Striking Effect of Hydroxamic Acid Substitution on the Phosphodiesterase Type 4 (PDE4) and TNFα Inhibitory Activity of Two Series of Rolipram Analogues: Implications for a New Active Site Model of PDE4†

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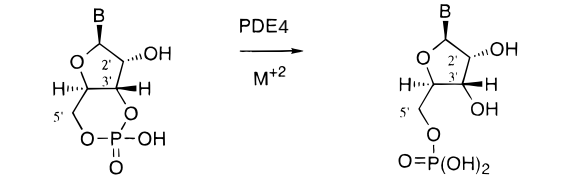
Received October 9, 1997

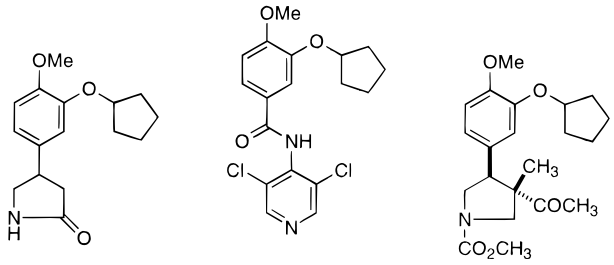
Cyclic nucleotide phosphodiesterases (PDEs) are a family of at least seven isoenzymes which degrade the second messengers adenosine 3′,5′-cyclic monophosphate (cAMP) and guanosine 3′,5′-cyclic monophosphate (cGMP) by hydrolyzing the P−$O^{3'}$ phosphodiester bond to generate 5′-AMP and 5′-GMP, respectively, as inactive products.[1] The association between cAMP elevation in proinflammatory cells with airway smooth muscle relaxation and inhibition of mediator release has led to widespread interest in the design of inhibitors of phosphodiesterase type 4 (PDE4),[2a−d] a cAMP-specific and the principal PDE in these cells, as antiasthmatic and antiinflammatory agents. PDE4, which requires a divalent metal ion for catalysis, exists as four gene subtypes (PDE4A−D),[3] and recently a recombinant form of PDE4A has been purified to homogeneity.[4] Presumably the further availability and study of pure enzyme subtypes will contribute to our presently limited understanding of the catalytic mechanism, role of the metal, and three-dimensional structure of the active site.



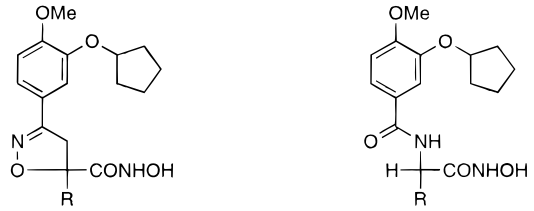
3,'5'-cAMP (B = adenosine)
3,'5'-cGMP (B = guanisine)

5'-AMP (B = adenosine)
5'-GMP (B = guanisine)

Owing to its high specificity toward PDE4 (vs other PDEs), the archetypal PDE4 inhibitor rolipram has been the starting point for numerous SAR studies aimed at optimizing selectivity and potency against inhibition of PDE4[2a,b,d] as well as release of TNFα,[5a,b] a cytokine whose overproduction has been associated with arthritis, endotoxic shock, and AIDS. With the three-dimensional structure of the active site unknown, PDE4 inhibitors have been designed principally by empirical methods, and among the most potent analogues are those in which the pyrrolidinone ring has been replaced by the pharmacophores depicted in RP73401[6] and GW3600.[7] Despite evidence of a zinc binding domain (His-His-Glu) in the active site,[8] and that rolipram is a competitive inhibitor of PDE4A[4] and thus binds in the active site, no SAR study has yet to consider possible interactions between the inhibitor and the metal. We now report on two novel series of rolipram analogues, 3-aryl-2-isoxazoline-5-hydroxamic acids **1** and their acyclic counterparts *N*-aroyl amino hydroxamic acids **2**, whose SARs demonstrate that *the hydroxamic acid group, a well-known metal chelator, makes a unique, tight, and highly stereospecific interaction with PDE4*. This effect not only results in a dramatic increase in PDE4 inhibitory activity over that of rolipram, but further translates into potent inhibition of TNFα-release in purified human monocytes (HM) and in the endogenous milieu—human whole blood (HWB). The close structural resemblance of the non-catechol regions of these series to the ribose 3′,5′-phosphate group of cAMP as is putatively bound to a metal in the active site, furthermore, provides circumstantial evidence that they bind to PDE4, in part, as substrate analogues, which has interesting implications for developing a new active site model of PDE4.[9a,b]



rolipram RP73401 GW3600



1 **2**

Chemistry

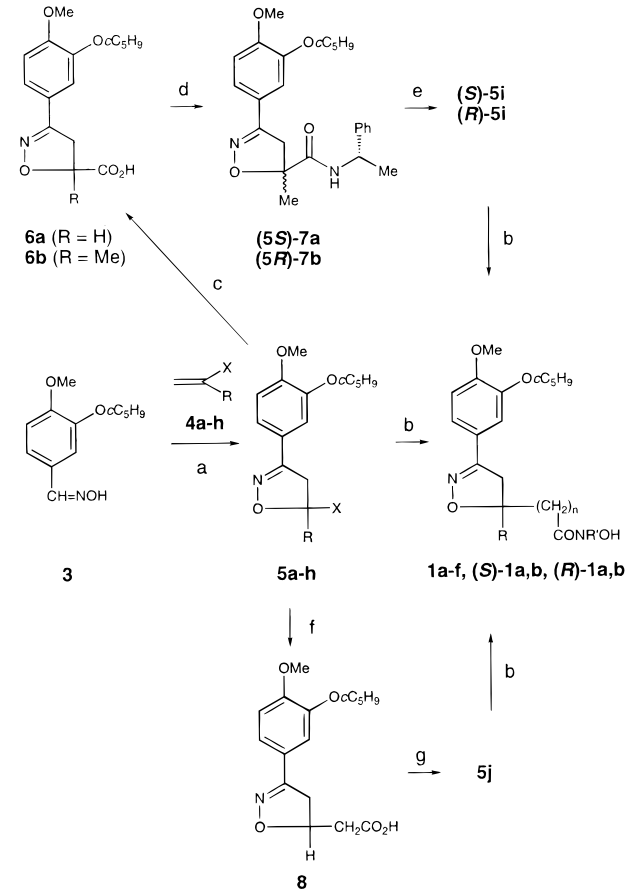
Syntheses of 2-isoxazoline- and isoxazole-containing analogues are shown in Schemes 1 and 2, respectively. Oxime **3**[10] is condensed via the nitrile oxide cycloaddition reaction with olefins **4a**−**h** to give 2-isoxazolines **5a**−**h**, respectively. The ester and acylsultam functions of **5a**−**d**, **(5*S*)-5e**, and **(5*R*)-5f**, respectively, are condensed with hydroxylamine to afford hydroxamates **1a**−**d**, **(*S*)-1a**, and **(*R*)-1a**. Similarly, *N*-methylhydroxamate **1e** is obtained from **5a** using *N*-methylhydroxylamine. Saponification of **5a** and **5b** gives acids **6a** and **6b**, respectively, of which the latter is condensed with (*S*)−(−)-α-methylbenzylamine to furnish a separable mixture of diastereomeric amides **(5*S*)-7a** and **(5*R*)-7b**,

† Dedicated to Dr. Hans-J. Hess on the occasion of his retirement and for his valued contributions to medicinal chemistry.

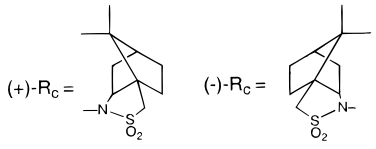
‡ To whom correspondence should be submitted concerning the X-ray structure of **(5*S*)-7a**.

S0022-2623(97)00685-7 CCC: $15.00 © 1998 American Chemical Society
Published on Web 01/13/1998

Scheme 1[a]



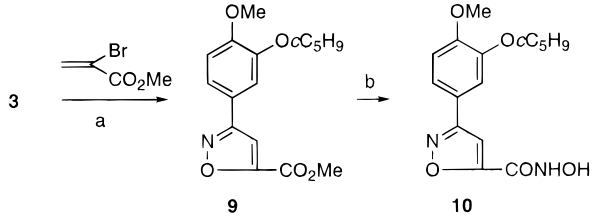
Comp	R	X	Comp	R	R'	n
5a	H	CO_2Me	**1a**	H	H	0
5b	Me	CO_2Et	**1b**	Me	H	0
5c	Et	CO_2Et	**1c**	Et	H	0
5d	Pr	CO_2Et	**1d**	Pr	H	0
(5*S*)-5e	H	CO-(+)-R_c	**(*S*)-1a**	H	H	0
(5*R*)-5f	H	CO-(-)-R_c	**(*R*)-1a**	H	H	0
5g	H	$CONH_2$	**1e**	H	Me	0
5h	H	CH_2CH_2OH	**(*S*)-1b**	Me	H	0
(*S*)-5i	Me	CO_2Me	**(*R*)-1b**	Me	H	0
(*R*)-5i	Me	CO_2Me	**1f**	H	H	1
5j	H	CH_2CO_2Me				



[a] Reagents: (a) (i) NCS, pyr, **2**, CH_2Cl_2; (ii) add olefin, TEA; (b) $NH_2OH{\cdot}HCl$ or $N(Me)HOH{\cdot}HCl$, NaOR, ROH; (c) KOH, MeOH; (d) (i) oxalyl chloride, PhH, Δ; (ii) (*S*)-(−)-$NH_2CH(Me)Ph$; (e) (i) KH, THF; (ii) CS_2; (iii) HCl, MeOH; (f) Jones; (g) HCl, MeOH.

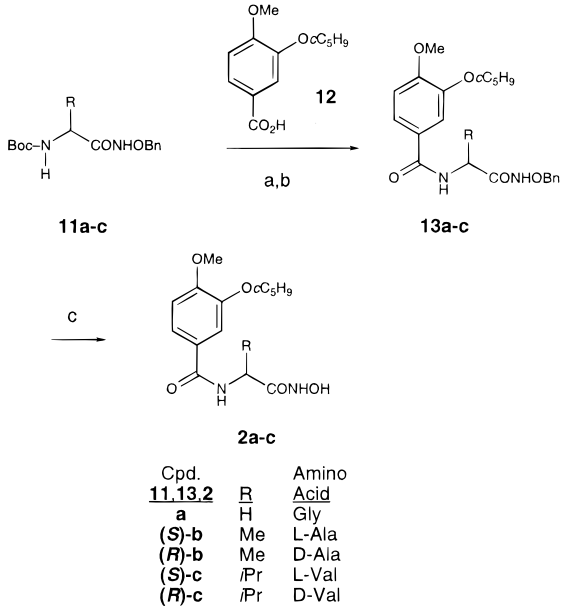
which are cleaved[11] and esterifed to afford **(*S*)-5i** and **(*R*)-5i**, and converted to hydroxamates **(*S*)-1b** and **(*R*)-1b**, respectively. Acid **8**, obtained by oxidation of alcohol **5h**, is esterified to furnish ester **5j**, which is converted to the homologous hydroxamate **1f**. The absolute stereochemistries of **(5*S*)-5e** and **(5*R*)-5f** are assigned based on literature precedent,[12] whereas that of **(5*S*)-7a** is determined by X-ray. Isoxazole **10** is prepared by hydroxylamine exchange of ester **9**, which, in turn, is formed by the one-pot nitrile oxide cycloaddition reaction of **3** and methyl α-bromoacrylate in the presence of excess triethylamine.

Scheme 2[a]



[a] Reagents: (a) (i) NCS, pyr, CH_2Cl_2; (ii) add olefin, TEA; (b) KOH, MeOH.

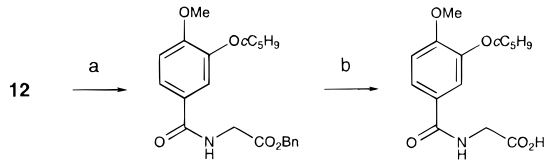
Scheme 3[a]



Cpd. 11,13,2	R	Amino Acid
a	H	Gly
(*S*)-b	Me	L-Ala
(*R*)-b	Me	D-Ala
(*S*)-c	*i*Pr	L-Val
(*R*)-c	*i*Pr	D-Val

[a] Reagents: (a) (i) 4 N HCl−dioxane; (ii) **12**, DEC, TEA, CH_2Cl_2; (b) H_2, $Pd(OH)_2$, MeOH.

Scheme 4[a]



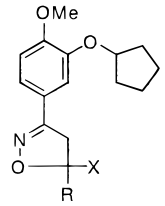
[a] Reagents: (a) $HCl{\cdot}NH_2CH_2CO_2Bn$, DEC, TEA, CH_2Cl_2; (b) H_2, $Pd(OH)_2$, MeOH.

N-Aroyl amino hydroxamic acids **2a**−**c** are synthesized by the sequence shown in Scheme 3, which involves (1) N_α-deprotection of *O*-benzyl hydroxamates **11a**−**c**;[13a,b] (2) coupling with acid **12**[6] to give amides **13a**−**c**; and (3) deprotection of the *O*-benzyl group via catalytic hydrogenation. The corresponding carboxylic acid **15** derived from glycine is synthesized analogously using Gly·OBn (Scheme 4).

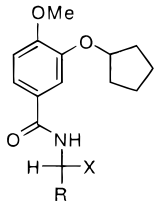
Results and Discussion

In vitro data for the inhibition of HM cytosol PDE[5b] and blockade of LPS-induced TNFα-release in HM[5b] is shown in Table 1 for analogues of series **1** and **2**, some related compounds, (±)-rolipram, and RP73401. In addition, data for the inhibition of unpurified recombinant PDE4A−D isozymes (expressed in baculovirus)[14] and LPS-induced TNFα-release in HWB[15] is reported

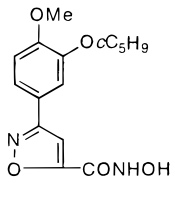
Table 1. Inhibition of Human Monocyte Cytosol PDE, Recombinant Human Isozyme PDE4A−D, and TNFα-Release from Human Monocytes and Human Whole Blood[a]



1a-f, (*R*)-1a,b, (*S*)-2a,b, 5g, 6a



2a, (*R*)-2b,c, (*S*)-2b,c, 15



10

compd	R	X	human monocyte PDE	IC$_{50}$ (μM) PDE4A	PDE4B	PDE4C	PDE4D	human monocyte TNFα	human whole blood TNFα
6a	H	CO_2H	2.54					35.1	
5g	H	$CONH_2$	3.93					4.24	
1a	H	CONHOH	0.054					0.010	
(*R*)-1a	H	CONHOH	0.024	0.0022	0.0021	0.018	0.0027	0.003	0.030
(*S*)-1a	H	CONHOH	4.33					1.36	
10			1.65					0.848	
(*R*)-1b	Me	CONHOH	0.028	0.0098	0.0109	0.0952	0.01	0.034	0.074
(*S*)-1b	Me	CONHOH	0.603					0.566	
1b	Me	CONHOH	0.059					0.039	
1c	Et	CONHOH	0.178					0.230	
1d	Pr	CONHOH	0.567					1.02	
1e	H	CONMeOH	3.96					10.2	
1f	H	$CH_2CONHOH$	0.221					0.138	
15	H	CO_2H	1.76					>50	
2a	H	CONHOH	0.249	0.0272	0.0179	0.0153	0.0156	0.032	0.066
(*R*)-2b	Me	CONHOH	0.224	0.0507	0.0222	0.1259	0.0202	0.049	0.050
(*S*)-2b	Me	CONHOH	33.0					11.9	
(*R*)-2c	iPr	CONHOH	1.32					0.917	
(*S*)-2c	iPr	CONHOH	36.2					>20	
(±)-rolipram			3.69	0.0107	0.0357	2.63	0.0316	0.379	0.66
RP73401			0.02	0.0016	0.001	0.0038	0.0008	0.002	0.080

[a] Results are the average of at least three assays.

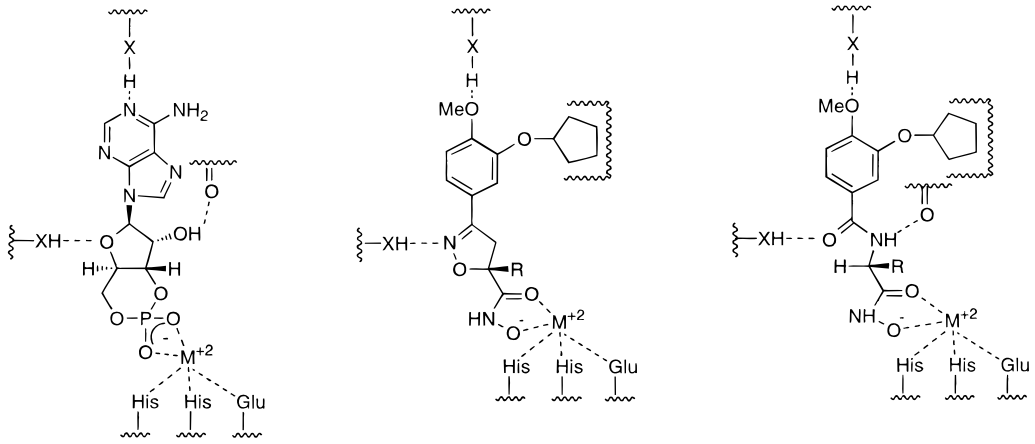


Figure 1. Comparison of the structures of series **(*R*)-1** and **(*R*)-2** with cAMP bound in the hypothetical active site of PDE4. Hypothetical regions of the enzyme are represented by wavy lines.

for selected compounds. The parent acid **6a** and amide **5g**[16a,b] of series **1** display comparable HM-PDE4 IC$_{50}$ values to that of (±)-rolipram. However, simple replacement of the N−H proton of **5g** by hydroxyl, giving hydroxamic acid **1a**, leads to a dramatic 75-fold increase in potency (IC$_{50}$ = 0.054 μM). The interaction of the hydroxamic acid with PDE4 is highly stereospecific as demonstrated by the 180- and 70-fold separations in potency between **(*R*)-1a** and its enantiomer **(*S*)-1a** and the planar isoxazole **10**, respectively. The preference for *R*-stereochemistry is also seen among the 5-methyl enantiomers **(*R*)-1b** and **(*S*)-1b**. Activity with respect to substitution at the 5-position of the 2-isoxazoline ring (**1a**−**d**) follows the trend: H ≥ Me > Et > Pr. Direct attachment of the hydroxamic acid to C5 of the 2-isoxazoline ring and its NH proton are important, since activity declines by inserting a methylene spacer (**1f**) and replacing the NH proton by methyl (**1e**). **(*R*)-1a** (CP-293,121), with an IC$_{50}$ of 0.024 μM which approaches that of RP73401, is the most potent compound in the series and its activity is reflected in the PDE4A−D isozyme assay.

Designed as conformationally flexible analogues of series **1**, the *N*-aroyl amino hydroxamic acid series **2**,

though somewhat less potent, exhibits strikingly similar SAR. Thus, hydroxamic is preferred over carboxylic acid (**2a** vs **15**), *R*-stereochemistry is preferred over *S*-((***R*)-2b,c** vs **(*S*)-2b,c**), and activity declines upon increasing steric bulk at the α-position ((***R*)-2b** vs **(*R*)-2c**). These results are explained by the ability of series **2** to adopt a conformation similar to **1**, in which the trans amide bond overlaps with the C3−C4 bond of the 2-isoxazline ring and the α-hydrogen eclipses the amide carbonyl to make a pseudo five-membered ring.[17] The most potent analogue, **(*R*)-2b**, has an IC_{50} of 0.224 μM in the HM-PDE assay, which is 15-fold lower than that of rolipram.

The ability of series **1** and **2** analogues to inhibit the release of HM-TNFα correlates well with their relative ability to inhibit HM-PDE4, paralleling a previous study,[5b] with the most potent compounds, **(*R*)-1a**, **(*R*)-1b**, **2a**, and **(*R*)-2b**, having IC_{50} values between 3 and 49 nM.[18] In the HWB-TNFα assay, which best reflects their therapeutic potential in TNFα-related diseases, these compounds retain excellent potency, exhibiting IC_{50} values < 80 nM. Interestingly, **(*R*)-1a**, with an IC_{50} of 30 nM, is nearly 3-fold more potent than RP73401 in this assay, suggesting that the hydroxamic acid has the added beneficial effect of reducing protein binding. To our knowledge, **(*R*)-1a** is the most potent PDE4 inhibitor of TNFα release in HWB so far discovered.

The hydroxamic effect described above leads us to compare the structures of series **(*R*)-1** and **(*R*)-2** with cAMP as is hypothetically bound via the phosphate[19] to the divalent metal in the active site (Figure 1). Indeed, some strikingly common structural features exist. First, the oxygen anions of the metal binding ligands and the aromatic base or catechol are connected by a five-membered ring template and separated by a distance of six atoms. Second, within the template a hydrogen-bonding acceptor is located at one of the adjacent positions to the aromatic base or catechol substituent, and a hydrogen-bonding donor is located at the alternate position (cAMP and **2** only). Third, the metal ligand is oriented on the *re* face of the template. Fourth, small R substituents (i.e., hydrogen for cAMP) are preferred at the position adjacent to the metal binding ligand in the template. Inherent in this model is the prediction that the catechol and adenosine groups bind in the same region of the enzyme. Despite the structural difference, there is a common hydrogen-bonding acceptor four atoms removed from the corresponding template, namely the methoxy oxygen of the catechol and N1 of the adenosine, respectively. As illustrated, this manner of overlap predicts that the cyclopentyl group of the catechol binds in an auxiliary lipophilic binding pocket, which, if absent in other PDEs and PDE4 mutants,[20] could account for the PDE4 specificity of rolipram. Clearly, this aspect of the model is less developed, and it is conceivable that the catechol and adenosine groups, respectively, are oriented differently within the active site.

In conclusion, we have shown that several analogues within series **1** and **2** are potent inhibitors of PDE4 and release of TNFα in HWB, thus raising the potential of these series for use as anti-TNFα therapy. By overlapping these series with cAMP and taking into account their ability to bind to a metal in the active site of PDE4, the early stages of a model of the PDE4 active site can be built. This model predicts that by incorporating structural features of cAMP and a site for metal coordination within the rolipram pharmacophore, more potent and structurally diverse analogues can be obtained. Ultimately, the true manner in which PDE4 inhibitors bind in the active site may be obtained by X-ray crystallography, and we view **(*R*)-1a** as an excellent candidate for cocrystallization studies in order to confirm our model and provide insight into the role of the divalent metal in the mechanism of cAMP hydrolysis and the binding of other inhibitors.

Acknowledgment. We would like to thank Drs. Alan J. Duplantier, Douglas A. Fisher, and John Lowe III for helpful discussions and Dr. Thomas J. Carty and Mr. Francis J. Sweeney for measurement of TACE inhibition. We also thank Drs. Kelvin Cooper and John W. Watson for their encouragement.

Supporting Information Available: Experimental procedures and data for new analogues and intermediates, mean standard errors for in vitro data, detailed results of the Sybil force field calculations of series **2**, and details of the X-ray determination of compound **(5*S*)-7a** (19 pages). Ordering information is given on any current masthead page.

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(17) Sybil force field calculations show that the conformation in which the amide carbonyl is eclipsed with the Cα-hydrogen (see Figure 1) lies in a energy well within 1 kcal/mol of the four lowest energy conformations about rotation of the amide carbonyl−aryl bond.
(18) Compounds **(*R*)-1a**, **2a**, and **(*R*)-2b** do not significantly inhibit recombinant TNFα converting enzyme (TACE), nor does **(*R*)-1a** significantly inhibit human PDE3; IC_{50} values are >100 μM.
(19) This assumption is based on the ability of divalent metal ions in related enzymes with similar functions to those of PDEs to also bind phosphate groups. For several examples, see: Sträter, N.; Lipscomb, W. N.; Klabunde, T.; Krebs, B. Two-metal ion catalysis in enzymatic acyl- and phosphoryl-transfer reactions. *Angew. Chem., Int. Ed. Engl.* **1996**, *35*, 2024−2055.
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JM970685M