monomethyl ether/water (16 g/84 g), with a Hewlett-Packard UV/VIS 8450A spectrophotometer.

Biology. Measurement of $^{86}Rb^+$ Efflux and Spontaneous Activity of Rat Portal Veins. Male Wistar rats were anesthetized with CO_2 and exsanguinated. The portal vein was exposed and attached at either end to a cotton thread. After removal of the surrounding connective tissue it was cut along the length axis. The lumen was washed free of blood, the vessel excised, and a tension of 500 mg was applied. The vein was then incubated for 30 min in a HEPES-buffered physiological salt solution (PSS) gassed with 95% O_2/5% CO_2 at 32 °C, pH 7.4. The PSS contained NaCl (120 mM), KCl (5 mM), $NaHCO_3$ (15 mM), NaH_2PO_4 (1.2 mM), $MgCl_2$ (1.2 mM), $CaCl_2$ (2.5 mM), glucose (11 mM), and HEPES (20 mM) and had pH 7.4 at 37 °C. For loading with $^{86}Rb^+$, the vein was incubated for an additional 80 min in PSS to which 5 μCi/mL $^{86}RbCl$ had been added.

After loading with $^{86}Rb^+$, the vein was shortly dipped into PSS to remove excess radioactivity and mounted in a thermostated perfusion chamber. A preload of 500 mg was applied and the chamber was perfused with PSS at 37 °C at a rate of 2.5 mL/min. The upper cotton thread was attached to an isometric force transducer (Gould cell, Statham) that was connected to a custom-built amplifier from which the signal was given to a recorder and to an integrator for quantitation of myogenic activity.

For measurement of $^{86}Rb^+$ efflux, the perfusate was collected at a sampling rate of 2 min and counted for radioactivity in the Cerenkov mode at 50% efficiency. The radioactivity remaining in the portal vein at the end of the assay was determined by dissolving the vessel in 0.5 mL of Lumasolve (Lumac) at 50 °C overnight. The sample was then supplemented with 0.5 mL of 1 M HCl and 10 mL of Optifluor (Packard) and counted in the ^{32}P channel at 100% efficiency.

The rate constant, k, of $^{86}Rb^+$ efflux, defined as the radioactivity released from the vessel per minute divided by the concurrent radioactivity of the vessel,[62] was calculated as described.[36] Drug effects on the efflux rate constant were calculated as the peak value of k obtained during drug application compared to the basal value of k averaged over 6–10 min before drug application and are expressed as a percentage scale (Δk%). Concentration–effect curves were fitted to the law of mass action or to the Hill equation by nonlinear least-squares analysis.

Acknowledgment. We thank Mme. J. Dosogne for pharmacological testing, R. Labhart for assistance in the synthetic work, Frau T. Zardin for interpretation of NMR spectra, and H. U. Gremlich for pK_a measurements.

(62) Bolton, T. B.; Clark, J. P. Actions of Various Muscarinic Agonists on Membrane Potential, Potassium Efflux, and Contraction of Longitudinal Muscle of Guinea-Pig Intestine. *Br. J. Pharmacol.* **1981**, *72*, 319–334.

Communications to the Editor

7-Oxabicycloheptylprostanoic Acids: Potent, Time-Dependent Cyclooxygenase Inhibitors That Induce a Conformational Change in the Prostaglandin Endoperoxide Synthase Protein

Inhibition of the cyclooxygenase activity of prostaglandin endoperoxide (PGH) synthase is believed to be the basis for the pharmacological action of nonsteroidal antiinflammatory agents.[1,2] Understanding the structural basis of cyclooxygenase inhibition by diverse classes of inhibitors has taken on added significance with the recent discovery of a second cyclooxygenase gene that is expressed in response to mitogenic stimulation of cells.[3-5] The protein coded by this gene is 60% similar to the constitutively expressed cyclooxygenase protein but its substrate specificity, sensitivity to inhibitors, etc., is not known.

The Squibb group recently described a novel series of 7-oxabicycloheptylprostanoic acid derivatives that inhibit arachidonic acid-induced platelet aggregation and arachidonic acid oxygenation by platelet and bovine seminal vesicle microsomes.[6-8] The most potent compound among a series of structural analogs is **1**, which possesses bis-exo

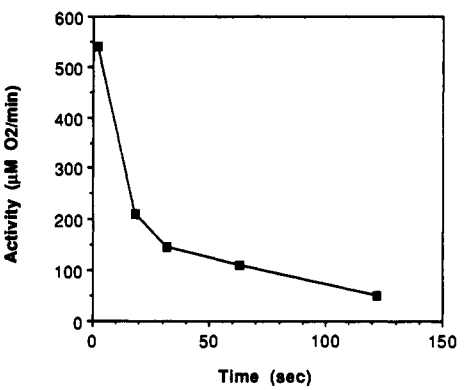


Figure 1. Time course of loss of cyclooxygenase activity following addition of **2**. The assay mixture contained 0.20 μM protein (specific activity 17 μmol O_2 min^{-1} mg^{-1} protein), 1 μM hematin, 1 mM phenol, and 0.075 μM **2** in 1.25 mL Tris-HCl (100 mM, pH 7.4). At varying times after addition of **2**, 97 μM arachidonic acid was added and the rate of O_2 uptake determined. The O_2 uptake rate in the absence of **2** was 629 μM O_2/min.

(1) Vane, J. R. Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. *Nature New Biol.* **1971**, *231*, 232–235.

(2) Robinson, H. J.; Vane, J. R. *Prostaglandin synthetase inhibitors–Their effects on physiological functions and pathological states*; Raven: New York, 1974.

(3) Xie, W.; Chipman, J.; Robertson, D. L.; Simmons, D. L. Cloning and characterization of a pp60v-src-inducible prostaglandin synthase. *FASEB J.* **1991**, *5*, 602.

(4) Xie, W.; Chipman, J. G.; Robertson, D. L.; Erikson, R. L.; Simmons, D. L.; Expression of a mitogen-responsive gene encoding prostaglandin synthase is regulated by mRNA splicing. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 2692–2696.

(5) Kujubu, D. A.; Fletcher, B. S.; Varnum, B. C.; Lim, R. W.; Herschman, H. R.; TIS10, a phorbol ester tumor promoter-inducible mRNA from Swiss 3T3 cells, encodes a novel prostaglandin synthase/cyclooxygenase homologue. *J. Biol. Chem.* **1991**, *266*, 12866–12872.

(6) Harris, D. N.; Phillips, M. B.; Michel, I. M.; Goldenberg, H. J.; Steinbacher, T. E.; Ogletree, M. L.; Hall, S. E. Inhibition of prostaglandin biosynthesis by SQ 28,852, a 7-oxabicyclo-[2.2.1]heptane analog. *Prostaglandins* **1986**, *31*, 651–667.

(7) Hall, S. E.; Han, W.-C.; Haslanger, M. F.; Harris, D. H.; Ogletree, M. L.; 9,11-Epoxy-9-homo-14-oxaprosta-5-enoic acid derivatives. Novel inhibitors of fatty acid cyclooxygenase. *J. Med. Chem.* **1986**, *29*, 2335–2347.

(8) The Squibb numbers for compounds 1 and 2 are SQ 28,852 and SQ 29,535, respectively.[6]

0022-2623/92/1835-2340$03.00/0

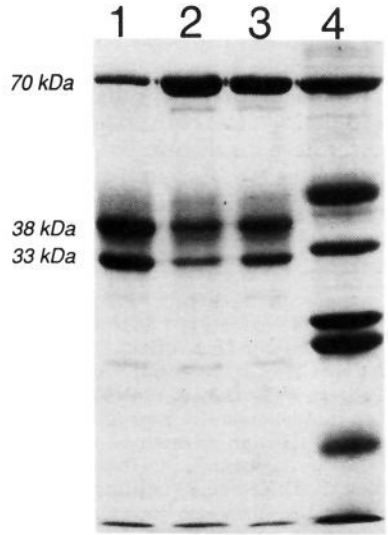
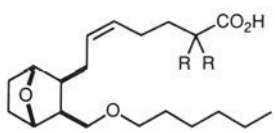


Figure 2. Protection of apoPGH synthase from cleavage by trypsin. Assays contained 9.4 μM apoPGH synthase with a specific activity of 58 μmol of O_2 min^{-1} mg^{-1} protein. It was incubated at room temperature with 1.25 μg of bovine pancreatic trypsin (Sigma Chemical lot 38F-8140). Hematin or **2** was added at a concentration of 9.4 μM. Incubations were stopped after 30 min by addition of 430 μg of soybean trypsin inhibitor (Sigma Chemical lot 126F-8190). Lane 1, apoPGH synthase + trypsin; lane 2, apoPGH synthase + hematin + trypsin; lane 3, apoPGH synthase + **2** + trypsin; lane 4, molecular weight markers.

substitution of the oxabicycloheptane ring. In addition to inhibiting cyclooxygenase activity in vitro, **1** prevents arachidonic acid-induced sudden death in the mouse and arachidonic acid-induced changes in lung mechanics in the guinea pig at doses significantly below those required for similar effects by indomethacin.[7] Analog **2** exhibits the same activities as **1** but has a longer duration of action in vivo.



1 R = H
2 R = CH_3

We find that compound **2** is a potent, time-dependent inhibitor of the cyclooxygenase activity of PGH synthase purified from ram seminal vesicles.[9] At an enzyme concentration of 140 nM, **2** exhibited an IC_{50} of 80 nM and completely inhibited activity at 150 nM. At a ratio of arachidonic acid to **2** of 2000:1, minimal inhibition was observed when substrate and inhibitor were added simultaneously to the enzyme preparation. If **2** was added 15–120 s before arachidonate, it displayed potent inhibitory activity (Figure 1).

Halogenated aromatic compounds such as indomethacin are time-dependent inhibitors of PGH synthase.[10,11] Indomethacin has been demonstrated to bind directly to PGH synthase by virtue of its ability to protect apoprotein from tryptic cleavage at Arg^{277}.[10,13] ApoPGH synthase (9.4 μM) was incubated with 1 equiv of **2** for 5 min and then treated with 2.9 μM trypsin for 30 min. Proteolysis was stopped by addition of a 20-fold excess of soybean trypsin inhibitor. For comparison, similar incubations were conducted with apoprotein alone and apoprotein plus 1 equiv of hematin.[15] Trypsin-treated apoPGH synthase demonstrated major bands on SDS-PAGE at 33 and 38 kDa corresponding to the cleavage fragments in addition to the band of the uncleaved protein at 70 kDa (Figure 2). Under the conditions of the present experiment, 81% of apoprotein was cleaved on incubation with trypsin. In contrast, only 47% of the protein was cleaved in incubations of trypsin with PGH synthase and **2**. Control experiments indicate that **2** did not directly inhibit the proteolytic activity of trypsin. Similar experiments conducted with apoPGH synthase and heme led to 31% cleavage on treatment with trypsin. These experiments indicate that binding of **2** to apoPGH synthase induces a conformational change in the protein in the region of Arg^{277}. Although binding of **2** or heme induce resistance to trypsin, the two agents do not appear to bind to the same site on the protein. Compound **2** inhibited the cyclooxygenase activity of fully reconstituted PGH synthase and did not inhibit heme binding to apoprotein. Furthermore, addition of **2** to holoPGH synthase did not cause release of heme from the protein as judged by the intensity of the heme Soret absorbance.

The present experiments demonstrate that **2** is a potent, time-dependent cyclooxygenase inhibitor that induces a conformational change in the PGH synthase protein. In fact, the potency of **2** in biochemical and pharmacological assays ranks it among the most potent cyclooxygenase inhibitors ever developed. Preliminary structure–activity studies of compounds related to **2** reveal considerable sensitivity to the stereochemistry of substitution on the oxabicycloheptane ring and to the position of oxygen substitution in the ω side chain.[7] The ability to demonstrate direct binding of these compounds to PGH synthase makes 7-oxabicycloheptylprostanoic acids powerful probes of the structural basis of protein–inhibitor association and attractive templates for the design of isozyme-specific inhibitors.

Acknowledgment. This work was supported by a research grant from the National Institutes of Health (Grant

(9) PGH synthase was purified to apparent electrophoretic homogeneity from ram seminal vesicles. It exhibited a specific activity of 40 μmol O_2 min^{-1} mg^{-1} protein. The assay mixture contained 0.14 μM protein, 1 μM hematin, 1 mM phenol, and 96 μM arachidonic acid in 1.25 mL of Tris-HCl (100 mM, pH pH 7.4).

(10) Rome, L. H.; Lands, W. E. M.; Structural requirements for time-dependent inhibition of prostaglandin biosynthesis by anti-inflammatory drugs. *Proc. Natl. Acad. Sci. U.S.A.* **1975**, *72*, 4863–4865.
(11) Kulmacz, R. J.; Lands, W. E. M.; Stoichiometry and kinetics of the interaction of prostaglandin H synthase with anti inflammatory agents. *J. Biol. Chem.* **1985**, *260*, 12572–12578.
(12) Kulmacz, R. J.; Topography of prostaglandin H synthase. Antiinflammatory agents and the protease-sensitive arginine 253 region. *J. Biol. Chem.* **1989**, *264*, 14136–14144.
(13) The numbering system refers to the translational start site.[14]
(14) DeWitt, D. L.; Smith, W. L. Primary structure of prostaglandin G/H synthase from sheep vesicular gland determined from the complementary DNA sequence. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 1412–1416.
(15) The heme prosthetic group also protects apoPGH synthase from tryptic cleavage at Arg^{277}.[16,17]
(16) Kulmacz, R. J.; Lands, W. E. M.; Protection of prostaglandin H synthase from trypsin upon binding of heme. *Biochem. Biophys. Res. Commun.* **1982**, *104*, 758–764.
(17) Chen, Y.-N. P.; Bienkowski, M. J.; Marnett, L. J. Controlled tryptic digestion of prostaglandin H synthase. *J. Biol. Chem.* **1987**, *252*, 16892–16899.

CA47479). We are grateful to Steven Hall (Bristol Myers-Squibb) for a gift of 1 and 2 and helpful discussions.

Indrani Pal, Rebecca Odenwaller
Lawrence J. Marnett*
A.B. Hancock Jr. Memorial Laboratory for Cancer Research
Center in Molecular Toxicology
Departments of Biochemistry and Chemistry
Vanderbilt University School of Medicine
Nashville, Tennessee 37232
Received April 13, 1992

(*E*)-1,3-Dialkyl-7-methyl-8-(3,4,5-trimethoxystyryl)xanthines: Potent and Selective Adenosine A_2 Antagonists

Adenosine receptors are localized virtually in all tissues, and modulate a wide range of physiological functions.[1] Adenosine receptors are divided into two major subtypes, designated as A_1 and A_2. The two receptor subtypes can be distinguished by the structure–activity relationships of adenosine agonists and have opposite effects on adenylate cyclase.[2,3]

The methylxanthines theophylline (1, Figure 1) and caffeine (2) exhibit a variety of pharmacological actions primarily through blockade of adenosine receptors.[4] However, they are virtually nonselective antagonists and have weak affinity for A_1 and A_2 receptors. Efforts to develop more potent and highly selective antagonists[5-17] have focused on the modification at the 1-, 3-, 7-, and 8-position of xanthines. Introduction of the propyl group to the 1- and 3-position increases the affinity at A_1 and A_2 receptors.[7-10] The discovery[8,10,15] that cycloalkyl substituents at the 8-position markedly enhanced the affinity at the A_1 receptor have resulted in potent and selective A_1 antagonists such as 8-cyclopentyl-1,3-dipropylxanthine (4)[16] and 1,3-dipropyl-8-(3-noradamantyl)xanthine (5).[17b,c]

(1) Daly, J. W. Adenosine Receptors: Targets for Future Drugs. *J. Med. Chem.* **1982**, *25*, 197-207.
(2) Londos, C.; Wolff, J. Two Distinct Adenosine-Sensitive Sites on Adenylate Cyclase. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 5482-5486.
(3) Van Calker, D.; Müller, M.; Hamprecht, B. Adenosine Regulates via Two Different Types of Receptors, the Accumulation of Cyclic AMP in Cultured Brain Cells. *J. Neurochem.* **1979**, *33*, 999-1005.
(4) Fredholm, B. B. Are Methylxanthine Effects due to Antagonism of Endogenous Adenosine? *Trends Pharmacol. Sci.* **1980**, *1*, 129-132.
(5) Williams, M. Adenosine Antagonists. *Med. Res. Rev.* **1989**, *9*, 219-243.
(6) Bruns, R. F. Structure Activity Relationships for Adenosine Antagonists. In *Purines in Cellular Signaling: Targets for New Drugs*; Jacobson, K. A., Daly, J. W., Manganiello, V., Eds.; Springler: New York, 1990; pp 126-135.
(7) Bruns, R. F.; Daly, J. W.; Snyder, S. H. Adenosine Receptor Binding: Structure–Activity Analysis Generates Extremely Potent Xanthine Antagonists. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 2077-2080.
(8) Bruns, R. F.; Lu, G. H.; Pugsley, T. A. Characterization of the A_2 Adenosine Receptor Labeled by [^{3}H]NECA in Rat Striatal Membranes. *Mol. Pharmacol.* **1986**, *29*, 331-346.
(9) Schwabe, U.; Ukena, D.; Lohse, M. J. Xanthine Derivatives as Antagonists at A_1 and A_2 Adenosine Receptors. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **1985**, *330*, 212-221.
(10) Martinson, E. A.; Johnson, R. A.; Wells, J. N. Potent Adenosine Receptor Antagonists that are Selective for the A_1 Receptor Subtype. *Mol. Pharmacol.* **1987**, *31*, 247-252.
(11) (a) Hamilton, H. W.; Ortwine, D. F.; Worth, D. F.; Badger, E. W.; Bristol, J. A.; Bruns, R. F.; Haleen, S. J.; Steffen, R. P. Synthesis of Xanthines as Adenosine Antagonists, a Practical Quantiative Structure–Activity Relationship Application. *J. Med. Chem.* **1985**, *28*, 1071-1079. (b) Daly, J. W.; Padgett, W.; Shamim, M. T.; Butts-Lamb, P.; Waters, J. 1,3-Dialkyl-8-(*p*-sulfophenyl)xanthines: Potent Water-Soluble Antagonists for A_1- and A_2-Adenosine Receptors. *J. Med. Chem.* **1985**, *28*, 487-492. (c) Daly, J. W.; Padgett, W. L.; Shamim, M. T. Analogues of 1,3-Dipropyl-8-phenylxanthines: Enhancement of Selectivity at A_1-Adenosine Receptors by Aryl Substituents. *J. Med. Chem.* **1986**, *29*, 1520-1524.
(12) (a) Jacobson, K. A.; Kirk, K. L.; Padgett, W. L.; Daly, J. W. Functionalized Congeners of 1,3-Dialkylxanthines: Preparation of Analogues with High Affinity for Adenosine Receptors. *J. Med. Chem.* **1985**, *28*, 1334-1340. (b) Jacobson, K. A.; Kirk, K. L.; Padgett, W. L.; Daly, J. W. A. Functionalized Congener Approach to Adenosine Receptor Antagonists: Amino Acid Conjugates of 1,3-Dipropylxanthine. *Mol. Pharmacol.* **1986**, *29*, 126-133. (c) Jacobson, K. A.; Ukena, D.; Padgett, W.; Daly, J. W.; Kirk, K. L. Xanthine Functionalized Congeners as Potent Ligands at A_2-Adenosine Receptors. *J. Med. Chem.* **1987**, *30*, 211-214.
(13) (a) Daly, J. W.; Padgett, W. L.; Shamim, M. T. Analogues of Caffeine and Theophylline: Effect of Structural Alterations on Affinity at Adenosine Receptors. *J. Med. Chem.* **1986**, *29*, 1305-1308. (b) Ukena, D.; Shamim, M. T.; Padgett, W.; Daly, J. W. Analogs of Caffeine: Antagonists with Selectivity for A_2 Adenosine Receptors. *Life Sci.* **1986**, *39*, 743-750. (c) Shamim, M. T.; Ukena, D.; Padgett, W. L.; Daly, J. W. Effects of 8-Phenyl and 8-Cycloalkyl Substituents on the Activity of Mono-, Di-, and Trisubstituted Alkylxanthines with Substitution at the 1-, 3-, and 7-Positions. *J. Med. Chem.* **1989**, *32*, 1231-1237. (d) Daly, J. W.; Hide, I.; Müller, C. E.; Shamim, M. Caffeine Analogs: Structure–Activity Relationships at Adenosine Receptors. *Pharmacology* **1991**, *42*, 309-321.
(14) Bruns, R. F.; Fergus, J. H.; Badger, E. W.; Bristol, J. A.; Santay, L. A.; Hays, S. J. PD 115,199: An Antagonist Ligand for Adenosine A_2 Receptors. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **1987**, *335*, 64-69.
(15) (a) Shamim, M. T.; Ukena, D.; Padgett, W. L.; Daly, J. W. 8-Aryl- and 8-Cycloalkyl-1,3-dipropylxanthines: Further Potent and Selective Antagonists for A_1-Adenosine Receptors. *J. Med. Chem.* **1988**, *31*, 613-617. (b) Katsushima, T.; Nieves, L.; Wells, J. N. Structure–Activity Relationships of 8-Cycloalkyl-1,3-dipropylxanthines as Antagonists of Adenosine Receptors. *J. Med. Chem.* **1990**, *33*, 1906-1910. (c) Jacobson, K. A.; Kiriasis, L.; Barone, S.; Bradbury, B. J.; Kammula, U.; Campagne, J. M.; Secunda, S.; Daly, J. W.; Neumeyer, J. L.; Pfleiderer, W. Sulfur-Containing 1,3-Dialkyl xanthine Derivatives as Selective Antagonists at A_1-Adenosine Receptors. *J. Med. Chem.* **1989**, *32*, 1873-1879. (d) Erickson, R. H.; Hiner, R. N.; Feeney, S. W.; Blake, P. R.; Rzeszotarski, W. J.; Hicks, R. P.; Costello, D. G.; Abreu, M. E. 1,3,8-Trisubstituted Xanthines. Effects of Substitution Pattern upon Adenosine Receptor A_1/A_2 Affinity. *J. Med. Chem.* **1991**, *34*, 1431-1435.
(16) (a) Bruns, R. F.; Fergus, J. H.; Badger, E. W.; Bristol, J. A.; Santay, L. A.; Hartman, J. D.; Hays, S. J.; Huang, C. C. Binding of the A_1-Selective Adenosine Antagonist 8-Cyclopentyl-1,3-dipropylxanthine to Rat Brain Membranes. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **1987**, *335*, 59-63. (b) Lohse, M. J.; Klotz, K.-N.; Lindenborn-Fotinos, J.; Reddington, M.; Schwabe, U.; Olsson, R. A. 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX)—A Selective High Affinity Antagonist Radioligand for A_1 Adenosine Receptors. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **1987**, *336*, 204-210.
(17) (a) Shimada, J.; Suzuki, F.; Nonaka, H.; Karasawa, A.; Mizumoto, H.; Ohno, T.; Kubo, K.; Ishii, A. 8-(Dicyclopropylmethyl)-1,3-dipropylxanthine: A Potent and Selective Adenosine A_1 Antagonist with Renal Protective and Diuretic Activities. *J. Med. Chem.* **1991**, *34*, 466-469. (b) Suzuki, F.; Shimada, J.; Ishii, A.; Ohno, T.; Karasawa, A.; Kubo, K.; Nonaka, H. Xanthine Compounds. Eur. Patent 415 456, 1990. (c) Shimada, J.; Suzuki, F.; Nonaka, H.; Ishii, A. 8-Polycycloalkyl-1,3-dipropylxanthines as Potent and Selective Antagonists for A_1-Adenosine Receptors. *J. Med. Chem.* **1992**, *35*, 924-930.

0022-2623/92/1835-2342$03.00/0