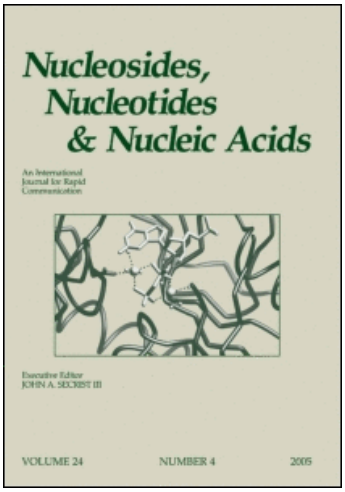

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Cis (IS,3R)-1-(9-Adenyl)-3-Hydroxycyclopentane Inhibits the Respiratory Burst from Polymorphonuclear Leukocytes and Has *In Vivo* Efficacy in an Acute and Chronic Model of Inflammation

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CIS (1S,3R)-1-(9-ADENYL)-3-HYDROXYCYCLOPENTANE INHIBITS THE RESPIRATORY BURST FROM POLYMORPHONUCLEAR LEUKOCYTES AND HAS *IN VIVO* EFFICACY IN AN ACUTE AND CHRONIC MODEL OF INFLAMMATION

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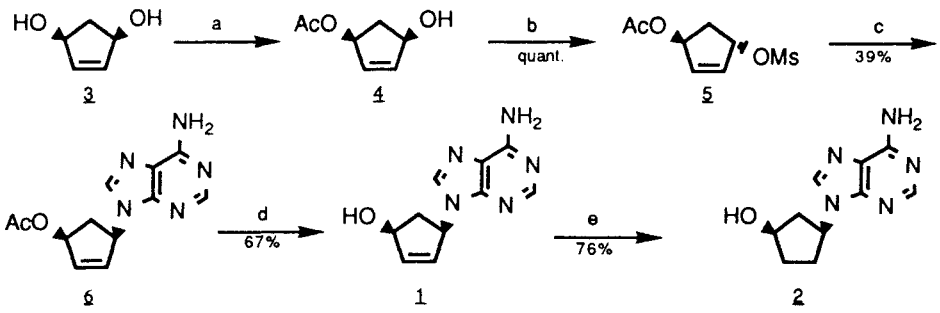
Abstract: The adenine-containing carbocyclic nucleosides 1 and 2 were synthesized in optically active form and have been shown to effectively inhibit interferon-gamma priming of Lewis (LEW/N) rat air pouch-derived PMNs. Compound 2 was also shown to have *in vivo* activity in a LEW/N rat antigen-induced arthritis model which assesses acute and chronic inflammation.

It has been demonstrated that macrophages (Mϕ) obtained from normal animals can be activated to produce oxygen metabolites upon stimulation with particulate or soluble products, and this phenomenon is known as "priming".[1-3] These activated Mϕ are characterized by their increased production of reactive oxygen intermediates, such as O_2^-, $HO\cdot$, and H_2O_2, and this phenomenom is termed the "respiratory burst". Activated polymorphonuclear leukocytes (PMN), another phagocytic cell type shown to be important in the early stages of inflammation, have also been shown to undergo similar priming and activation steps.[4-6] The most thoroughly characterized substance produced by mammalian cells that can prime both Mϕ and PMNs in both *in vitro* and *in vivo* systems is interferon-γ (IFN-γ).[7,8] Triggering of phagocytic cell activation and the accompanying respiratory burst can cause considerable harm to host tissues,[9] especially in autoimmune disease states such as rheumatoid arthritis (RA) which is characterized by progressive joint and tissue destruction.[10-12] Synthetic agents which inhibit the priming of PMNs by Mϕ-derived cytokines can potentially act as therapeutic agents to decrease the recruitment and destruction caused by PMNs in inflammatory diseases. Recent literature has suggested that



a)ref. 16; b)CH_3SO_2Cl (3eq),TEA,CH_2Cl_2; c)adenine(3eq),NaH,DMF; d)K_2CO_3,CH_3OH; e)PtO_2,H_2,CH_3OH

Scheme 1

adenosine (ADO) and some closely related adenosine analogues regulate the respiratory burst pathway in phagocytic cells.[13-15] The carbocyclic nucleoside analogs 1 and 2 (Scheme 1) were synthesized and tested for their ability to inhibit rat IFN-γ and human IL-1β priming of rat air pouch-derived PMNs *in vitro*. In addition, compound 2 was evaluated for its *in vivo* effects in a rat antigen-induced arthritis model.

Chemistry

The compounds 1 and 2 were synthesized according to Scheme 1. The starting optically active material 4 was prepared from 3 using a previously described method.[16] Compound 4 was converted to the trans-substituted mesylate 5 using 3.0 eq. of methanesulfonyl chloride and 3 eq. of triethylamine (TEA) in quantitative yield.[17] Compound 5 was used without further purification and was added to a stirring solution of 3 eq. of sodium adenide in DMF at 60°C to give the chiral protected nucleoside 6 in 39% yield. The acetate protecting group was removed with potassium carbonate in methanol and the free base was purified on silica gel using 9:1 methylene chloride/methanol to give the optically active nucleoside 1 in 67% yield ($[\alpha]_D$=+80° (c=1.03,MeOH)).[18] Compound 1 was then reduced with PtO_2 in methanol and the product was purified on silica gel using 4:1 methylene chloride/methanol to give 2 in 76% yield ($[\alpha]_D$=+7.8° (c=0.135,MeOH)). Hydrogenolysis product, 9-adenylcyclopentane, was the major side-product

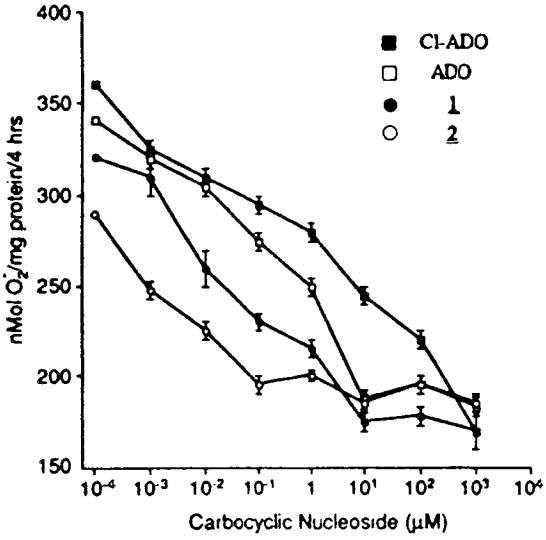


Figure 1 Rat air pouch PMN, obtained from pre-formed air pouches on Lewis (LEW/N) female rat (20,21), were cultured as outlined by Edwards et al.(3). After a 30 minute incubation, rRaIFN-γ (100 mg/ml) and either ADO , 2-ClADO, 1, or 2 (all at 0.0001 μM to 1000 μM) were added to the PMNs and incubated for 4 hours. SOD-inhibitable O_2^- production using PMA (100ng) as soluble stimulus was used. Results shown are from one representative experiment; three experiments using two replicates at each dose of compound were performed. Control non-stimulated PMNs treated for 4 hrs. with IFN-γ-released 100 ±13 nmol O_2^-/mg protein/4hr. PMNs treated with PMA released 312±13 nmol O_2^- /mg protein/4hr.

(approx. 20%) in the reduction. The final products 1 and 2 were synthesized in optically active form in 26 and 20 percent overall yields with ee's greater than 99%, respectively, from 4.

Biology

The ability of adenosine (ADO), 2-chloroadenosine (2-Cl-ADO), 1 and 2 to inhibit the recombinant rat IFN-γ (rRaIFN-γ) and recombinant human interleukin-1β (rHuIL-1β) priming of rat PMNs *in vitro* was determined by measuring the inhibition of the respiratory burst in which the production of superoxide anion (O_2^-) is monitored using a previously published procedure.[1,3,19] The rat air pouch model of inflammation was used to obtain the rat PMNs using 25 ng/pouch of rHuIL-1β.[20,21] The PMNs obtained in this

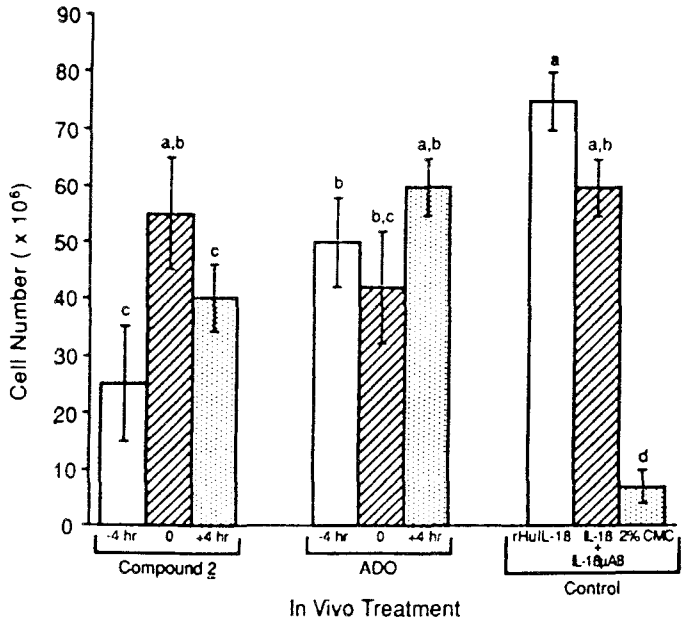


Figure 2 Rat air pouches (21,22) were treated with rHuIL-1β (25ng/pouch) with or without various treatment groups. After 18 hours, PMNs were counted using a Coulter Counter (Hialeah, Fl). Compound 2, 10 mg/kg/rat, s.c.; ADO, 10mg/kg/rat, s.c.; Controls: rHuIL-1β [5ng/rat]; IL-1β + mouse μ-human IL-1β MAB [1mg/rat]; 2% CMC vehicle. Bars with different superscripts are different at $p<0.05$ by Duncan's new multiple range test [SAS Institute(1987). SAS User's Guide. Statistics. SAS Institute, Cary, North Carolina, 43506].

manner release relatively low levels of oxygen radicals *in vitro* and can be further activated *in vitro* when primed with either rRaIFN-γ or rHuIL-1β. ADO, 2-Cl-ADO, 1, and 2 showed a dose-dependent inhibition of O_2^- production after rRaIFN-γ-induced priming of rat PMNs (Figure 1). Compounds 1 and 2 are significantly more potent at 1 μM than ADO and 2-Cl-ADO (N=6 from pooled data; $p<0.05$ by Duncans Multiple Range Test). Similar results were obtained with rHuIL-1β priming of rat PMNs (data not shown). In another experiment, ADO and 2 (10mg/kg/rat; 4 hours post-rHuIL-1β; Figure 2) were used to determine if the migration of PMNs into a rat air pouch treated with 25ng of rHuIL-1β was inhibited using the method described by Esser et al.[21] Compound 2 was more effective at inhibiting PMN migration into the air pouch than ADO four hours post-rHuIL-1β. Compound 1 was not active in this *in vivo* model (data not shown). It has been shown that S-adenosylhomocysteine hydrolase

(SAHase) inhibitors can inhibit PMN chemotaxis through the inhibition of phospholipid methylation.[22] Therefore, compounds 2 was evaluated as a potential SAHase inhibitor to determine if this could account for the inhibition of leukocyte migration into the air pouch model. It was determined that neither compound 1 or 2 significantly inhibited rat liver SAH hydrolase according to the procedure of Mehdi et al. (data not shown).[23] It has been demonstrated that ADO receptors (A_1 and A_2) regulate the expression of adhesion molecules and the respiratory burst in PMNs which may also account for the biological activity seen with these compounds.[15] Therefore, compounds 1 and 2 were evaluated for their ability to displace [^{3}H]CGS 21680 from A_2 receptors and [^{3}H]N6-cyclohexyladenosine from A_1 receptors using rat brain membrane preparations.[24,25] These compounds had measurable but weak potency (≥15 μM) for displacing the radioligand from A_2 receptors which may be partially responsible for their activity.[15b] Another possible mechanism for the inhibition of PMN chemotaxis and release of oxygen radicals may result from inhibiting cytokine release from activated PMNs or macrophages. Compound 2 has been shown to specifically inhibit tumor necrosis factor-alpha (TNF-α) production from macrophages by selectively inhibiting TNF-α mRNA transcription.[26] The inhibition of PMN priming and chemotaxis may be a result of the compounds ability to inhibit TNF-α production since TNF-α has been shown to induce the production of oxygen radicals in PMNs.[27-29]

Since respiratory burst products, such as O_2^-, are involved in promoting the tissue destruction observed in rheumatoid arthritis, we tested the effects of ADO or compound 2 in a LEW/N rat model of antigen-induced arthirits (AIA).[30] Knee joints of LEW/N rats pretreated with compound 2 (25 mg/kg/rat, S.C.) but not ADO (25 mg/kg/rat S.C.), significantly ($P<0.01$) reduced knee joint swelling (which is a measurement for acute inflammation) responses (Figure 3, panel A). No decrease in knee joint swelling was observed when ADO or 2 were given post-mBSA challenge (Figure 3 panel B). AIA rats receiving pretreatment or post-treatments with either ADO or 2 were observed to have improved histopathological incidence and severity lesion scores, including reduction of knee joint fibrosis/fibroplasia and synovial cell hyperplasia/hypertrophy, compared to disease controls during chronic inflammation (Figure 4).

We have shown that the carbocyclic nucleoside 2 is more potent than ADO in inhibiting rRaIFN-γ priming of rat PMNs and has potent anti-

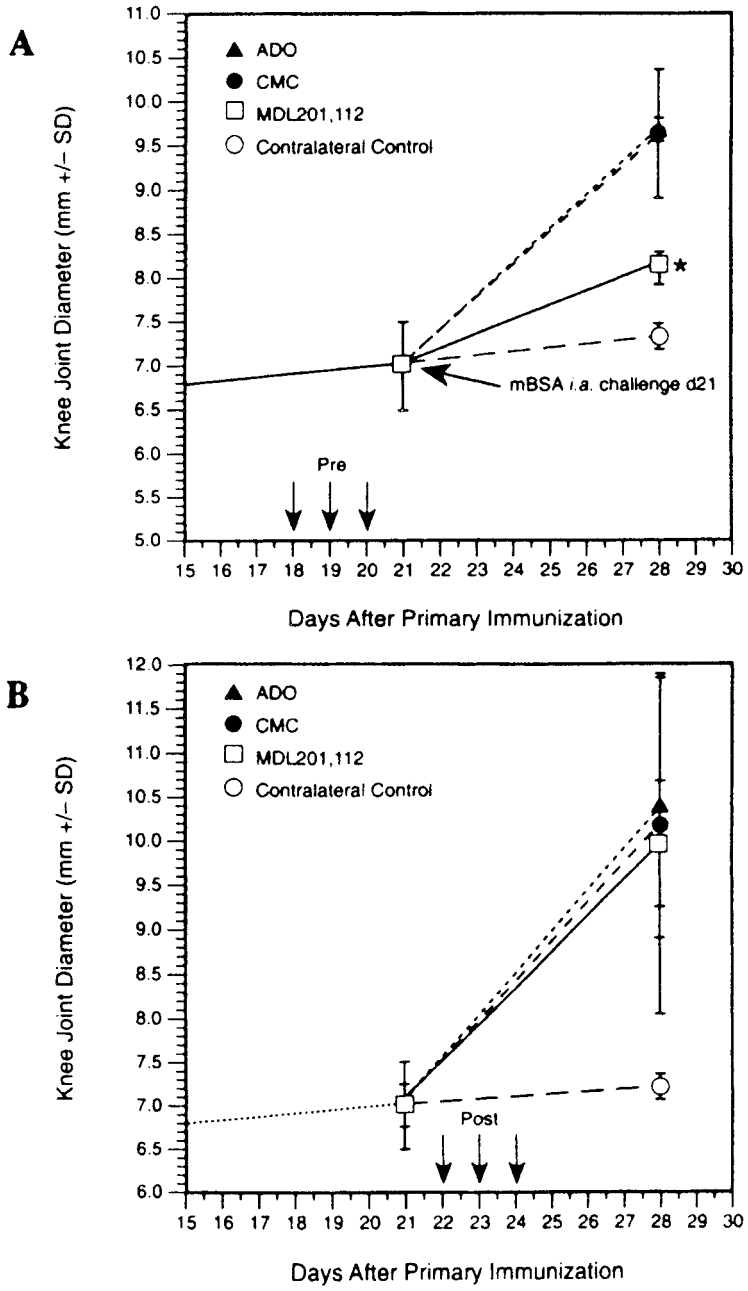


Figure 3 LEW/N rats (N=10/group) were immunized with mBSA (1 mg/ml) emulsified in CFA and challenged I.A. 21 d later with mBSA (250 µg) in right knee joint. Left knee joints (contralateral control) were injected I.A. with pyrogen-free saline (100 µl). Panel A and Panel B were pretreated or post-treated with either ADO (25 mg/kg/rat qD; 3d S.C.) or 2 (25 mg/kg/rat qD; 3d S.C.). Control animals recieved 2% CMC 200 µl/rat qD; 3d S.C. Knee joint swelling was measured using calibrated calipers on d+28 after immunization with mBSA.

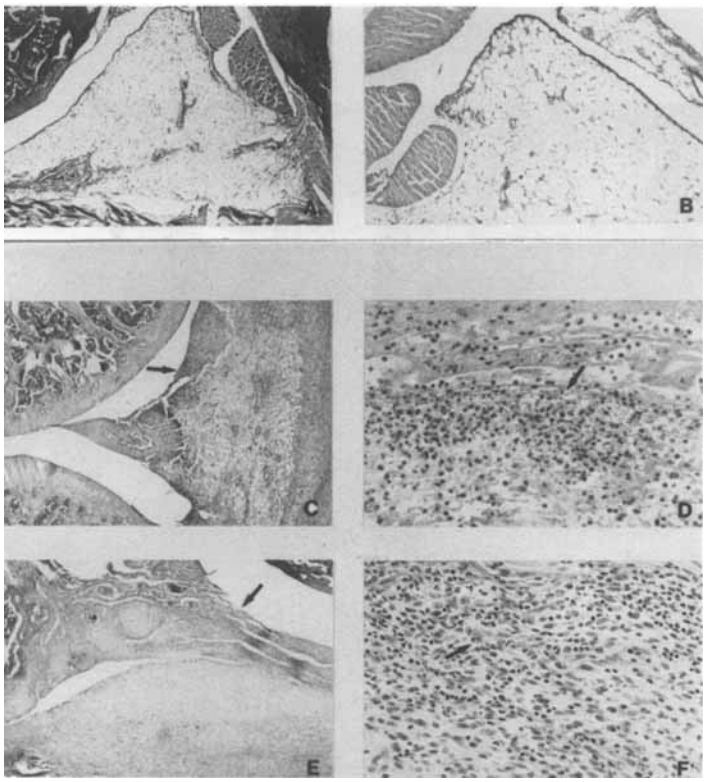


Figure 4 Hematoxyllin and eosin stains of histopathological grading of inflammatory dieases in LEW/N female rats. Panel A: normal knee joint at 10x mag; Panel B: normal knee joint 25x mag; Panel C and D: disease control at day+25 showing acute reaction, exemplified by PMN infilitration (arrow) of the joint space and some fibroplasia (10x): Panel E and F: chronic disease at day+39, note proliferation of fibrous connective tissue and mononuclear cell inflitrate (F,100x); Panel G and H: pretreatment with 2 (25 mg/kg,qD, 3d;S.C.)-mild chronic reaction at day+40 with minimal proliferative fibrous connective tissue (G,10x) and minimal synovial cell hyperplasia arrow (H,25x); Panel I and J: posttreatment with 2 (25 mg/k,qD, 3d; S.C.)-at day+42 no reaction at 10x and 25x; Panel K and L: pretreatment with ADO (25 mg/kg,qD, 3d; S.C.) at day+28 moderate chronic lesion with extensive fibrosis of connective tissue in joint fat pad (K, arrow)(10x) also synovial cell hyperplasia and minimal mononuclear inflitrate seen in L at 100x mag; Panel M and N: post-treatment with ADO (25 mg/kg,qD, 3d; S.C.) note fibrosis of joint pad (M, arrow,10x) and synovial cell hyperplasia (N, arrow,20x).

(continued)

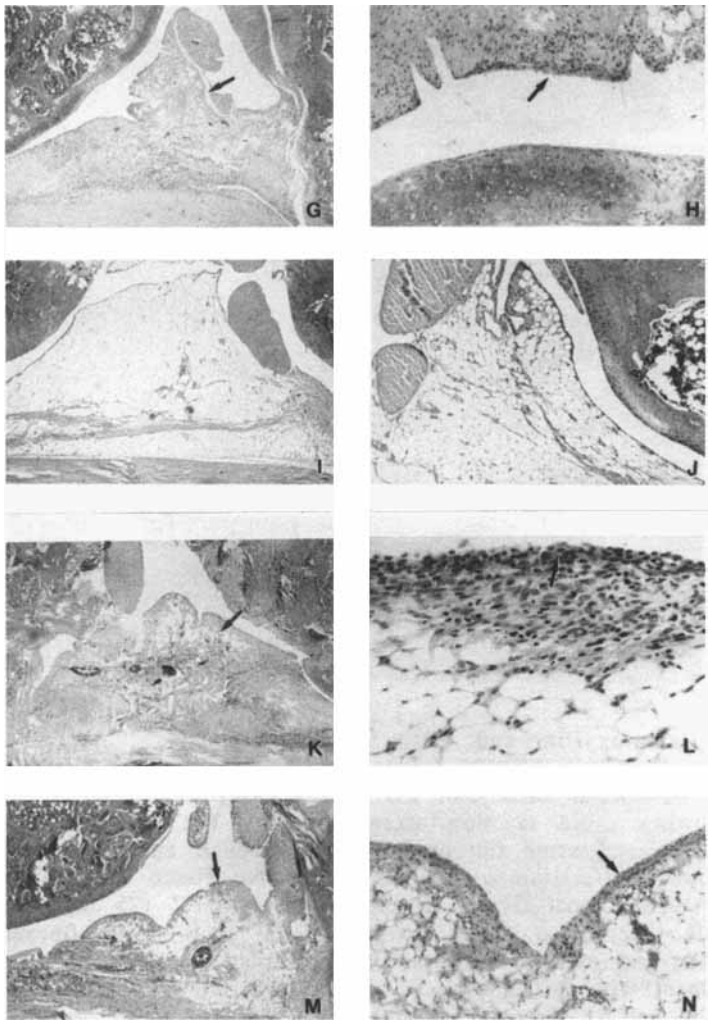


Figure 4 Continued.

inflammatory effects *in vivo* in the LEW/N rat antigen-induced arthritis model. These data suggest that 2 may regulate PMN cell activation *in vitro* and *in vivo* through the NADPH-oxidase respiratory burst pathway. Compound 2 has also been shown to protect mice against a lethal dose of LPS and D-galactosamine in an animal model of septic shock.[26] This protective effect may be due to the binding of these compounds to an adenosine A_3 receptor[31] and the subsequent inhibition of macrophage-derived TNF-α production and PMN cell activation, followed by the inhibition of biochemical mechanisms important in mediating the oxidative burst pathway in phagaocytic cells.

Experimental

Melting points were determined with a Thomas-Hoover capillary melting point apparatus and are uncorrected. TLC analysis were preformed with Merck Kieselgel 60 F254 plates, with visualization by UV irradiation or acidic p-anisaldehyde. Flash chromatography was performed with Merck silica gel 60 (0.040-0.063 mm). NMR spectra were recorded on Varian VXR-300 or Gemini-300 spectrometers in CDCl3, unless otherwise stated. 1H-NMR signals are reported in ppm from tetramethylsilane and coupling constants are reported in hertz (Hz). IR spectra were recorded on a Perkin-Elmer Model 1800 or a Mattson Galaxy 5020 FT-IR spectrophotometer. MS data were collected at 70 eV on a Finnigan MAT 4600, MAT TSQ-700 or VG Analytical Limited ZAB2-SE mass spectrophotometer. All reactions were run under an inert atmosphere. Starting materials and solvents were purchased form Aldrich Chemical Company.

(1R,4S)-4-Acetoxy-1-(9-adenyl)cyclopent-2-ene (6): To a 250 mL flask was added (1S,4R)-1-acetoxy-4-hydroxycyclopent-2-ene[16] (4, 1.42g, 10.0 mmol), methylene chloride (40 mL) and methanesulfonyl chloride (3.72g, 30 mmol) which was stirred and cooled with an ice bath to 0-5° C. Neat triethylamine (5.1 mL, 30 mmol) was added dropwise over a 3-minute period and after the addition was complete the ice bath was removed. The reaction mixture was allowed to come to room temperature and stir for six hours. Progress of the reaction was monitored by thin layer chromatography using EtOAc/hexane (4:1) and the reaction was judged to be complete ($R_f$prod=0.75; R_fS.M.=.25). The mixture was poured into a 250 mL separatory funnel and extracted with water (2x25 mL), and brine (1x50 mL). The aqueous layers were

combined and extracted once with 50 mL of methylene chloride and the combined organic layers were dried over sodium sulfate and concentrated to give 5 as a yellow oil (2.2 grams, 100% yield). This material was used immediately without further purification. ^{1}H-NMR ($CDCl_3$) δ 6.13(ddd,1H, J=5,2,2Hz), 6.05(ddd,1H,J=5,2,1Hz), 5.85(m,1H), 5.08(m,1H), 3.71(s,3H), 2.59(ddd,1H,J=16,8,4Hz), 2.38(ddd,1H,J=16,8,4Hz), 2.04(s,3H): ^{13}C-NMR ($CDCl_3$) δ 170.7, 137.5, 133.8, 78.4, 61.3, 41.5, 31.6, 21.0.

Sodium adenide was prepared by adding adenine (4.1g, 3.0 mmol) to a 250 mL round bottom three-neck flask fitted with a mechanical stirrer and 50 mL of DMF was added to form a slurry, then NaH (60% in mineral oil, 0.12g, 3.0 mmol) was added and the reaction mixture was stirred for three hours at 55-60°C. The (1S,4S)-1-acetoxy-4-methanesulfonyloxy-cyclopent-2-ene (5) (2.2g, 10 mmol) was poured into the reaction mixture and the reaction was stirred for forty-eight hours at 55-60°C. The product formation (R_f=.9) was monitored by thin layer chromatography with methylene chloride/methanol (4:1). The reaction mixture was cooled to 10°C with an ice bath and the solid which formed was filtered and washed thoroughly with methylene chloride. The dimethylformamide/ methylene chloride containing mixture was concentrated and the residue was dissolved in methylene chloride (200 mL) and filtered again to removed undissolved solids. The filtrate was extracted with brine (2x25 mL) and the aqueous layer was extracted with methylene chloride (3x100 mL). The combined organic layers were dried over sodium sulfate and concentrated to dryness. The residue was purified on silica gel (30 g) using methylene chloride/methanol (19:1) to give a total of 1.07 g of 6 (39% yield). mp=68°C(soften), 257°C (decomp); ^{1}H-NMR ($CDCl_3$) δ 8.35(s,1H), 7.84(s,1H), 6.32(m,1H), 6.18(m,1H), 6.07(br s, 2H), 5.7(m,2H), 3.11(ddd,1H, J=16, 8, 8 Hz), 2.08(s,3H), 1.95(ddd,1H,J=16, 2, 2 Hz); ^{13}C-NMR ($CDCl_3$) δ170.4, 155.6, 153.1, 149.6, 138.6, 135.5, 133.7, 119.5, 77.2, 56.7, 38.7, 21.0; MS (CI, methane) 260(base, M^{+1}), 200, 173, 136, 125; $[\alpha]_D$=+9.84° (c=1.05, MeOH); Anal. (C12H13N5O2·0.25H2O) C, H, N.

(1R,4S)-1-(9-adenyl)-4-hydroxycyclopent-2-ene (1): Compound 6 (1.0g, 3.8 mmol) was dissolved in methanol (20mL) and K_2CO_3 (1.0 g) was added and the reaction was stirred for 2 hours at room temperature. The mixture was filtered and concentrated to dryness. The material was dissolved in methylene chloride/methanol (9:1) and purified on a silica gel column (10 grams) using the same solvent system. The fractions containing product were concentrated to dryness. The product was dissolved in H_2O and the pH of the solution was

adjusted to 2.5 with 6N HCl. The solution was lyophilized to give 650 mg of 1 (67% yield). mp=204-205°C; ^{1}H-NMR (DMSO-d_6) δ 9.6(brs, 1H), 9.0(brs,1H), 8.57(s,1H), 8.49(s,1H), 6.26(m,1H), 6.03(m,1H), 5.54(m,1H), 4.75(m,1H), 2.95(ddd,1H, 16, 8, 8 Hz), 1.79(ddd,1H, 16, 2,2 Hz); ^{13}C-NMR (DMSO-d_6) δ 150.5, 147.8, 144.8, 142.2, 140.3, 129.9, 118.0, 73.5, 57.8, 41.3; MS (EI) m/z 217 (M^+), 188, 173, 135(base) 108; $[\alpha]_D$=+80° (c=1.03, MeOH); UV(MeOH) λ=261nm, ε=14,500; Anal. (C10H11N5O·0.8HCl) C, H, N, Cl.

(1S,3R)-1-(9-adenyl)-3-hydroxycyclopentane (2): Compound 1 (100mg, 0.4 mmol) was dissolved in methanol (50 mL) and PtO_2 was added and the mixture was hydrogenated at 35 psi of hydrogen for 3 hours. The catalyst was removed by filtering through celite and the mixture was concentrated. The material was purified on a silica gel column (10 g) using methylene chloride/methanol (9:1). The fractions containing product were concentrated and then dissolved in water which was adjusted to pH 2.5 with 6N HCl. The solution was lyophilized to give 76 mg of compound 2 (76% yield). mp=231°C (decomp); ^{1}H-NMR (DMSO-d_6+D_2O) δ 8.62(s,1H), 8.44(s,1H), 5.03(p,1H, J=9Hz), 4.30(p,1H, J=2Hz), 2.49(m,1H), 2.26(m,1H), 2.13(m,1H), 1.99-1.8(m,3H); ^{13}C-NMR (DMSO-d_6) δ 150.5, 148.2, 144.9, 142.5, 120.0, 70.3, 53.9, 41.5, 34.0, 31.0; MS (EI) m/z 219 (M^+), 202, 162, 135(base) 108; $[\alpha]_D$=+7.78° (c=0.135,MeOH); Anal. (C10H14ClN5O·0.75H2O) C, H, N.

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