S)/(T), flow rate of 0.5 mL/min, and using a UV detector at an absorption of 256 nm. Tucaresol and MIMP were synthesized according to literature procedures.[23,24] All noncommercial 6-substituted purines were prepared by standard procedures.[25] All amino acids were obtained from Bachem Bioscience Inc. (King of Prussia, PA), and all other reagents were obtained from Aldrich Chemical Co. (Milwaukee, WI), Fluka (Ronkonkoma, NY), or VWR Inc. (Montreal, Canada). All nonaqueous reactions were carried out under an inert atmosphere. Standard workup consisted of pouring the reaction solution into the indicated solvent system (1:1 mixture) followed by extraction with CH_2Cl_2 (2 ×). The organic phase was dried over $MgSO_4$, solids were filtered, and the solvent was removed *in vacuo*.

General Procedure for the Synthesis of Monoprotected Diols. Compounds **4d**, **12d**, **13d**, **14d**, **15d**, **16f**, **18d**, **20b**, and **21b** were prepared from commercially available diols. Compounds **17g**, **21f**, **22f**, **23e** and **32b** were prepared as outlined in Scheme 2 (synthetic details are available as Supporting Information). All commercial diols were protected using the same procedure as described for **16f**; however, sodium hydride was substituted for imidazole in the protection of diols **4d**, **12d**, **13d**, **20b**, and **21b**.

***N*-(Carbobenzyloxy)diethanolamine (16e).** To a solution of **16d** (2.0 g, 19.0 mmol) in acetonitrile (19 mL) were

added successively powdered K_2CO_3 (5.3 g, 38 mmol) and benzyl chloroformate (2.9 mL, 20.0 mmol). The white suspension was stirred vigorously for 3 h and treated by standard workup (H_2O/CH_2Cl_2) to give **16e**, as a clear oil that was used without purification or characterization.

***N*-(Carbobenzyloxy)diethanolamine *tert*-Butyldiphenylsilyl Ether (16f).** To a solution of **16e** (1.29 g, 19.0 mmol) in dry CH_2Cl_2 (190 mL) at 0 °C were added imidazole (1.29 g, 19.0 mmol) and *tert*-butylchlorodiphenylsilane (5 mL, 19.0 mmol). The solution was stirred at 0 °C for 45 min and rt for 90 min. It was then treated by standard workup (saturated NH_4Cl/CH_2Cl_2). The crude product was purified by silica gel column chromatography, eluting with 20% ethyl acetate/toluene to give **16f** (2.09 g, 4.37 mmol, 23%) as a clear oil: ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.65−7.63 (4H, m, aromatic), 7.46−7.22 (11H, m, aromatic), 5.16 (2H, AB quartet, CH_2Ph), 5.06 (2H, AB quartet, CH_2Ph), 3.86−3.74 (4H, m, CH_2), 3.55−3.45 (4H, m, CH_2), 1.59 (1H, br s, OH), 1.05 (9H, s, *t*-Bu).

General Procedure for the Preparation of Alkylpurines (Scheme 1). Purine derivatives **IV** were prepared by Mitsunobu coupling of 6-chloropurine and monoprotected diols. The sequence is illustrated for **6c**.

5-(6-Chloropurin-9-yl)-1-[(*tert*-butyldiphenylsilyl)oxy]pentane (II). To a stirred solution of triphenylphosphine (4.7 g., 17.9 mmol) and 6-chloropurine (2.3 g., 15.1 mmol) in THF (100 mL) was added diethyl azodicarboxylate (2.8 mL, 17.9 mmol). After 10 min, **4d** (4.7 g., 13.7 mmol) dissolved in THF (20 mL) was added dropwise to the reaction, which was then stirred at ambient temperature overnight. The solvent was removed *in vacuo*, and the crude product was purified by silica gel column chromatography eluting with 30% ethyl acetate/hexane to give **II** (3.7 g, 7.6 mmol, 55%) as a colorless oil: TLC (K) R_f 0.30; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.74 (1H, s, purine); 8.07 (1H, s, purine); 7.62 (5H, m, aromatic); 7.39 (5H, m, aromatic); 4.27 (2H, t, NCH_2); 3.65 (2H, t, CH_2O); 2.0−1.3 (6H, m, $(CH_2)_3$); 1.05 (9H, s, *t*-Bu).

5-(6-Chloropurin-9-yl)pentanol (6c). To a stirred solution of **II** (2.3 g, 4.4 mmol) in THF (40 mL) was added tetrabutylammonium fluoride (5.3 mL, 5.1 mmol), and the reaction was stirred overnight. To the solution was added glacial acetic acid (0.3 mL, 5.1 mmol) and solvent removed *in vacuo*. The crude product was purified by silica gel column chromatography, eluting with 10% methanol/ethyl acetate. The solvent was removed *in vacuo* to give **6c** (1.0 g., 4.2 mmol, 95%) as a white solid: mp 84 °C; TLC (K) R_f 0.20; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.75 (1H, s, purine); 8.13 (1H, s, purine); 4.32 (2H, t, NCH_2); 3.65 (2H, t, CH_2O); 2.1−1.3 (7H, m, $(CH_2)_3$, OH).

Alternate Procedure for the Preparation of Alkylpurines (Scheme 2). Compound **32d** was prepared by coupling of 6-chloropurine and alkyl iodide.

5-(6-Chloropurin-9-yl)-*N*-(carbobenzyloxy)pentanamine (32d). To a solution of 6-chloropurine (1.55 g, 10.0 mmol) and Cs_2CO_3 in DMF (45 mL) was added **32c** (4.18 g, 12.0 mmol). The reaction was stirred for 16 h. The solvent was evaporated, and the crude product was purified by silica gel column chromatography, eluting with 80% ethyl acetate/hexane to give **32d** (2.53 g, 6.77 mmol, 67.7%) as a pale yellow oil: TLC (J) R_f 0.41; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.08 (1H, s, purine), 7.27 (5H, m, aromatic), 5.15 (1H, br s, NH), 5.03 (2H, s, $PhCH_2$), 4.22 (2H, t, purine-NCH_2), 3.18−3.10 (2H, m, NCH_2), 1.93−1.83 (2H, p, CH_2), 1.57−1.47 (2H, p, CH_2), 1.35−1.25 (2H, p, CH_2).

General Procedure for Coupling of Alcohol with Amino Acids or Peptides. To a stirred solution of 5-(6-hydroxypurin-9-yl)pentanol, **1c** (1.0 g, 4.2 mmol) in THF (75 mL) was added toluenic phosgene (4.4 mL, 8.8 mmol), and the reaction was stirred for 6−8 h. Formation of the chloroformate intermediate was monitored by TLC (A). The solvent and unreacted phosgene were removed *in vacuo*, and the residue was taken up in THF (75 mL). In a separate beaker, D-arginine (0.94 g, 5.4 mmol) was dissolved in minimal water, and added to the chloroformate suspension. After 10 min $NaHCO_3$ (1.06 g,12.6 mmol) was added, and the reaction was stirred overnight. The solvent was removed *in vacuo*, and the residue was purified by silica gel column chromatography, eluting with 80% MeOH/EtOAc. The combined product fractions were dried *in vacuo*, and the product was taken up in minimal methanol. Trace amounts of silica were removed by either of two methods: (1) the solution was cooled (4 °C) for 2−4 h and then centrifuged (375 g, 10 min), and the supernatant was removed, or (2) the solution was passed through a micron filter The solution was then added dropwise to vigorously stirred ether (500 mL). The product was collected on sintered glass, washed with ether, and dried *in vacuo* to give **1a** (0.85 g, 2.01 mmol, 48%) as a white solid: mp 182 °C; TLC (Q) R_f 0.18; ^{1}H NMR (DMSO-d_6, 300 MHz) δ 9.28 (1H, br, COOH), 8.10 (1H, s, purine), 8.04 (1H, s, purine), 7.8−7.2 (4H, br, guanidine), 6.38 (1H, d, NH), 4.32 (1H, br, OH), 4.13 (2H, t, NCH_2), 3.87 (2H, t, CH_2O), 3.61 (1H, m, $C^{\alpha}H$), 3.04 (2H, br, $C^{\delta}H_2$), 1.8−1.1 (10H, m, $C^{\beta}H_2$, $C^{\gamma}H_2$, $(CH_2)_3$); HRMS *m/z* calcd for $C_{17}H_{27}N_8O_5$ (MH^+) 423.2104, found 423.2124; HPLC 99%.

***N*$^{\alpha}$-[[5-(6-Hydroxypurin-9-yl)pentoxy]carbonyl]-L-arginine (1b, ST 789):**[9] yield 42%; mp 184 °C; TLC (A) R_f 0.20; spectral properties were identical with **1a**; HPLC 98%.

***N*$^{\alpha}$-[[5-(6-Aminopurin-9-yl)pentoxy]carbonyl]-D-arginine (2a):** yield 52%; mp 150 °C; TLC (A) R_f 0.20; ^{1}H NMR (DMSO-d_6, 300 MHz) δ 9.40(1H, br, COOH), 8.14 (1H, s, purine), 8.13 (1H, s, purine), 8.0−7.0 (6H, m, guanidine, NH_2), 6.36 (1H, br, NH), 4.13 (2H, t, NCH_2), 3.87 (2H, t, CH_2O), 3.65 (1H, m, $C^{\alpha}H$), 3.03 (2H, b, $C^{\delta}H_2$), 1.9−1.2 (10H, m, $C^{\beta}H_2$, $C^{\gamma}H_2$, $(CH_2)_3$); HRMS *m/z* calcd for $C_{17}H_{28}N_9O_4$ (MH^+) 422.2264, found 422.2242; HPLC 97%.

***N*$^{\alpha}$-[[5-(6-Aminopurin-9-yl)pentoxy]carbonyl]-L-arginine (2b):** yield 49%; mp 153−155 °C; TLC (A) R_f 0.22; spectral properties were identical with **2a**; HRMS *m/z* (MH^+) found 422.2259; HPLC 100%.

***N*$^{\alpha}$-[[5-[6-(*N*-Methylamino)purin-9-yl]pentoxy]carbonyl]-D-arginine (3a):** yield 61%; mp 130 °C; TLC (A) R_f 0.20; ^{1}H NMR (DMSO-d_6, 300 MHz) δ 9.30(1H, br, COOH), 8.21 (1H, s, purine), 8.13 (1H, s, purine), 7.8−7.2 (4H, br, guanidine), 6.29 (1H, d, NH), 4.13 (2H, t, NCH_2), 3.86 (2H, t, CH_2O), 3.61 (1H, m, $C^{\alpha}H$), 3.17 (1H, m, CH_3NH), 3.02 (3H, br, $NHCH_3$), 2.97 (2H, br, $C^{\delta}H_2$), 1.9−1.2 (10H, m, $C^{\beta}H_2$, $C^{\gamma}H_2$, $(CH_2)_3$); HRMS *m/z* calcd for $C_{18}H_{30}N_9O_4$ (MH^+) 436.2421, found 436.2400; HPLC 97%.

***N*$^{\alpha}$-[[5-[6-(*N*-Methylamino)purin-9-yl]pentoxy]carbonyl]-L-arginine (3b):** yield 48%; mp 130 °C; TLC (A) R_f 0.20; spectral properties were identical with **3a**; HRMS *m/z* (MH^+) found 436.2430; HPLC 98%.

***N*$^{\alpha}$-[[5-[6-(*N*,*N*-Dimethylamino)purin-9-yl]pentoxy]carbonyl]-D-arginine (4a, BCH 1393):** yield 43%; mp 123−125 °C; TLC (Q) R_f 0.23; ^{1}H NMR (DMSO-d_6, 300 MHz) δ 9.40 (1H, br, COOH), 8.20 (1H, s, purine), 8.15 (1H, s, purine), 8.0−7.3 (4H, br, guanidine), 6.33 (1H, d, NH), 4.13 (2H, t, NCH_2), 3.86 (2H, t, CH_2O), 3.63 (1H, m, $C^{\alpha}H$), 3.36 (6H, br s, $N(CH_3)_2$), 3.02 (2H, br, $C^{\delta}H_2$), 1.8−1.2 (10H, m, $C^{\beta}H_2$, $C^{\gamma}H_2$, $(CH_2)_3$); HRMS *m/z* calcd for $C_{19}H_{32}N_9O_4$ (MH^+) 450.2577, found 450.2578; HPLC 99%.

***N*$^{\alpha}$-[[5-[6-(*N*,*N*-Dimethylamino)purin-9-yl]pentoxy]carbonyl]-L-arginine (4b):** yield 47%; mp 123−125 °C; TLC (Q) R_f 0.25; spectral properties were identical with **3a**; HRMS *m/z* (MH^+) found 450.2575; HPLC 98%.

***N*$^{\alpha}$-[[5-(6-Hydrazinopurin-9-yl)pentoxy]carbonyl]-D-arginine (5a):** yield 65%; mp 134 °C; TLC (A) R_f 0.27; ^{1}H NMR (DMSO-d_6, 300 MHz) δ 9.20 (1H, br, COOH), 8.23 (1H, s, purine), 8.14 (1H, s, purine), 8.0−7.3 (4H, br, guanidine), 6.60 (2H, br, NH_2), 6.43 (1H, d, NH), 4.14 (2H, t, NCH_2), 3.87 (2H, t, CH_2O), 3.64 (1H, m, $C^{\alpha}H$), 3.04 (2H, br, $C^{\delta}H_2$), 1.8−1.3 (10H, m, $C^{\beta}H_2$, $C^{\gamma}H_2$, $(CH_2)_3$); HRMS *m/z* calcd for $C_{17}H_{29}N_{10}O_4$ (MH^+) 437.2373, found 437.2350; HPLC 99%.

***N*$^{\alpha}$-[[5-(6-Hydrazinopurin-9-yl)pentoxy]carbonyl]-L-arginine (5b):** yield 68%; mp 134 °C; TLC (A) R_f 0.26; spectral properties were identical with **5a**; LRMS *m/z* (MH^+) found 437; HPLC 98%.

***N*$^{\alpha}$-[[5-(6-Chloropurin-9-yl)pentoxy]carbonyl]-D-arginine (6a):** yield 41%; mp 145−148 °C; TLC (Q) R_f 0.26; ^{1}H NMR (DMSO-d_6, 300 MHz) δ 9.40 (1H, br, COOH), 8.77 (1H, s, purine), 8.73 (1H, s, purine), 8.1−7.2 (4H, br, guanidine), 6.35 (1H, d, NH), 4.29 (2H, t, NCH_2), 3.87 (2H, t, CH_2O), 3.64 (1H, m, $C^{\alpha}H$), 3.02 (2H, br, $C^{\delta}H_2$), 1.9−1.2 (10H, m, $C^{\beta}H_2$, $C^{\gamma}H_2$, $(CH_2)_3$); LRMS *m/z* (MH^+) found 441; HPLC 100%.

***N*$^{\alpha}$-[[5-(6-Chloropurin-9-yl)pentoxy]carbonyl]-L-arginine (6b):** yield 54%; mp 143−146 °C; TLC (Q) R_f 0.28; spectral

properties were identical with **6a**; LRMS *m/z* (MH^+) found 441; HPLC 94%.

***N*$^\alpha$-[[5-(6-Mercaptopurin-9-yl)pentoxy]carbonyl]-D-arginine (7a):** yield 61%; mp 200 °C; TLC (A); R_f 0.50; ^{1}H NMR (DMSO-d_6, 300 MHz) δ 9.15 (1H, b, COOH), 8.29 (1H, s, purine), 8.18 (1H, s, purine), 7.5−7.3 (4H, br, guanidine), 6.39 (1H, d, NH), 4.13 (2H, t, NCH_2), 3.87 (2H, t, CH_2O), 3.65 (1H, m, $C^\alpha H$), 3.04 (2H, br, $C^\delta H_2$), 1.90−1.23 (10H, m, $C^\beta H_2$, $C^\gamma H_2$, $(CH_2)_3$); HRMS *m/z* calcd for $C_{17}H_{27}N_8O_4S$ (MH^+) 439.1876, found 439.1858; HPLC 100%.

***N*$^\alpha$-[[5-(6-Mercaptopurin-9-yl)pentoxy]carbonyl]-L-arginine (7b):** yield 60%; mp 200 °C; TLC (A); R_f 0.50; spectral properties were identical with **7a**; LRMS *m/z* (MH^+) found 439; HPLC 100%.

***N*$^\alpha$-[[5-(Purin-9-yl)pentoxy]carbonyl]-D-arginine (8a):** yield 68%; hygroscopic solid; TLC (A); R_f 0.23; ^{1}H NMR (DMSO-d_6, 300 MHz) δ 9.10 (1H, s, purine), 8.93 (1H, s, purine), 8.64 (1H, s, purine), 7.8−7.2 (4H, br, guanidine), 6.25 (1H, br s, NH), 4.28 (2H, t, NCH_2), 3.87 (2H, t, CH_2O), 3.58 (1H, m, $C^\alpha H$), 3.09 (2H, m, $C^\delta H_2$), 1.20−1.90 (10H, m, $C^\beta H_2$, $C^\gamma H_2$, $(CH_2)_3$); HRMS *m/z* calcd for $C_{17}H_{27}N_8O_4$ (MH^+) 407.2155, found 407.2179; HPLC 99%.

***N*$^\alpha$-[[5-(Purin-9-yl)pentoxy]carbonyl]-L-arginine (8b):** yield 64%; hygroscopic solid; TLC (A); R_f 0.23; spectral properties were identical with **8a**; HRMS *m/z* (MH^+) found 407.2180; HPLC 92%.

***N*$^\alpha$-[[5-[6-(*N*-Cyclopropylamino)purin-9-yl]pentoxy]carbonyl]-D-arginine (9a):** yield 58%; mp 151 °C; TLC (A); R_f 0.34; ^{1}H NMR (DMSO-d_6, 300 MHz) δ 8.22 (1H, s, purine), 8.14 (1H, s, purine), 8.0−7.0(4H, br s, guanidine), 6.28 (1H, d, NH), 4.13 (2H, t, NCH_2), 3.87 (2H, m, CH_2O), 3.62 (1H, m, $C^\alpha H$), 3.02 (2H, m, $C^\delta H_2$), 1.8−1.2 (11H, m $C^\beta H_2$, $C^\gamma H_2$, $(CH_2)_3$), CH), 0.69 (2H, m, CH_2), 0.67 (2H, m, CH_2); HRMS *m/z* calcd for $C_{20}H_{32}N_9O_4$ (MH^+) 462.2577, found 462.2588; HPLC 100%.

***N*$^\alpha$-[[5-[6-(*N*-Cyclopropylamino)purin-9-yl]pentoxy]carbonyl]-L-arginine (9b):** yield 55%; mp 144−146 °C; TLC (A); R_f 0.35; spectral properties were identical with **9a**; HRMS *m/z* (MH^+) found 462.2578; HPLC 99%.

***N*$^\alpha$-[[5-[6-(*N*-Azetidinyl)purin-9-yl]pentoxy]carbonyl]-D-arginine (10a):** yield 74%; mp 190−192 °C; TLC (A); R_f 0.25; ^{1}H NMR (CD_3OD, 300 MHz) δ 7.96 (1H, s, purine), 7.89 (1H, s, purine), 4.27 (4H, m, 2 × CH_2-N-azetidine), 4.02 (2H, t, NCH_2), 3.79 (3H, m, CH_2O and $C^\alpha H$), 2.99 (2H, m, CH_2-azetidine), 2.32 (2H, m, CH_2), 1.71−1.17 (10H, m, $C^\beta H_2$, $C^\gamma H_2$, $(CH_2)_3$); HRMS *m/z* calcd for $C_{20}H_{32}N_9O_4$ (MH^+) 462.2577, found 462.2593; HPLC 100%.

***N*$^\alpha$-[[5-[6-(*N*-Azetidinyl)purin-9-yl]pentoxy]carbonyl]-L-arginine (10b):** yield 60%; mp 187 °C; TLC (A); R_f 0.27; spectral properties were identical with **10a**; LRMS *m/z* (MH^+), found 462; HPLC 100%.

***N*$^\alpha$-[[5-[6-Methylaziridin-*N*-yl)purin-9-yl]pentoxy]carbonyl]-D-arginine (11a):** yield 64%; mp 200 °C; TLC (A); R_f 0.40; ^{1}H NMR (CD_3OD, 300 MHz) δ 8.29 (1H, s, purine), 8.14 (1H, s, purine), 4.10 (2H, t, NCH_2), 3.79 (3H, m, CH_2O, $C^\alpha H$), 2.97 (2H, m, $C^\delta H_2$), 2.62 (1H, m, CH-aziridine), 2.45 (2H, d, CH_2-aziridine), 1.76−1.2 (13H, m, $C^\beta H_2$, $C^\gamma H_2$, $(CH_2)_3$), CH_3); HRMS *m/z* calcd for $C_{20}H_{32}N_9O_4$ (MH^+) 462.2604, found 462.2602; HPLC 95%.

***N*$^\alpha$-[[6-[6-(*N*,*N*-Dimethylamino)purin-9-yl]hexoxy]carbonyl]-D-arginine (12a):** yield 60%; mp 123 °C; TLC(A); R_f 0.48; ^{1}H NMR (CD_3OD, 300 MHz) δ 7.98 (1H, s, purine), 7.81 (1H, s, purine), 3.98 (2H, t, NCH_2), 3.78 (3H, m, CH_2O and $C^\alpha H$), 3.27 (6H, br s, $N(CH_3)_2$), 2.96 (2H, m, $C^\delta H_2$), 1.78−1.10 (12H, m, $C^\beta H_2$, $C^\gamma H_2$, $(CH_2)_4$); HRMS *m/z* calcd for $C_{20}H_{34}N_9O_4$ (MH^+) 464.2734, found 464.2749; HPLC 100%.

***N*$^\alpha$-[[6-[6-(*N*,*N*-Dimethylamino)purin-9-yl]hexoxy]carbonyl-L-arginine (12b):** yield 61%; mp 131 °C; TLC (A); R_f 0.47; spectral properties were identical with **12a**; HRMS *m/z* (MH^+) found 464.2754; HPLC 100%.

***N*$^\alpha$-[[4-[6-(*N*,*N*-Dimethylamino)purin-9-yl]butoxy]carbonyl]-D-arginine (13a):** yield 86%; mp 140−142 °C; TLC (P) R_f 0.20; ^{1}H NMR (DMSO-d_6, 300 MHz) δ 8.20 (1H, s, purine), 8.16 (1H, s, purine), 8.1−7.3 (4H, br, guanidine), 6.40 (1H, d, NH), 4.16 (2H, t, NCH_2), 3.91 (2H, t, CH_2O), 3.65 (1H, m, $C^\alpha H$), 3.40 (6H, br s, $N(CH_3)_2$), 3.02 (2H, m, $C^\delta H_2$), 1.9−1.3 (8H, m, $C^\beta H_2$, $C^\gamma H_2$, $(CH_2)_2$); HRMS *m/z* calcd for $C_{18}H_{30}N_9O_4$ (MH^+) 436.2421, found 436.2392; HPLC 99%.

***N*$^\alpha$-[[4-[6-(*N*,*N*-Dimethylamino)purin-9-yl]butoxy]carbonyl]-L-arginine (13b):** yield 75%; mp 139−142 °C; TLC (P) R_f 0.20; spectral properties were identical with **13a**; LRMS *m/z* (MH^+) found 436.2421; HPLC 100%.

***N*$^\alpha$-[[[6-(*N*,*N*-Dimethylamino)purin-9-yl]ethoxy]ethoxy]carbonyl]-D-arginine (14a):** yield 33%, mp 140−145 °C; TLC (A) R_f 0.35; ^{1}H NMR (DMSO-d_6, 300 MHz) δ 8.25 (1H, s, purine), 8.14 (1H, s, purine), 6.5 (1H, br, NH), 4.37 (2H, t, NCH_2), 4.03 (2H, m, CH_2), 3.81 (2H, m, CH_2), 3.72 (1H, m, $C^\alpha H$), 3.60 (2H, m, CH_2), 3.72 (6H, m, $N(CH_3)_2$), 3.05 (2H, m, $C^\delta H_2$), 1.78−1.39 (4H, m, $C^\beta H_2$, $C^\gamma H_2$); HRMS *m/z* calcd for $C_{18}H_{30}N_9O_5$ (MH^+) 452.2370, found 452.2400; HPLC 98%.

***N*$^\alpha$-[[[6-(*N*,*N*-Dimethylamino)purin-9-yl]ethoxy]ethoxy]carbonyl]-L-arginine (14b):** yield 41%; mp 130−135 °C; TLC (A) R_f 0.35; spectral properties were identical with **14a**; HRMS *m/z* (MH^+) found 452.2361; HPLC 100%.

***N*$^\alpha$-[[[[6-(*N*,*N*-Dimethylamino)purin-9-yl]ethyl]thio]ethoxy]carbonyl]-D-arginine (15a):** yield 26%; mp 150−155 °C; TLC (Q) R_f 0.38; ^{1}H NMR (DMSO-d_6) δ 8.26 (1H, s, purine), 8.22 (1H, s, purine), 6.61 (1H, br, NH), 4.39 (2H, t, NCH_2), 4.09 (2H, t, CH_2O), 3.72 (1H, m, $C^\alpha H$), 3.48 (8H, m, $N(CH_3)_2$, CH_2), 3.06 (2H, t, CH_2), 2.78 (2H, t, $C^\delta H_2$), 1.75−1.40 (4H, m, $C^\beta H_2$, $C^\gamma H_2$); HRMS *m/z* calcd for $C_{18}H_{30}N_9O_4S$ (MH^+) 468.2141, found 468.2114; HPLC 99%.

***N*$^\alpha$-[[[[6-(*N*,*N*-Dimethylamino)purin-9-yl]ethyl]thio]ethoxy]carbonyl]-L-arginine (15b):** yield 57%; mp 145−150 °C; TLC (A) R_f 0.35; spectral properties were identical with **15a**; HRMS *m/z* (MH^+) found 468.2129; HPLC 99%.

***N*$^\alpha$-[[[[6-(*N*,*N*-Dimethylamino)purin-9-yl]ethyl]amino]ethoxy]carbonyl]-D-arginine (16a):** yield 56%; mp 160−165 °C; TLC (A) R_f 0.10; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 9.36 (m, 1H), 8.19 (1H, s, purine), 8.11 (1H, s, purine), 6.38 (1H, d, NH), 4.17 (2H, t, CH_2), 3.91−3.87 (2H, m, CH_2), 3.65 (1H, m, $C^\alpha H$), 3.43 (6H, br s, $N(CH_3)_2$), 3.03−3.01 (2H, m, $C^\delta H_2$), 2.90 (2H, t, CH_2), 2.68 (2H, t, CH_2), 1.64−1.44 (4H, m, $C^\beta H_2$, $C^\gamma H_2$); HRMS *m/z* calcd for $C_{18}H_{31}N_{10}O_4$ (MH^+) 451.2530, found 451.2519; HPLC 96%.

***N*$^\alpha$-[[[[6-(*N*,*N*-Dimethylamino)purin-9-yl]ethyl]amino]ethoxy]carbonyl]-L-arginine (16b):** yield 54%; mp 125−130 °C; TLC (R) R_f 0.30; spectral properties were identical with **16a**; HRMS *m/z* (MH^+) found 451.2551; HPLC 100%.

***N*$^\alpha$-[[[6-(*N*,*N*-Dimethylamino)purin-9-yl]-3-pentynyl]oxy]carbonyl]-D-arginine (17a):** yield 30%; mp 130−135 °C; TLC (Q) R_f 0.32; ^{1}H NMR (DMSO-d_6) δ 8.27 (1H, s, purine), 8.26 (1H, s, purine), 6.55 (1H, m, NH), 5.05 (2H, br, CH_2N), 4.02 (2H, m, CH_2O), 3.69 (1H, m, $C^\alpha H$), 3.60−3.35 (8H, m, $N(CH_3)_2$, CH_2), 3.06 (2H, m, $C^\delta H_2$), 1.70−1.40 (4H, m, $C^\beta H_2$, $C^\gamma H_2$); HRMS *m/z* calcd for $C_{19}H_{27}N_9O_4$ (MH^+) 446.2264, found 446.2280; HPLC 100%.

***N*$^\alpha$-[[[6-(*N*,*N*-Dimethylamino)purin-9-yl]-3-pentynyl]oxy]carbonyl]-L-arginine (17b):** yield 17%; mp 130−135 °C; TLC (A) R_f 0.30; spectral properties were identical with **17a**; HRMS *m/z* (MH^+) found 446.2263; HPLC 100%.

***N*$^\alpha$-[[[*m*-[[6-(*N*,*N*-Dimethylamino)purin-9-yl]methyl]benzyl]oxy]carbonyl]-D-arginine (18):** yield 25%; mp 165−170 °C; TLC (A) R_f 0.30; ^{1}H NMR (DMSO-d_6) δ 8.31 (1H, s, purine), 8.26 (1H, s, purine), 7.36−7.19 (4H, m, aromatic), 6.67 (1H, br, NH), 5.41 (2H, s, CH_2N), 4.98 (2H, s, CH_2O), 3.73 (1H, m, $C^\alpha H$), 3.41 (6H, br s, $N(CH_3)_2$), 3.07 (2H, m, $C^\delta H_2$), 1.69−1.50 (4H, m, $C^\beta H_2$, $C^\gamma H_2$); HRMS *m/z* calcd for $C_{22}H_{30}N_9O_4$ (MH^+) 484.2421, found 484.2410; HPLC 100%.

***trans*-*N*$^\alpha$-[[[4-[[(6-(*N*,*N*-Dimethylamino)purin-9-yl]methyl]cyclohexyl]methoxy]carbonyl]-D-arginine (19):** yield 25%; mp 164−166 °C; TLC (A) R_f 0.35, ^{1}H NMR (DMSO-d_6, 400 MHz) δ 9.4 (1H, br s, COOH), 8.19 (1H, s, purine), 8.11 (1H, s, purine), 8.0−7.2 (4H, br, guanidine), 6.28 (1H, d, NH), 3.98 (2H, d, NCH_2), 3.69 (2H, d, CH_2O), 3.61 (1H, m, $C^\alpha H$), 3.43 (6H, br s, $N(CH_3)_2$), 3.00 (2H, br, $C^\delta H_2$), 1.9−0.8 (14H, m, $C^\beta H_2$, $C^\gamma H_2$, 2 × CH, 4 × CH_2); HRMS *m/z* calcd for $C_{22}H_{36}N_9O_4$ (MH^+) 490.2890, found 490.2899; HPLC 100%

***cis*-*N*$^\alpha$-[[[4-[[(6-(*N*,*N*-Dimethylamino)purin-9-yl]methyl]cyclohexyl]methoxy]carbonyl]-D-arginine (20):** yield 27%; mp 168−170 °C; TLC (A) R_f 0.35; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 9.28 (1H, br s, COOH), 8.19 (1H, s, purine), 8.13 (1H, s, purine), 8.0−7.2 (4H, br, guanidine), 6.34 (1H, d, NH), 4.10 (2H, d, NCH_2), 3.85 (2H, d, CH_2O), 3.65 (1H, m, $C^\alpha H$), 3.44 (6H, br s, $N(CH_3)_2$), 3.02 (2H, m, $C^\delta H_2$), 2.09 (1H, m, CH),

1.8−1.2 (14H, m, $C^{\beta}H_2$, $C^{\gamma}H_2$, 2 × CH, 4 × CH_2); HRMS *m/z* calcd for $C_{22}H_{36}N_9O_4$ (MH^+) 490.2890, found 490.2885; HPLC 100%.

(1*S*,3*R*)- and (1*R*,3*S*)-1-[[(6-(*N*,*N*-Dimethylamino)purin-9-yl]methyl]-3-[[(D-arginin-N^{α}-ylcarbonyl)oxy]methyl]cyclopentane (21): yield 33%; mp 140−155 °C; TLC (A) R_f 0.32; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.19 (1H, s, purine), 8.15 (1H, s, purine), 6.32 (1H, br, NH), 4.09 (2H, d, NCH_2), 3.81 (2H, d, CH_2O), 3.65 (1H, m, $C^{\alpha}H$), 3.39 (6H, m, $N(CH_3)_2$), 3.02 (2H, m, $C^{\delta}H_2$), 2.45 (1H, m), 2.10 (1H, m), 1.72−1.30 (9H, m), 0.95 (1H, m). HRMS *m/z* calcd for $C_{21}H_{34}N_9O_4$ (MH^+) 476.2738, found 476.2734; HPLC 100%.

(2*S*,4*S*)-2-[[(6-(*N*,*N*-Dimethylamino)purin-9-yl]methyl]-4-[[(D-arginin-N^{α}-ylcarbonyl)oxy]methyl]-1,3-dioxolane (22): yield 41%; mp 155−160 °C; TLC (A) R_f 0.30; ^{1}H NMR (DMSO-d_6) δ 8.43 (1H, s, purine), 8.11 (1H, s, purine), 6.6 (1H, br, NH), 5.28 (1H, m, H-2-dioxolane), 4.39 (2H, m, NCH_2), 4.26 (1H, m, H-4-dioxolane), 3.97−3.81 (3H, m, $C^{\alpha}H$, CH_2O), 3.71 (2H, m, H-5-dioxolane), 3.39 (6H, br s, $N(CH_3)_2$), 3.07 (2H, m, $C^{\delta}H_2$), 1.70−1.45 (4H, m, $C^{\beta}H_2$, $C^{\gamma}H_2$); HRMS *m/z* calcd for $C_{19}H_{30}N_9O_6$ (MH^+) 480.2319, found 480.2306; HPLC 99%.

(2*S*,4*S*)- and (2*R*,4*R*)-2-[[(6-(*N*,*N*-Dimethylamino)purin-9-yl]methyl]-4-[[(D-arginin-N^{α}-ylcarbonyl)oxy]methyl]-1,3-oxathiolane (23): yield 30%; mp 160−165 °C; TLC (Q) R_f 0.32; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.49 (1H, s, purine), 8.47 (1H, s, purine), 6.60 (1H, br, NH), 6.22 (1H, m, H-2-oxathiolane), 4.26−4.03 (3H, m), 3.63−3.00 (11H, m), 2.78−2.69 (2H, m, H-5-oxathiolane), 1.53−1.40 (4H, m, $C^{\beta}H_2$, $C^{\gamma}H_2$); HRMS *m/z* calcd for $C_{19}H_{30}N_9O_5S$ (MH^+) 496.2091, found 496.2100; HPLC 97%.

***N*-[[5-[6-(*N*,*N*-Dimethylamino)purin-9-yl]pentoxy]carbonyl]glycine (24):** yield 14%; mp 93 °C; TLC (O) R_f 0.; ^{1}H NMR (DMSO-d_6, 300 MHz) δ 8.20 (s, 1H, purine), 8.16 (s, 1H, purine), 6.05 (1H, d, NH), 4.14 (2H, t, NCH_2), 3.86 (2H, t, CH_2O), 3.33 (6H, br s, $N(CH_3)_2$), 3.18 (2H, d, $C^{\alpha}H_2$), 1.80 (2H, m, CH_2), 1.55 (2H, m, CH_2), 1.26 (2H, m, CH_2); HRMS *m/z* calcd for $C_{15}H_{23}N_6O_4$ (MH^+) 351.1780, found 351.1768; HPLC 100%.

***N*$^{\alpha}$-[[5-[6-(*N*,*N*-Dimethylamino)purin-9-yl]pentoxy]carbonyl]-D-ornithine (25):** yield 19%; mp 189−190 °C; TLC (A) R_f 0.20; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 8.20 (1H, s, purine), 8.12 (1H, s, purine), 6.21 (1H, d, NH), 4.10 (2H, t, NCH_2), 3.87 (2H, t, CH_2O), 3.59 (1H, m, $C^{\alpha}H$), 3.4 (8H, br, $N(CH_3)_2$, NH_2), 2.70 (2H, m, $C^{\delta}H_2$), 1.9−1.2 (10H, m, $(CH_2)_3$, $C^{\beta}H_2$, $C^{\gamma}H_2$); HRMS *m/z* calcd for $C_{18}H_{30}N_7O_4$ (MH^+) 408.2359, found 408.2379; HPLC 100%.

***N*-[[5-[6-(*N*,*N*-Dimethylamino)purin-9-yl]pentoxy]carbonyl]-D-citruline (26):** yield 30%; mp 178−181 °C; TLC (O) R_f 0.2; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 8.19 (1H, s, purine), 8.15 (1H, s, purine), 6.21 (1H, d, NH), 6.11 (1H, s, NCO-NH), 5.42 (2H, s, NH_2), 4.14 (2H, t, NCH_2), 3.86 (2H, m, CH_2O), 3.56 (1H, m, $C^{\alpha}H$), 3.35 (6H, br s, $N(CH_3)_2$), 2.87 (2H, m, $C^{\delta}H_2$), 1.9−1.2 (10H, m, $(CH_2)_3$, $C^{\gamma}H_2$, $C^{\beta}H_2$); HRMS *m/z* calcd for $C_{19}H_{31}N_8O_5$ (MH^+) 451.2417, found 451.2437; HPLC 100%.

2-[[5-[6-(*N*,*N*-Dimethylamino)purin-9-yl]pentoxy]carbonyl]ethylamine (27): yield 87%; hygroscopic solid; TLC (A) R_f 0.3; ^{1}H NMR (DMSO-d_6, 300 MHz) δ 8.43 (1H, s, purine), 8.40 (1H, s, purine), 8.04 (3H, br s, NH_3), 4.24 (2H, t, NCH_2), 4.02 (2H, t, CH_2O), 3.69 (6H, br s, $N(CH_3)_2$), 2.99 (2H, m, CH_2), 2.67 (2H, m, CH_2), 1.83 (2H, m, CH_2), 1.62 (2H, m, CH_2), 1.28 (2H, m, CH_2); LRMS *m/z* (MH^+) found 321; HPLC 100%.

5-[6-(*N*,*N*-Dimethylamino)purin-9-yl]pentanol (28): yield 80%; mp 57−59 °C; TLC (N) R_f 0.48; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.35 (1H, s, purine), 7.71 (1H, s, purine), 4.19 (2H, t, NCH_2), 3.64 (2H, t, CH_2O), 3.54 (6H, br s, $N(CH_3)_2$), 1.92 (2H, m, CH_2), 1.62 (2H, m, CH_2), 1.44 (2H, m, CH_2); HRMS *m/z* calcd for $C_{12}H_{20}N_5O$ (MH^+) 250.1668, found 250.1682; HPLC 100%.

***N*-[[5-[6-(*N*,*N*-Dimethylamino)purin-9-yl]pentoxy]carbonyl]-L-isoleucyl-L-prolyl-L-isoleucine (29):** yield 12%; mp 93 °C; TLC (O) R_f 0.35; ^{1}H NMR (CD_3OD, 400 MHz) δ 8.21 (1H, s, purine), 8.03 (1H, s, purine), 4.62 (1H, t, $C^{\alpha}H$), 4.3−3.6 (8H, m, NCH_2, $C^{\delta}H_2$, CH_2O, 2 × $C^{\alpha}H$), 3.50 (6H, br s, $N(CH_3)_2$), 2.1−1.0 (16H, m, $(CH_2)_3$, $C^{\beta}H_2$, 2 × $C^{\beta}H$, 3 y $C^{\gamma}H_2$), 0.95 (6H, d, 2 × $C^{\beta}CH_3$), 0.87 (6H, t, 2 × $C^{\gamma}CH_3$); HRMS *m/z* calcd for $C_{30}H_{49}N_8O_6$ (MH^+) 617.3775, found 617.3783; HPLC 100%.

***N*-[[5-[6-(*N*,*N*-Dimethylamino)purin-9-yl]pentoxy]carbonyl]-L-valyl-L-prolyl-L-leucine (30):** yield 11%; mp 168 °C; TLC (O) R_f 0.40; ^{1}H NMR (CD_3OD, 400 MHz) δ 8.22 (1H, s, purine); 8.05 (1H, s, purine), 4.60 (1H, t, $C^{\alpha}H$), 4.3−3.6 (8H, m, NCH_2, $C^{\delta}H_2$, CH_2O, 2 × $C^{\alpha}H$), 3.51 (6H, br s, $N(CH_3)_2$), 2.2−1.2 (14H, m, $(CH_2)_3$, 2 × $C^{\beta}H_2$, $C^{\beta}H$, $C^{\gamma}H_2$, $C^{\gamma}H$), 1.0−0.8 (12H, m, 2 × $C^{\beta}CH_3$, 2 × $C^{\gamma}CH_3$); LRMS *m/z* (MH^+) found 603.5; HPLC 97%.

6-[6-(*N*,*N*-Dimethylamino)purin-9-yl]hexanoic Acid 5-[[6-(*N*,*N*-dimethylamino)purin-9-yl]pentyl Ester (31): yield 73%; mp 70−72 °C; TLC (L) R_f 0.42; ^{1}H NMR ($CDCl_3$ 300 MHz) δ 8.33 (2H, s, purine), 7.70 (2H, s, purine), 4.17 (4H, m, 2 × NCH_2), 4.02 (2H, m, CH_2O), 3.53 (12H, br s 2 × $N(CH_3)_2$), 2.25 (2H, t, $COCH_2$), 1.92 (4H, m, 2 × CH_2), 1.64 (4H, m, 2 × CH_2), 1.37 (4H, m, 2 × CH_2); HRMS *m/z* calcd for $C_{25}H_{37}N_{10}O_2$ (MH^+) 509.3101, found 509.3072; HPLC 99%.

***N*-[5-[6-(*N*,*N*-Dimethylamino)purin-9-yl]pentyl]carbamic Acid 5-[6-(*N*,*N*-dimethylamino)purin-9-yl]pentyl Ester (32):** yield 64%; oil; TLC (M) R_f 0.40; ^{1}H NMR ($CDCl_3$ 300 MHz) δ 8.34 (2H, s, purine), 7.71 (2H, s, purine), 4.93 (1H, br s, NH), 4.18 (4H, 2t, 2 × NCH_2), 4.02 (2H, t, CH_2O), 3.53 (12H, br s, 2 × $N(CH_3)_2$); 3.15 (2H, t, CH_2) 1.96−1.2 (12H, 3m, 3 × CH_2), HRMS *m/z* calcd for $C_{25}H_{38}N_{11}O_2$ (MH^+) 524.3210, found 524.3233; HPLC 99%.

[[[5-[6-(*N*,*N*-Dimethylamino)purin-9-yl]-pentoxy]carbonyl]amino]acetic Acid 5-[6-(*N*,*N*-dimethylamino)purin-9-yl]pentyl Ester (33): yield 60%; oil; TLC (M) R_f 0.4; ^{1}H NMR ($CDCl_3$ 300 MHz) δ 8.33 (2H, s, purine), 7.71 (2H, s, purine), 5.42 (1H, t, NH), 4.19−3.90 (10H, m, 2 × NCH_2, 2 × CH_2O, CH_2NH), 3.52 (12H, br s, 2 × $N(CH_3)_2$), 1.91 (4H, m, 2 × CH_2), 1.70 (4H, m, 2 × CH_2), 1.40 (4H, m, 2 × CH_2); HRMS *m/z* calcd for $C_{27}H_{41}N_{11}O_4$ (MH^+) 582.3265, found 582.3280; HPLC 99%.

Biology. Cytotoxic T-Lymphocyte/Mixed Lymphocyte Reaction (CTL/MLR) Assay: Splenocyte cell suspensions were prepared by homogenization of spleens from 6- to 8-week-old female C57BL/6 (H-2^b) and DBA/2 (H-2^d) mice in tissue culture medium [RPMI 1640 (Gibco, Burlington, Canada) supplemented with 10% fetal calf serum (Hyclone, Logan, UT) and 2 μM L-glutamine]. The homogenate was centrifuged on a density gradient (Lympholyte M, Cedarlane, Hornby, Canada) (800 g, 20 min), and the mononuclear leukocytes were collected and washed three times in phosphate buffered saline, pH = 7.4 (PBS) and resuspended in RPMI for evaluation of cell viability by the use of trypan blue. DBA/2 spleen cells were irradiated (3000 rads) for the unidirectional MLR and to prime CTLs. The assay was performed as follows: Splenocytes (10^7 cells from C57BL/6 and DBA/2 mice) were incubated in a 5% CO_2 humidified incubator at 37 °C for 120 h and collected, and an aliquot was pulsed with 0.1 μCi of [^{3}H]thymidine (Amersham, Oakville, Canada). After 6 hours these cells were collected by filtration, and newly synthesized DNA was measured by determination of the uptake of tritiated thymidine.

Cells were simultaneously assayed for cell-mediated cytolysis by incubation of 5 × 10^5 cells from the culture for 4 h with sodium chromate labeled ($^{51}CrO_4$; Amersham, Oakville, Canada) P815 mastocytoma cells (5 × 10^3). After the incubation, chromium in the lysate was quantified and the percent specific release or lysis calculated as follows:

$$\text{percent specific } {}^{51}\text{Cr release} = (\text{ER} - \text{SR})/(\text{TR} - \text{SR}) \times 100\%;$$

where ER = experimental release, TR = total release, and SR = spontaneous release

Due to variability between individual experiments, data is compared to the positive control (human IL 2, 15 ng/mL, R&D Systems, Minneapolis, MN) and presented in a semiquantitative (+) format. Therefore, the percent specific lysis induced by IL 2 in each experiment is taken as 100% or the maximum stimulation. As such, 0 to 20% activation of CTLs relative to IL 2 is assigned 0; 20 to 40%, +; 40 to 60%, ++; 60 to 80%, +++; and 80 to 100%, ++++.

Immunophenotyping Assay. Female, 6- to 8-week-old, C57BL/6 mice were injected intraperitoneally for one, four, or nine consecutive days with BCH-1393, dissolved in physiological saline, at different concentrations. Mice given one and four

doses of BCH-1393 were sacrificed on day five by cardiac puncture, while mice given nine doses were sacrificed in the same manner on day ten. Gross pathological observations were recorded at the end of the experiment. Blood and spleens were collected and cell suspension prepared and lysed in ACK buffer (155 mM NH_4Cl, 12 mM $NaHCO_3$, 0.1 mM EDTA, pH = 7.3) for five minutes. The cells were washed three times in phosphate buffered saline, pH = 7.4 (PBS) and resuspended in tissue culture medium. The cells were then incubated for 45 min on ice with fluorescein (FITC) or phycoerythrin (PE) conjugated cell surface marker monoclonal antibodies according to the manufacturer's (Gibco/BRL, Cedarlane, Boehringer Mannheim) recommendation. The cells were then washed in PBS, fixed with 1% paraformaldehyde, and analyzed with a Coulter XL flow cytometer. Analysis of the cell subsets was undertaken by determination of standard cell surface markers which were as follows: CD3 (T-cells), TCR (T-cell receptor), CD4 (T helper), CD8 (T cytotoxic/suppressor), CD45 (tyrosine phosphatase; activation marker), CD11b (macrophage), NK (NK cells), and Ly5 (B-cells).

Statistical Analysis. The immunophenotyping data are presented as ± standard error. The means are compared using an unpaired student's *t* test. The differences are considered significant when $P < 0.05$.

Acknowledgment. We gratefully acknowledge the excellent technical assistance of Sylvie Taillon. The participation of J. Duchaine, J. Heibein, D. Jutras, and M. Lagraoui in the immunophenotyping experiments is appreciated. The preparation of gram quantities of BCH-1393 for *in vivo* studies by Dr. T. Mansour and his group is also acknowledged, as is the preliminary toxicity study by Dr. R. Martel and his group. We appreciate the thoughtful reading of this manuscript by Dr. T. Bowlin and Dr. S. Abbott, and a useful discussion with Dr. J. W. Hadden. We also thank Ms. Sandy MacLellan for the preparation of this document.

References

(1) Hadden, J. W. Immunostimulants. *Immunol. Today* **1993**, *14*, 275−280.
(2) Rhodes, J.; Chen, H.; Hall, S. R.; Beesley, J. E.; Jenkins, D. C.; Collins, P.; Zheng, B. Therapeutic Potentiation of the Immune System by Costimulatory Schiff-base Forming Drugs. *Nature* **1995**, *377*, 71−75.
(3) Reitz, A.; Goodman, M. G.; Pope, B. L.; Argentieri, D. C.; Bell, S. C.; Burr, L.E.; Chourmouzis, E.; Come, J.; Goodman, J. H.; Klaubert, D. H.; Maryanoff, B. E.; McDonnell, M. E.; Rampulla, M. S.; Schott, M. S.; Chen, R. Small Molecule Immunostimulants. Synthesis and Activity of 7,8-Disubstituted Guanosines and Structurally Related Compounds. *J. Med. Chem.* **1994**, *37*, 3561−3578.
(4) Hadden, J. W.; Cornaglia-Ferraris, P.; Coffey, R. G. Purine Analogs as Immunomodulators. *Progr. Immunol.* **1983**, *V*, 1393−1408.
(5) Hadden, J. W.; Giner-Sorolla, A. Isoprinosine and NPT 15392: Modulators of Lymphocyte and Macrophage Development and Funtion. *Augmenting Agents Cancer Ther.* **1981**, 497−522.
(6) Sosa, M.; Saha, A. R.; Wang, Y.; Coto, J.; Giner-Sorolla, A.; Hadden, E. M.; Hadden, J. W. Potentiation of Immune Responses in Mice by a New Inosine Derivative - Methyl Inosine Monophosphate (MIMP). *Int. J. Immunopharmacol.* **1992**, *14*, 1259−1266.
(7) De Simone, C.; Martelli, E. A. ST 789: A New Synthetic Immunomodulator. *Thymus* **1992**, *19* (Suppl. 1), S1−S5 and the rest of the articles in this issue.
(8) Cornaglia-Ferraris, P.; Biano, A; Corrias, M. V.; Stradi, R. A New Peptide Analog (RM06) Modulates the Growth of Hematopoietic Cells. *Int. J. Immunopharmacol.* **1991**, *13*, 1005−1012.
(9) Stradi, R.; Rossi, E.; Perezzani, L.; Migliorati, G.; Riccardi, C.; Cornaglia-Ferraris, P. Synthesis and Immunomodulatory Properties of Some N^2-(ω-(Hypoxanthin-9-yl)Alkoxycarbonyl) L-arginines. *Il Farmaco* **1990**, *45*, 39−47.
(10) For a similar reaction see: Claus, R. E.; Schreiber, S. L. *Org. Synth.* **1985**, *64*, 150.
(11) Zacharie, B.; Connolly, T. P.; Rej, R.; Attardo, G.; Penney, C. L. A Short Synthesis of 4-substituted 1-(Hydroxyalkyl)-1H-pyrazolo[3,4-d] pyrimidines. *Tetrahedron* **1996**, *52*, 2271−2278.
(12) For an example, see: Corey, E. J.; Niimura, K.; Konishi, Y.; Hashimoto, S.; Hamada. Y. *Tetrahedron Lett.* **1986**, *27*, 2199.
(13) For the preparation of the linker see: (a) Oku, A.; Harada, T.; Kita, K. *Tetrahedron Lett.* **1982**, *23*, 681. (b) Forth, M. A.; Smith, S. *Synth. Commun.* **1994**, *24*, 951. (c) Jung, M. E.; Hatfield, G. L. *Tetrahedron Lett.* **1978**, *46*, 4483.
(14) Schon, E.; Born, I.; Demuth, H.; Faust, J.; Neubert, K.; Steinmetzer, T.; Barth, A.; Ansorge, S. Dipeptidyl Peptidase IV in the Immune System. *Biol. Chem. Hopper-Seyler* **1991**, *372*, 305−311.
(15) Held, H. R.; Landi, S. Binding of Toxic Metabolites of Isoniazid by Aconiazide. *J. Pharm. Sci.* **1980**, *69*, 1284−1287.
(16) Spreafico, F. Problems and Challenges in the Use of Immunomodulating Agents. *Int. Arch. Allergy Appl. Immunol.* **1985**, *76*, Suppl. 1, 108−118.
(17) Foresta, P.; Ruggiero, V.; Albertoni, C.; Pacello, L.; Leoni, B.; Martelli, E. A. *In Vitro* Activation of Murine Peritoneal Exudate Cells and Peritoneal Macrophages by ST 789. *Int. J. Immunopharmacol.* **1992**, *14*, 1061−1068.
(18) Toes, R. E. M.; Offringa, R.; Blom, R. J. J.; Melief, C. J. M.; Kast, W. M. Peptide Vaccination Can Lead to Enhanced Tumor Growth Through Specific T-cell Tolerance Induction. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 7855−7860.
(19) Morimoto, C.; Lord, C. I.; Zhang, C.; Duke-Cohan, J. S.; Letvin, N. L.; Schlossman, S. F. Role of CD26/Dipeptidyl Peptidase IV in Human Immunodeficiency Virus Type I Infection and Apoptosis. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 9960−9964.
(20) The effect of BCH-1393 on the expression and activation of CD26 was examined *in vitro* in enriched human T-cells. Expression of CD26 was not affected by BCH-1393, as ascertained by immunophenotyping. However, a modulation of the enzyme activity in excess of 100% was observed at 10^{-7} M to 10^{-5} M BCH-1393 on IL 2/PHA stimulated cells. Further details of this work, as well as the stimulation of human CTLs by BCH-1393, will be published elsewhere.
(21) Zheng, L.; Fisher, G.; Miller, R. E.; Peschon, J.; Lynch, D. H.; Lenardo, M. J. Induction of Apoptosis in Mature T-Cells by Tumor Necrosis Factor. *Nature* **1995**, *377*, 348−351.
(22) Parmley, M. J.; Zhou, W. W.; Edwards, C. K.; Borcherding, D. B.; Silverstein, R.; Morrison, D. C. Adenosine and a Related Carbocyclic Nucleoside Analogue Selectively Inhibit Tumor Necrosis Factor-α Production and Protect Mice Against Endotoxin Challenge. *J. Immunol.* **1993**, *151*, 389−396.
(23) Zacharie, B.; Attardo, G.; Barriault, N.; Penney, C. *J. Chem. Soc., Perkin Trans. 1*, in press.
(24) Revankar, G. R.; Huffman, J. H.; Allen, L. B.; Sidwell, R. W.; Robins, R. K.; Tolman, R. L. Synthesis and Antiviral Activity of Certain 5′-Monophosphates of 9-D-Arabinofuranosyladenine and 9-D-Arabinofuranosylhypoxanthine. *J. Med. Chem.* **1975**, *18*, 7, 721−726; as modified by Hadden, J. W.; Giner-Sorolla, A. Immunopotentiating Inosine Monophosphate 5′-Nucleotidase Resistant Derivatives and Uses. World Patent Application WO 9633203 A1, October 24, 1996.
(25) (a) *Synthetic Procedures in Nucleic Acid Chemistry*; Zorbach, W. W.; Tipson, R. S., Eds.; John Wiley and Sons: New York, 1968. (b) Giner-Sorolla, A.; Segarra, J. T.; Brooks, M. H. *J. Med. Chem.* **1978**, *21*, 344; and references cited therein.

JM960844M