Antiviral Chemistry & Chemotherapy **13**:39–59

Coumarins and pyranocoumarins, potential novel pharmacophores for inhibition of measles virus replication

Dale L Barnard[1]*, Ze-Qi Xu[2], Valerie D Stowell[1], Hongwei Yuan[2], Donald F Smee[1], Raghu Samy[2], Robert W Sidwell[1], M Kim Nielsen[3], Lihui Sun[2], Hua Cao[2], Ailing Li[2], Clay Quint[2], Jeffrey Deignan[2], Jennifer Crabb[2] and Michael T Flavin[2]

[1]Institute for Antiviral Research, Utah State University, Logan, UT, USA
[2]MediChem Research Inc., Woodridge, Ill., USA
[3]Cache Valley Specialty Hospital, Logan, UT, USA

*Corresponding author: Tel: +1 435 797 2696; Fax: +1 435 797 3959; E-mail: honery@cc.usu.edu

A series of coumarin and pyranocoumarin analogues were evaluated *in vitro* for antiviral efficacy against measles virus (MV), strain Chicago. Of the 22 compounds tested for inhibition, six were found to have selectivity indices greater than 10. These were compounds 5-hydroxy-7-propionyloxy-4-propylcoumarin (2a), 5,7-bis(tosyloxy)-4-propylcoumarin (7); 5-hydroxy-4-propyl-7-tosyloxy-coumarin (8); 6,6-dimethyl-9-propionyloxy-4-propyl-2*H*,6*H*-benzo[1,2-*b*:3,4-*b*′]dipyran-2-one (9); 6,6-dimethyl-9-pivaloyloxy-4-propyl-2*H*,6*H*-benzo[1,2-*b*:3,4-*b*′]dipyran-2-one (10); and 7,8-*cis*-10,11,12-*trans*-4-propyl-6,6,10,11-tetramethyl-7,8,9-trihydroxy-2*H*,6*H*,12*H*-benzo[1,2-*b*:3,4-*b*′:5,6-*b*″]tripyran-2-one (18). Three of the active drugs were propyl coumarin analogues (2a, 7 and 8), two were dipyranone or chromeno-coumarins (9 and 10), and one was a benzotripyranone with a coumarin nucleus (18). Some appeared to be rather specific and potent inhibitors of MV with EC_{50} values ranging from 0.2 to 50 μg/ml and the majority of the EC_{50} values being less than 5 μg/ml. The compounds inhibited an additional nine strains of MV, and in virucidal tests the drugs did not physically disrupt the virion to inhibit virus replication. The inhibitory activity for one of the compounds tested (7) was somewhat dependent on virus concentration and it was still active when added to cells up to 24 h after virus exposure. When used in combination with ribavirin, compound 7 appeared not to profoundly affect the antiviral efficacy of ribavirin or its cell-associated toxicity. However, a slightly antagonistic MV-inhibitory effect was observed at the highest concentration of ribavirin used in combination with most concentrations of compound 7 tested. This and related compounds may be valuable leads in the development of a potent and selective class of MV inhibitors that could be used in future in the clinic.

Keywords: coumarin, pyranocoumarin, benzodipyranone, benzotripyranone, measles virus, inhibition, antiviral, structure–activity relationships

Introduction

Measles virus (MV) is a ubiquitous pathogen that still ranks as one of the leading causes of infectious disease-induced morbidity and mortality in childhood throughout the world. Currently, 30–40 million measles infections occur worldwide each year leading to 1–2 million deaths (Manchester *et al.*, 2000). In addition to causing an acute respiratory infection, this disease is also associated with a serious, but transient suppression of cell-mediated immunity (Wyde *et al.*, 2000), apparently mediated by virus proteins resistant to heat treatment (Marttila *et al.*, 2001). This immunosuppression can lead to secondary infections, and major complications such as pneumonia and diarrhoea in children, especially in those suffering from malnutrition (Griffin & Bellini, 1996). In rare cases, it can also cause encephalitis and persistent central nervous system infections (Griffin & Bellini, 1996).

Although the use of a live attenuated virus has nearly eradicated the endemic circulation of measles in the USA and in other highly industrialized nations, re-introduction of the virus can still cause repeated limited outbreaks (Gay, 2000). In addition, vaccine safety has occasionally been questioned because of tenuous links to Crohn's disease and particularly to autism (Kawashima *et al.*, 2000). No studies to date, however, have conclusively established a causal relationship with vaccine viruses and those two diseases (Afzal *et al.*, 2000). Therefore, campaigns need to be

maintained that establish high routine coverage of the vaccine to prevent the re-accumulation of susceptible hosts that will permit sustained measles transmission (Gay, 2000). Those who may be especially vulnerable are the non-immunized or immunized individuals with waning immunity, such as those living in extended care facilities (Clements & Cutts, 1995). In addition, with an increasing number of health care professionals having only received vaccine or never having been exposed to measles infection in infancy, nosocomially-acquired and -transmitted MV infections in health care facilities may also contribute to a potential increase in MV infections (Steingart *et al.*, 1999). Thus, there always exists the potential for limited measles outbreaks until indigenous virus transmission is eliminated in most populations of a given country (reviewed by De Serres *et al.*, 2000). Furthermore, the efforts for the elimination of measles virus outbreaks is challenged significantly by the fact that measles virus isolates are not of single genotype or even serotype. As with other RNA viruses, MV has demonstrated considerable heterogeneity as measured by monoclonal antibody reactivities and nucleotide sequencing (Rota *et al.*, 1998). The RNA sequences of the strains of MV currently circulating have changed considerably from those isolated earlier.

Thus, there is a need to supplement global immunization with non-toxic therapeutic agents that can be used to treat unexpected outbreaks of measles virus infections quickly and effectively. No chemotherapeutic agents have yet been approved for treatment of measles virus infections, although ribavirin has been reported to be efficacious in patients when administered intravenously and orally alone (Gururangan *et al.*, 1990) or in combination with immune serum globulin (Stogner *et al.*, 1993). No other clinical trials have been carried out to support these findings; to the contrary, a number of studies have shown that ribavirin is of no obvious clinical efficacy in measles virus infections when given as small-particle aerosol or intravenously (reviewed by Wyde *et al.*, 2000). Recently, Barnard *et al.* (2001) reported several 5′-norcarbocyclic adenosine analogues that potently and selectively inhibited MV replication *in vitro*.

Historically, natural products have been a great resource to find novel and biologically active agents, included among which are coumarin-type compounds. Naturally occurring coumarin analogues have been reported to exhibit anti-tumor and antiviral properties (McKee *et al.*, 1996; Pengsuparp *et al.*, 1999; Semple *et al.*, 1999). These lead compounds have stimulated interest in the synthesis of more potent, less toxic analogues targeted to specific viral functions (Zhao *et al.*, 1997; Martyanov *et al.*, 1999; Yang *et al.*, 1999). Coumarin compounds have been shown to inhibit HIV-1 reverse transcriptase (Bourinbaiar *et al.*, 1993), HIV-1 integrase (Zhao *et al.*, 1997), HIV-1 protease (Skulnick *et al.*, 1997), protein kinase C (Yang *et al.*, 1999), and vaccinia DNA topoisomerase (Sekiguchi *et al.*, 1996) among other viral targets. In addition, a number of di-substituted (3′R, 4′R)-*cis*-khellactone analogues with a coumarin nucleus have been synthesized (Xie *et al.*, 1999). These derivatives possess potent anti-HIV inhibitory activity involving an as yet unknown mechanism and have been studied for structure-activity relationships (Xie *et al.*, 2001). Moreover (+)-calanolide A, one of the coumarin compounds studied in depth, has been evaluated for safety and pharmacokinetic profiles in healthy human volunteers (Creagh *et al.*, 2001) and is currently being investigated for anti-HIV efficacy in HIV-infected volunteers (R Sherer, CORE Center, Cook County Hospital, Chicago, Ill., personal communication).

Thus, coumarin analogues represent a novel class of compounds that warrant further investigation as potential antiviral agents. Herein we wish to present some of the first data on the anti-MV activity of coumarin and pyranocoumarin analogues.

Materials and methods: chemistry

General

Melting points were uncorrected. Unless otherwise stated, the ^{1}H NMR and ^{13}C NMR spectra were recorded on a Varian Gemini 2300 at 300 and 75.5 MHz, respectively, or a Varian Inova NMR spectrometer at 400 and 100.5 MHz, in the indicated deuterated solvent with chemical shifts reported in parts per million (ppm) using tetramethylsilane as the internal standard. FTIR was performed on a MIDAC Model M12000 and mass spectra were recorded on a Finnegan MAT 90 or a Finnegan LCQ-MS spectrometer. TLC analyses were performed on analytical thin plates coated with silica gel 60 F_{254} (Merck) and components were visualized under UV or stained with iodine. Column chromatography was performed using silica gel 60 (70–230 mesh from EM Sciences). Elemental analyses were carried out at Midwest Microlab (Indianapolis, Ind., USA). All commercial reagents and solvents were used without further purification. Ribavirin, used as a positive control, was obtained from ICN Pharmaceuticals Inc. (Costa Mesa, Calif., USA). Compounds **1**, **4**, **5**, **6** and **14** were synthesized by the published procedures (Flavin *et al.*, 1996) while compounds **7** and **8** prepared according to the literature method with some modifications. All the compounds reported herein were fully characterized and were in agreement with the assigned structures.

*5-hydroxy-7-propionyloxy-4-propylcoumarin (**2a**).* To a 2 l, three-neck round-bottom flask equipped with a stir bar, addition funnel and a N_2 inlet and outlet were added 50 g (2.27 mol) of 5,7-dihydroxy-4-propylcoumarin (1)

(Flavin *et al.*, 1996), 500 ml of anhydrous THF and 1 ml of pyridine. To this reaction mixture, 33 g (2.27 mol) of propionic anhydride was added dropwise at room temperature under constant stirring. After 90 min, the reaction was stopped and reaction mixture washed with 5% aqueous $NaHCO_3$ solution. The organic layer was separated and washed with 1 N HCl and brine. The aqueous layers were extracted with dichloromethane. The organic layers were combined and washed with brine. The crude product was dried over Na_2SO_4, rotary evaporated and concentrated *in vacuo* to give 62 g crude product. TLC (1:1 hexane/ethyl acetate) analysis indicated that the crude material contained the desired product **2a**, coumarin (**1**) and a small amount of 5,7-diester **3a**. The crude product was then purified by silica gel column chromatography on a Biotage column by eluting with 2:1 hexane/ethyl acetate and resulted in 12 g of **2a** (36% yield) as white solid: melting point: 166–168°C (170–171°C, Flavin *et al.*, 1996); ^{1}H NMR (DMSO-d_6), δ:0.97 (3H, t, J = 7.2 Hz), 1.14 (3H, t, J = 7.4 Hz), 1.62 (2H, sextet, J = 7.5 Hz), 2.62 (2H, q, J = 7.5 Hz), 2.92 (2H, t, J = 7.5 Hz), 6.09 (1H, s), 6.57 (1H, d, J = 2.4 Hz), 6.67 (1H, d, J = 2.4 Hz), 11.10 (1H, s); ^{13}C NMR ($CDCl_3$) δ: 8.6, 13.7, 22.4, 26.9, 37.2, 101.4, 105.3, 106.2, 111.9, 153.0, 155.9, 157.2, 158.0, 159.7, 172.2; IR (film) 3300-3075, 2968, 1758, 1676, 1610, 1433, 1126 cm^{-1}; MS m/e 277 (M+1); analysis calculated for $C_{15}H_{16}O_5$: C, 65.21; H, 5.84. Found: C, 64.61; H, 5.86.

*5-hydroxy-7-pivaloyloxy-4-propylcoumarin (**2b**).* Pyridine (2.02 ml, 25 mmol) was added to a solution of **1** (1.10 g, 5 mmol) in THF (10 ml) followed by the addition of pivaloyl chloride (0.612 ml, 5 mmol). The resulting mixture was stirred at room temperature for 6 days. The pyridinium hydrochloride was removed by filtration and the product washed a few times with ethyl acetate. The organic solutions were combined and washed successively with 1N HCl (2×25 ml), water (25 ml), aqueous saturated $NaHCO_3$ (25 ml). The product was then dried over Na_2SO_4 and concentrated under vacuum, the crude product was purified by silica gel chromatography (8:1 hexane/ethyl acetate to 2:1 hexane/ethyl acetate) to obtain **3b** (350 mg, 18% yield) and the corresponding monoester **2b** (550 mg, 36% yield) as a white solid. The analytical profile of compound **2b** was as follows: melting point: 158–160°C; R_f = 0.32 (4:1 hexane/ethyl acetate); ^{1}H NMR ($CDCl_3$) δ: 0.97 (3H, t, J = 7.8 Hz), 1.37 (9H, s), 1.56 (2H, sextet, J = 7.4 Hz), 2.84 (2H, t, J = 7.8 Hz), 6.07 (1H, s), 6.43 (1H, d, J = 2.4 Hz), 6.61 (1H, d, J = 2.1 Hz), 8.09 (1H, s); ^{13}C NMR ($CDCl_3$) δ: 13.8, 22.4, 26.9, 37.8, 39.3, 102.4, 105.8, 107.2, 112.1, 153.3, 156.2, 156.3, 159.3, 161.9, 178.0; IR (film) 3358, 2971, 2365, 1730, 1615, 1431, 1275, 1146 cm^{-1}; MS m/e 305 (M+1); analysis calculated for $C_{17}H_{20}O_5$: C, 67.09; H, 6.62. Found: C, 66.80; H, 6.70.

*5,7-bis(pivaloyloxy)-4-propylcoumarin (**3b**).* Pivaloyl chloride (0.673 ml, 5.5 mmol) was added to a solution of **1** (1.10 g, 5 mmol) in pyridine (12 ml) and THF (6 ml) at 0°C. The mixture was allowed to warm to room temperature and stirred at room temperature for 24 h. TLC indicated the presence of the unreacted **1**. In an effort to drive the reaction to completion, additional pivaloyl chloride (0.50 ml) was added and the reaction stirred at room temperature for another 72 h. The pyridinium hydrochloride was removed by filtration and washed a few times with ethyl acetate. The organic solutions were combined and washed successively with 1N HCl (2×25 ml), water (25 ml), and aqueous saturated $NaHCO_3$ (25 ml). The crude products were dried over Na_2SO_4 and concentrated under vacuum, the crude product was purified by silica gel chromatography (2:1 hexane/ethyl acetate) to obtain **3b** as a white solid (1.90g, 98% yield). Analysis of **3b** resulted in the following data: melting point 110–112°C; R_f = 0.49 (4:1 hexane/ethyl acetate); ^{1}H-NMR ($CDCl_3$) δ: 1.03 (3H, t, J = 7.8 Hz), 1.36 (9H, s), 1.41 (9H, s), 1.69 (2H, sextet, J = 7.4 Hz), 2.81 (2H, t, J = 7.8 Hz), 6.22 (1H, s), 6.60 (1H, d, J = 2.4 Hz), 7.03 (1H, d, J = 2.1 Hz); ^{13}C-NMR ($CDCl_3$) δ: 13.5, 20.7, 26.4, 26.8, 26.9, 36.4, 39.2, 39.4, 108.6, 111.5, 113.4, 114.2, 149.4, 152.7, 154.9, 155.4, 159.9, 177.1; IR (film) 3090, 2971, 2941, 2876, 1757, 1750, 1615, 1481, 1422, 1273 cm^{-1}; MS m/e 389 (M+1); analysis calculated for $C_{22}H_{28}O_6$: C, 68.02; H, 7.26. Found: C, 67.77; H, 7.18.

*7-TBDMS and 5,7-bis(TBDMS) substituted coumarins (**2c** and **3c**).* A mixture of coumarin **1** (5.0 g, 23 mmol), TBDMS-Cl (5.8 g, 27 mmol), and imidazole (4.7 g, 69 mmol) in 50 ml of dry DMF was stirred at room temperature under N_2 for 20 h, whereupon EtOAc (300 ml) was added to the reaction mixture. The precipitates formed were removed by filtration. The filtrate was washed successively with 1N HCl (2×100 ml), water (3×100 ml), and brine (200 ml). The organic layer was then dried with Na_2SO_4. After removal of the drying agent by filtration, the organic solution was kept at room temperature and crystals were formed. The solid was collected. The mother liquor was concentrated and the residue crystallized from EtOAc. This process was repeated two more times to give overall 2.2 g (28% yield) of **2c** as white crystals. The residue from the mother liquor was further purified by column chromatography to give 2.5 g (24% yield) of bis-TBDMS ether **3c**, additional 0.2 g of **2c** (a combined yield of 31%), and 0.2 g of unreacted starting material **1**. The analytical data of **2c** were: melting point 220–223°C; ^{1}H NMR (acetone-d_6) δ: 0.28 (6H, s), 1.00 (12H, s), 1.69 (2H, sextet, J = 7.5 Hz), 2.91 (2H, t, J = 7.5 Hz), 5.91 (1H, s), 6.33 (1H, d, J = 2.7 Hz), 6.42 (1H, d, J = 2.4 Hz), 9.55 (1H, s); ^{1}H NMR (DMSO-d_6) δ: 0.20 (3H, s), 0.40 (3H, s), 0.99

(9H, s), 1.01 (3H, s), 1.63 (2H, sextet, J = 7.5 Hz), 2.95 (2H, t, J = 7.5 Hz), 6.06 (1H, s), 6.26 (1H, d, J = 2.7 Hz), 6.52 (1H, d, J = 2.4 Hz); ^{13}C NMR (DMSO-d_6) δ: −4.7, −3.9, 13.7, 22.4, 25.4, 37.1, 99.2, 103.3, 103.5, 109.8, 156.7, 157.5, 158.4, 158.5, 160.1; IR (film) 3497-3021, 2957, 1684, 1616 cm^{-1}; MS m/e 335 (M+1); Analysis calculated for $C_{18}H_{26}O_4Si$: C, 64.64; H, 7.83. Found: C, 64.31; H, 7.78. For **3c**: melting point 78–79°C; ^{1}H NMR (DMSO-d_6) δ: 0.24 (6H, s), 0.36 (6H, s), 0.95–9.97 (21H, m), 1.59 (2H, q, J = 7.5 Hz), 2.91 (2H, t, J = 7.5 Hz), 6.02 (1H, s), 6.21 (1H, d, J = 2.7 Hz), 6.48 (1H, d, J = 2.4 Hz).

*5,7-bis(tosyloxy)-4-propylcoumarin (**7**).* A mixture of **1** (30 g, 0.14 mol), K_2CO_3 (76 g, 0.55 mol), p-toluenesulfonyl chloride (57 g, 0.3 mol) and acetone (450 ml) was refluxed for 4.5 h. After cooling to room temperature, the mixture was filtered and the filtrate evaporated to give a light-yellow solid. The solid residue was dissolved in a mixture containing 1.2 l of EtOAc and 1 l of water. The aqueous layer was removed and extracted with EtOAc (2×200 ml). The combined organic layers were washed with brine, dried over Na_2SO_4, filtered, and then concentrated under vacuum to afford 75 g of crude product as a light-yellow solid. The crude material was triturated with EtOAc to remove polar impurities and then filtered to give a white solid and an orange filtrate. The white solid was triturated with hexane to remove the less polar impurities and then filtered to give 51.8 g of **7** as a white powder. The orange filtrate was concentrated to give 22 g of residue as a dark-orange oil which was solidified by addition of hexane. The solid was collected by filtration, triturated with EtOAc, filtered, and washed with hexane to give an additional 11.7 g of product as a white powder. A total of 63.5 g (88% yield) of **7** was obtained with the following characteristics: melting point 110–112°C (112°C, Fox *et al.*, 2000); ^{1}H NMR (DMSO-d_6) δ 0.82 (3H, t, J = 7.2 Hz), 1.44 (2H, sextet, J = 7.5Hz), 2.44 (3H, s), 2.46 (3H, s), 2.72 (2H, t, J = 7.6 Hz), 6.36 (1H, s), 6.80 (1H, d, J = 2.4 Hz), 7.20 (1H, d, J = 2.4 Hz), 7.52 (2H, d, J = 8.4 Hz), 7.54 (2H, d, J = 7.8 Hz), 7.78 (2H, d, J = 8.4 Hz), 7.80 (2H, d, J = 8.4 Hz); ^{13}C NMR (DMSO-d_6) δ 13.3, 21.2, 21.5, 36.1, 110.1, 111.7, 112.1, 116.3, 128.4, 128.5, 130.7, 130.8, 130.9, 146.3, 146.8, 147.2, 149.8, 154.0, 155.1, 158.2; IR (film) 3094, 2965–2878, 1740, 1615, 1379, 1192 cm^{-1}; MS m/e 529 (M^+); analysis calculated for $C_{26}H_{20}O_8S_2$: C, 59.08; H, 4.58. Found: C, 58.97; H, 4.58.

*5-Hydroxy-4-propyl-7-tosyloxy-coumarin (**8**).* Sixty grams (0.113 mol) of the bistosylated compound **7** and 300 ml of THF were added to a 1 l, three-necked round-bottomed flask equipped with a mechanical stirrer, an addition funnel, a thermometer, and N_2 inlet/outlet. The solution was cooled to 0°C, and 125 ml (0.125 mol) of a 1.0 M solution of tetra-n-butylammonium fluoride in THF was added. The mixture was stirred at 0°C for 5 h. The solvent was then removed under vacuum to give a green-brown oil which was diluted with 1 l of EtOAc and washed with water (500 ml). The aqueous layer was extracted with EtOAc (2×250 ml). The organic layers were combined, washed with brine (300 ml), dried over Na_2SO_4 and filtered. The solvent was removed under vacuum to yield 100 g of a thick green-brown oil that was purified by filtering through a column of silica gel using EtOAc as the eluent. The EtOAc eluents were concentrated and the resulting solid was crystallized from EtOAc to afford 24 g (57% yield) of **8** as a white solid. The analytical data for this product were: melting point 214–215°C (210°C, Fox *et al.*, 2000); ^{1}H NMR (DMSO-d_6) δ 0.94 (3H, t, J = 7.2 Hz), 1.57 (2H, sextet, J = 7.4 Hz), 2.44 (3H, s), 2.88 (2H, t, J = 7.5 Hz), 6.11 (1H, s), 6.49 (1H, d, J = 2.4 Hz), 6.55 (1H, d, J = 2.4 Hz), 7.51 (2H, d, J = 8.1 Hz), 7.82 (2H, d, J = 8.1 Hz), 11.29 (1H, s); ^{13}C NMR (DMSO-d_6) δ 13.7, 21.2, 22.3, 37.0, 101.3, 105.0, 107.4, 112.6, 128.4, 130.5, 131.4, 146.3, 150.9, 155.7, 157.5, 157.6, 159.3; IR (film) 3092, 2967, 1692, 1611, 1379 cm^{-1}; MS m/e 375 (M+1); analysis calculated for $C_{19}H_{18}O_6S$: C, 60.95; H, 4.85. Found: C, 60.85; H, 4.83.

*6,6-Dimethyl-9-propionyloxy-4-propyl-2H,6H-benzo[1,2-b:3,4-b']dipyran-2-one (**9**).* $(n\text{-}Bu)_4NI$ (1.11 g, 3 mmol), K_2CO_3 (1.04 g, 7.5 mmol), and 3-chloro-3-methyl-1-butyne (1.11 g, 3 mmol) were added to a solution of 7-propionate **2a** (0.83 g, 3.0 mmol) in 2-butanone (40 ml) and DMF (4 ml). The reaction mixture was heated to 60°C for 1 h before $ZnCl_2$ (3.9 ml of 1.0 M solution in ether, 3.9 mmol) was added. The temperature was then raised to 70°C and the mixture stirred for 21 h at 70°C. The reaction mixture was cooled to room temperature and quenched with saturated aqueous NH_4Cl (100 ml). The mixture was extracted with EtOAc (2×100 ml) and the combined organic layers were washed with brine (100 ml) and dried over Na_2SO_4. Evaporation of the solvent gave 1.9 g of crude product. After column chromatographic purification, 280 mg (27% yield) of **9** was obtained as a waxy solid. Analysis of **9** gave the following results: melting point 85–88°C; ^{1}H NMR (DMSO-d_6) δ 1.00 (3H, t, J = 7.2 Hz), 1.16 (3H, t, J = 7.5 Hz); 1.47 (6H, s), 1.61 (2H, m), 2.71 (2H, q, J = 7.5 Hz), 2.89 (2H, t, J = 7.8), 5.84 (1H, d, J = 9.9 Hz), 6.17 (1H, s), 6.41 (1H, d, J = 10.2 Hz), 6.81 (1H, s); ^{13}C NMR (DMSO-d_6) δ 8.6, 13.7, 22.8, 26.6, 27.2, 37.4, 78.3, 103.6, 107.2, 110.8, 113.4, 115.5, 130.1, 148.5, 151.4, 154.4, 156.9, 159.3, 172.2; IR (film) 2972, 1767, 1723, 1616 cm^{-1}; MS m/e 343 (M+1); analysis calculated for $C_{20}H_{22}O_5$: C, 70.16; H, 6.47. Found: C, 70.37; H, 6.51.

6,6-Dimethyl-9-pivaloyloxy-4-propyl-2H,6H-benzo[1,2-b:3,4-b']dipyran-2-one (10). A suspension of **2b** (304 mg, 1 mmol) was prepared in 2-butanone (13 ml) and DMF (1.3 ml). K_2CO_3 (346 mg, 2.5 mmol), 3-chloro-3-methyl-1-butyne (0.56 ml, 5 mmol) and $(n\text{-}Bu)_4NI$ (360 mg, 1 mmol) were then added to the suspension. The reaction mixture was heated to 60°C for 1 h and then anhydrous $ZnCl_2$ (1.0 M solution in ether, 1.3 ml) was added. The reaction mixture was heated to 70°C for 26 h, then cooled to room temperature, quenched with saturated aqueous NH_4Cl (25 ml) and extracted with ethyl acetate (2×75 ml). The organic solutions were combined, washed with brine, dried over Na_2SO_4 and concentrated. The concentrated crude product was purified by silica gel chromatography (8:1 hexane/ethyl acetate) to yield **10** as a yellow solid (270 mg, 73% yield) with the following analytical profile: melting point: 65–68°C; R_f=0.54 (4:1 hexane/ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 1.04 (3H, d, J = 7.2 Hz), 1.39 (9H, s), 1.52 (6H, s), 1.66-1.71 (2H, m), 2.90 (2H, t, J = 7.8 Hz), 5.62 (1H, d, J = 10.2 Hz), 6.07 (1H, s), 6.30 (1H, d, J = 10.2 Hz), 6.62 (1H, s); ^{13}C NMR ($CDCl_3$) δ 13.8, 22.9, 26.9, 27.8, 38.3, 39.3, 78.1, 103.5, 107.9, 110.9, 113.4, 115.9, 129.1, 148.9, 152.0, 155.1, 157.4, 160.6, 176.2; IR (film) 2967, 1750, 1616, 1364, 1142 cm^{-1}; MS m/e 371 (M+1); analysis calculated for $C_{22}H_{26}O_5$: C, 71.33; H, 7.07. Found: C, 71.08; H, 7.35.

6,6-Dimethyl-4-propyl-9-tosyloxy-2H,6H-benzo[1,2-b:3,4-b']dipyran-2-one (11). To a 100 ml, three-necked round-bottomed flask equipped with a mechanical stirrer, a condenser, a thermometer and N_2 inlet/outlet were added 0.5 g (1.34 mmol) of **8**, 20 ml of 2-butanone, and 2 ml of DMF. This was followed by the addition of 0.46 g (3.34 mmol) of K_2CO_3, 0.49 g (1.33 mmol) of $(n\text{-}Bu)_4NI$. To this reaction mixture, 0.44 ml (4.0 mmol) of 3-chloro-3-methyl-1-butyne was added by syringe. The resulting solution was heated to 60°C for 1 h, and then 1.74 ml of 1 M solution of $ZnCl_2$ in ether was added. The reaction mixture was stirred for 40 h at 70°C. After cooling to room temperature, the mixture was diluted with EtOAc (100 ml) and quenched with saturated aqueous NH_4Cl. The aqueous layer was extracted with EtOAc (2×50 ml). The combined organic solution was washed with brine, dried over Na_2SO_4 and filtered. The solvent was removed under vacuum to provide 0.8 g of crude product as a yellowish solid. The crude product was then purified by column chromatography and the solid obtained was crystallized from EtOAc to afford 0.3 g (50% yield) of the desired product **11** as a white solid: melting point 150–151°C (148°C, Fox *et al.*, 2000); ^{1}H NMR (DMSO-d_6) δ 0.97 (3H, t, J = 7.1 Hz), 1.34 (6H, s), 1.57 (2H, m), 2.42 (3H, s), 2.84 (2H, t, J = 7.6 Hz), 5.67 (1H, d, J = 10.2 Hz), 6.20 (1H, d, J = 8.4 Hz), 6.21 (1H, s), 6.68 (1H, s), 7.47 (2H, d, J = 7.5 Hz), 7.79 (2H, d, J = 7.5 Hz); ^{13}C NMR (DMSO-d_6) δ 13.6, 21.1, 22.7, 27.0, 37.3, 78.5, 103.4, 108.4, 111.5, 114.1, 114.8, 128.6, 130.3, 130.6, 130.9, 146.1, 146.6, 151.6, 154.2, 156.6, 159.0. Analysis calculated for $C_{24}H_{24}O_6S$: C, 65.44; H, 5.49. Found: C, 65.32; H, 5.50.

6,6-dimethyl-9-hydroxy-4-propyl-2H,6H-benzo[1,2-b:3,4-b']dipyran-2-one (13). To a solution of ester **9** (223 mg, 0.65 mmol) in 15 ml of MeOH were added saturated aqueous solution of $NaHCO_3$ (7 ml) and water (7 ml). The reaction mixture was stirred at room temperature under N_2 for 7 h until TLC indicated complete consumption of the starting material. The reaction mixture was then acidified with 10% aqueous HCl (100 ml) and extracted with EtOAc (50 ml). The organic solution was washed with brine (100 ml) and dried over Na_2SO_4. Evaporation of the solvent yielded the crude product that was purified by preparative TLC to afford 97 mg (52% yield) of **13** as a solid. Analysis of that product yielded the following analytical data: melting point 190–192°C (190°C, Fox *et al.*, 2000); ^{1}H NMR (DMSO-d_6) δ 0.99 (3H, t, J =7.3Hz), 1.45 (6H, s), 1.59 (2H, sextet, J =7.5Hz), 2.83 (2H, t, J =7.6Hz), 5.66 (1H, d, J=9.9Hz), 5.92 (1H, s), 6.34 (1H, s), 6.57 (1H, d, J =9.9Hz), 10.77 (1H, s); ^{13}C NMR (DMSO-d_6) δ 13.7, 22.9, 27.2, 37.5, 77.5, 95.7, 102.2, 106.1, 109.9, 116.3, 127.1, 151.7, 155.7, 156.4, 157.8, 160.0; IR (film) 3185, 1686, 1582, 1381, 1157 cm^{-1}; MS m/e 287 (M+1); analysis calculated for $C_{17}H_{18}O_4$: C, 71.31; H, 6.34. Found: C, 71.39; H, 6.40.

Preparation of 13 from tosylate 11. Compound **13** can be also obtained from detosylation of **11**. To a 100 ml, three-necked round-bottomed flask equipped with a stir bar and N_2 inlet/outlet was added 1.8 g (4.09 mmol) of **11** and 40 ml of THF. The solution was cooled to 0°C, and 6.2 ml (6.2 mmol) of a 1.0 M solution of $(n\text{-}Bu)_4NF$ in THF was added by the syringe. The resulting mixture was stirred at 0°C for 7 h. The solvent was then removed under vacuum to furnish a green-brown oil which was diluted with EtOAc (500 ml) and washed with water (300 ml). The aqueous layer was extracted with EtOAc (2×100 ml). The organic layers were combined, washed with brine (100 ml), dried over Na_2SO_4 and filtered. The solvent was removed under vacuum to provide 2.1 g of crude product as a green-brown oil. The oil was further purified by column chromatography followed by crystallization from EtOAc to afford 0.5 g (43% yield) of **13** as a white solid, which was identical to the compound obtained from **9**.

6,6-dimethyl-9-(2-methyl-2-methoxycarbonylpropyloxy)-4-propyl-2H,6H-benzo[1,2-b:3,4-b']dipyran-2-one (15). To compound **13** (430 mg, 1.50 mmol), Ph_3P (590 mg, 2.25 mmol) and methyl 2,2-dimethyl-3-hydroxy

propionate (396 mg, 3 mmol) in dioxane (34 ml) was added DEAD (360 µl, 2.25 mmol) and stirred at reflux under N_2 for 2 h. The reaction mixture was cooled to room temperature and concentrated under vacuum. The residue was dissolved in ethyl acetate (100 ml) and washed with water (80 ml), dried (Na_2SO_4), concentrated under vacuum and purified by silica gel chromatography (3/1 hexane/ethyl acetate) to afford **15** (445 mg, 74% yield) as a white solid. The analytical data were as follows: melting point: 100–101°C; ^{1}H NMR ($CDCl_3$) δ 1.03 (3H, t, J = 7.4 Hz), 1.35 (6H, s), 1.49 (6H, s), 1.65 (2H, sextet, J = 7.5 Hz), 2.89 (2H, t, J = 7.8 Hz), 3.71 (3H, s), 4.00 (2H, s), 5.52 (1H, d, J = 9.9 Hz), 5.96 (1H, s), 6.37 (1H, s), 6.56 (1H, d, J = 10.2 Hz); ^{13}C NMR ($CDCl_3$) δ 13.8, 22.3, 23.0, 27.7, 38.3, 43.2, 52.1, 74.8, 77.7, 93.3, 104.3, 107.2, 111.2, 116.3, 127.1, 151.7, 156.4, 156.7, 158.1, 161.3, 176.2; IR (film) 2878, 1726, 1605, 1364, 1155 cm^{-1}; MS m/e 401 [M+1]; analysis calculated for $C_{23}H_{28}O_6$: C, 68.98; H, 7.05. Found C, 69.16; H, 7.12.

6,6-dimethyl-9-(2-methyl-2-carboxylpropyloxy)-4-propyl-2H,6H-benzo[1,2-b:3,4-b′]dipyran-2-one (16). To a solution of compound **15** (40 mg, 0.1 mmol) in methanol (2 ml) was added KOH (25 mg, 0.45 mmol) in water (1 ml). The resulting solution was stirred at room temperature for 3 h, whereupon it was concentrated under vacuum. Water was then added to the residue which was acidified with 1N HCl and extracted with ethyl acetate. The organic extracts were combined and washed with brine, dried (Na_2SO_4) and concentrated under vacuum to furnish **16** (30 mg, 78% yield) as a white solid. Analysis of the product revealed the following characteristics: melting point: 140–142°C; ^{1}H NMR ($CDCl_3$) δ 0.99 (3H, t, J = 7.6 Hz), 1.38 (6H, s), 1.48 (6H, s), 1.65 (2H, sextet, J = 7.4 Hz), 2.89 (2H, t, J = 7.7 Hz), 4.02 (2H, s), 5.51 (1H, d, J = 9.9 Hz), 5.98 (1H, s), 6.38 (1H, s), 6.59 (1H, d, J = 10.2 Hz); ^{13}C NMR ($CDCl_3$) δ 13.8, 22.1, 23.0, 27.7, 38.3, 43.1, 74.4, 77.7, 93.3, 104.3, 107.3, 111.2, 116.4, 127.1, 151.8, 156.4, 156.6, 158.2, 161.4, 181.4; IR (film) 3381–3073, 2963–2750, 1740, 1605, 1474, 1385, 1163 cm^{-1}; MS m/e 387 [M+1]; analysis calculated for $C_{22}H_{26}O_6 \cdot 0.2H_2O$: C, 67.75; H, 6.92. Found C, 67.75; H, 6.81.

7,8-cis-10,11,12-trans-4-propyl-6,6,10,11-tetramethyl-7,8,9-trihydroxy-2H,6H,12H-benzo[1,2-b:3,4-b′:5,6-b″]tripyran-2-one (18). To a round-bottom flask equipped with magnetic stirrer were added acetone (10 ml) (±)-calanolide A (**17**) (Flavin *et al.*, 1996) (0.5 g, 1.35 mmol), t-butylammonium acetate (88.4 mg, 0.34 mmol), and t-butylhydroperoxide (70%, 0.3 ml, 2.19 mmol). The solution was stirred at room temperature until a homogeneous solution was obtained. The temperature of the solution was then lowered to 0°C. A solution of OsO_4 (1 mg, 0.004 mmol) in t-butyl alcohol was added. The reaction mixture turned purple. After stirring for 1 h, the cold bath was removed and the reaction mixture was warmed up to room temperature and stirred for 30 h. Ether (10 ml) was added and the resulting mixture cooled to 0°C, followed by addition of freshly prepared 10% aqueous $NaHSO_3$ (15 ml). The cold bath was removed and stirring was continued for another hour to afford a two-layer solution. Solid NaCl was added to the aqueous layer until it was saturated. The two-phase solution was stirred for 10 mins; the organic layer was then separated and the aqueous phase extracted with ether (3×10 ml). The combined organic phase was washed with brine, dried (Na_2SO_4) and concentrated to yield a brown residue as the crude product. The crude product was purified by 2mm-Chromatotron with ethyl acetate/hexane (1:4) as the eluent to afford compund **18** (130 mg, 24% yield), a mixture of two isomers at a ratio of approximately 2.5:1. The analytical data were as follows: ^{1}H NMR (400 MHz, $CDCl_3$) δ [with minor isomer listed in brackets] 1.02 (3H, t, J = 7.4 Hz), 1.16 (3H, d, J = 6.8 Hz), 1.37 (3H, s), 1.50 (3H, d, J = 6.4 Hz), 1.52 (3H, s), 1.63 (2H, sextet, J = 7.2 Hz), 1.98 (1H, m), 2.80–2.95 (2H, m), 3.21 (1H, m), 3.59 (1H, broad-s), 3.82 (1H, m), 4.12 (1H, dq, J = 6.6, 8.4 Hz), 4.76 (1H, d, J = 7.2 Hz) [4.73 (d, J = 7.2 Hz)], 5.02 (1H, d, J = 5.2 Hz) [4.99 (m)], 5.98 (1H, s); ^{13}C NMR (100.5 MHz, $CDCl_3$) δ [with the minor isomer listed in bracket] 13.9 [14.2], 15.2 [15.0], 19.3 [19.2], 22.6, 23.2, 24.0, 39.1 [38.9], 40.0 [40.5], 62.4 [62.1], 66.6 [66.9], 69.9 [70.2], 77.9 [78.0], 78.9 [79.1], 104.4, 106.1, 106.3, 110.8 [110.9], 151.1, 155.0, 156.0, 159.3, 160.3; IR (film) 3441, 2971–2874, 1711, 1595, 1379, 1113 cm^{-1}; MS m/e 405 (M+1); analysis calculated for $C_{22}H_{28}O_7$: C, 65.33; H, 6.98. Found C, 65.54; H, 7.20.

7,8-trans-10,11,12-trans-8-(3-chlorobenzonyloxy)-7,12-dihydroxy-4-propyl-6,6,10,11-tetramethyl-2H,6H,12H-benzo[1,2-b:3,4-b′:5,6-b″]tripyran-2-one (19). To a solution of (±)-calanolide A (**17**) (0.4 g, 1.1 mmol) in CH_2Cl_2 (2 ml) at 0°C was added a solution of m-chloroperbenzoic acid (MCPBA) (0.5 g, 2.9 mmol). The reaction mixture was stirred at 0°C for 3 h. The reaction was quenched with 10% aqueous sodium sulfite solution (15 ml), followed by saturated $NaHCO_3$ solution (5 ml). The resulting two layers were separated and the aqueous layer was extracted with CH_2Cl_2 (3×5 ml). The combined organic phase was washed with brine and dried over Na_2SO_4. Concentration under reduced pressure yielded a yellow residue that was purified by 2-mm chromatotron with ethyl acetate/hexane (1:3) as the eluent to afford benzoate **19** (178.6 mg, 46% yield) which was a mixture of isomers at a ratio of 3:1. The compound had the

following physical and chemical data: ^{1}H NMR ($CDCl_3$) δ [with the minor isomer listed in bracket] 1.04 (3H, d, J = 6.4 Hz), 1.05 (3H, t, J = 7.2 Hz), 1.13 (3H, d, J = 7.16.4 Hz), [1.18 (d, J = 6.4 Hz)], 1.11 (3H, d, J = 6.3 Hz), 1.26 (3H, s), 1.46 (3H, s), 1.56 (3H, s), 1.67 (2H, sextet, J = 7.6 Hz), 1.90 (1H, m), 2.91 (2H, mt, J = 7.8 Hz), 3.83 (1H, dq, J = 6.4, 9.0 Hz) [4.08 (m)], 4.69 (1H, d, J = 7.8 Hz), 5.98 (1H, s), 6.18 (1H, d, J = 4.2 Hz) [6.41 (d, J = 5.1 Hz)], 7.34 (1H, t, J = 8.0 Hz), 7.66 (1H, dd, J = 2.1, 7.8 Hz), 7.86 (1H, dd, J = 2.2, 7.8 Hz), 7.96 (1H, t, J = 1.8 Hz); ^{13}C NMR ($CDCl_3$) δ [with minor isomer listed in brackets] 13.8 [14.0], 14.7 [14.8], 18.5, 23.2 [21.8], 24.1 [22.6], 29.3 [29.5], 31.8, 38.9, 40.4 [40.3], 67.0 [66.8], 69.2, 72.6 [71.6], 77.2, 79.2, 103.2, 104.0, 107.0, 110.6, 127.9, 129.9, 131.6, 133.4, 134.8, 152.0, 155.9, 157.1, 159.3, 160.5, 165.8; IR (film) 3434, 2965–2872, 1728, 1710, 1597, 1252, 1117 cm^{-1}; MS m/e 545 and 543 (M^+), 527 and 525 (M-H_2O), 387 (M-$C_7ClH_4O_2$), 369 (M-H_2O-$C_7ClH_4O_2$); analysis calculated for $C_{29}ClH_{31}O_8$: C, 64.15; H, 5.75. Found C, 63.82; H, 5.95.

3,4-trans-6,7,8-trans-10-(2-methoxycarbonyl-1-propylethenyl)-3,4,8,9-tetrahydroxy-2,2,6,7-tetramethyl-2H,8H-benzo[1,2-b:3,4-b']dipyrane (20) and 7,8-trans-10,11,12-trans-4-propyl-6,6,10,11-tetramethyl-7,8,12-trihydroxy-2H,6H,12H-benzo[1,2-b:3,4-b':5,6-b'']tripyran-2-one (21). To a solution of benzoate **19** (1.2 g, 2.2 mmol) was added a solution of NaOMe in MeOH (15 ml), freshly prepared by addition of a small piece of sodium to MeOH. The solution was stirred at room temperature for 3 days. The solvent was then removed under reduced pressure to yield a yellow solid, which was further purified by chromatography with ethyl acetate/hexane (25% to 40% gradient) as the eluent. Compound **20** (275.7 mg, 29% yield) was collected first followed by **21** (69.8 mg, 8% yield). Compound **20**, a mixture of isomers with a ratio of approximately 2.5:1. The analytical data were as follows: ^{1}H NMR (400 MHz, $CDCl_3$) δ [with the minor isomer listed in brackets] 1.02 (3H, t, J = 7.6 Hz), 1.16 (3H, d, J = 6.8 Hz), 1.45 (6H, s), 1.48 (3H, d, J = 6.4 Hz), 1.63 (2H, sextet, J = 7.5 Hz), 1.87–1.95 (1H, m), 2.85–2.95 (2H, m), 2.90 (1H, d, J = 8.0 Hz), 3.57 (1H, d, J = 3.4 Hz) [3.54 (d, J = 3.4 Hz)], 3.63 (3H, s) [3.66 (s)], 3.80 (1H, dd, J = 5.0, 8.5 Hz) [3.78 (dd, J = 5.1, 8.5 Hz)], 4.05 (1H, dq, J = 6.8, 8.1 Hz) [3.89 (dq, J = 6.8, 10.0 Hz)], 4.51 (1H, d, J = 4.8 Hz) [4.46 (d, J = 5.4 Hz)], 4.76 (1H, dd, J = 5.0, 8.6 Hz) [4.73 (dd, J = 3.8, 7.4 Hz)], 5.93 (1H, s); ^{13}C NMR (100.5 MHz, $CDCl_3$) δ [with minor isomer listed in brackets] 13.9, 15.1 [14.9], 18.9, 21.5 [20.6], 23.2 [23.3], 26.1 [26.5], 39.0 [39.1], 40.3 [40.6], 60.0 [60.6], 67.1 [67.3], 70.7 [71.1], 71.2, 77.2 [77.5], 79.1 [79.0], 104.0 [103.8], 105.8, 106.4 [106.5], 110.2, 151.6 [151.5], 155.4 [155.5], 156.9 [157.3], 159.4, 160.5; MS m/e 436 (M^+), 419 (M-H_2O), 401 (M-2 H_2O), 369 (M-H_2O-MeO).

The analytical data for compound **21** were as follows: ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.02 (3H, t, J = 7.4 Hz), 1.15 (3H, d, J = 6.8 Hz), 1.37 (3H, s), 1.50 (3H, d, J = 6.4 Hz), 1.51 (3H, s), 1.63 (2H, sextet, J = 7.5 Hz), 1.99 (1H, sextet, J = 7.5 Hz), 2.81–2.95 (2H, m), 3.22 (1H, d, J = 4.4 Hz), 3.65 (1H, broad-s), 3.81 (1H, t, J = 4.6 Hz), 3.93 (1H, d, J = 1.6 Hz), 4.12 (1H, dq, J = 6.6, 8.4 Hz), 4.76 (1H, dd, J = 2.4, 7.2 Hz), 5.03 (1H, dd, J = 1.8, 5.0 Hz), 5.98 (1H, s); ^{13}C NMR (100.5 MHz, $CDCl_3$) δ 13.9, 15.2, 19.2, 22.6, 23.2, 24.1, 39.1, 40.0, 62.4, 66.5, 70.0, 77.9, 78.9, 104.4, 106.2, 106.3, 110.7, 151.0, 155.0, 156.0, 159.4, 160.4; MS m/e 405 (M+1), 387 (M-OH), 369 (M-OH-H_2O).

7,8-trans-10,11-trans-8-(3-chlorobenzonyloxy)-7-hydroxy-4-propyl-6,6,10,11-tetramethyl-2H,6H,12H-benzo[1,2-b:3,4-b':5,6-b'']tripyran-2,12-dione (23). To a solution of trans-(±)-ketone **22** (Flavin *et al.*, 1996) (0.40 g, 1.1 mmol) in CH_2Cl_2 (2 ml) at 0°C was added a solution of MCPBA. The reaction mixture was stirred at 0°C for 3 h. The reaction was quenched with 10% aqueous sodium sulfite solution (15 ml), followed by saturated $NaHCO_3$ solution (5 ml). The resulting two layers were separated and the aqueous layer was extracted with CH_2Cl_2 (3×5 ml). The combined organic phase was washed with brine and dried over Na_2SO_4. Concentration under reduced pressure yielded a yellow residue which was purified by 2-mm chromatotron with ethyl acetate/hexane (1:3) as the eluent to furnish benzoate **23** as a white solid (353 mg, 59% yield), which is a mixture of isomers with a ratio of 2:1. The analytical profile was: ^{1}H NMR ($CDCl_3$) δ [with the minor isomer listed in brackets] 1.04 (3H, t, J = 7.4 Hz), 1.14 (3H, dd, J = 1.4, 7.1 Hz), 1.22 (3H, d, J = 6.3 Hz), 1.54 (3H, s), 1.59 (3H, s), 1.60–1.80 (2H, m), 2.31 (1H, d, J = 2.4 Hz), 2.40-2.55 (1H, m), 2.80–3.00 (2H, m), 4.01 (1H, dd, J = 2.3, 4.4 Hz), 4.20–4.30 (1H, m), 6.08 (1H, s), 6.23 (1H, d, J = 4.2 Hz) [6.48 (1H, d, J = 5.1 Hz)], 7.41 (1H, t, J = 8.0 Hz), 7.59 (1H, dd, J = 1.2, 8.1Hz), 7.90 (1H, dd, J = 2.2, 7.8 Hz), 7.98 (1H, t, J = 2.2 Hz); MS m/e 542 (M+1).

7,8-cis-10,11-trans-7,8-dihydroxy-4-propyl-6,6,10,11-tetramethyl-2H,6H,12H-benzo[1,2-b:3,4-b':5,6-b'']tripyran-2,12-dione (24). To a vigorously stirred solution of trans-(±)-ketone **22** (1.0 g, 2.7 mmol) in ethyl acetate/acetonitrile (1:1, 16 ml) at 0°C was added a solution of RuO_2 (42.7 mg, 0.2 mmol) and $NaIO_4$ (1.8667 g, 14.0 mmol) in distilled water. The resulting solution turned brown after it had been stirred vigorously at 0 °C for 3.5 h. Saturated Na_2SO_3 solution (25 ml) was then added. The layers were separated and the aqueous phase was extracted

with ethyl acetate (5×15 ml). The combined organic phase was dried over Na_2SO_4 and concentrated to yield a black residue which was then dissolved in ethyl acetate (10 ml) and passed through a short silica gel column (5 cm) with ethyl acetate as the eluent. The collected solution was concentrated to afford a brown solid which was further purified via 2-mm Chromatotron with ethyl acetate/hexane (1:2) as the eluent to provide **24** (91 mg, 8% yield), a mixture of two isomers at a ratio of approximately 1.2:1 with these characteristics: ^{1}H NMR (400 MHz, $CDCl_3$) δ [with the minor isomer listed in brackets] 1.01 (3H, t, J = 7.2 Hz), 1.24 (3H, d, J = 7.2 Hz) [1.23 (d, J = 6.0 Hz)], 1.44 (3H, s) [1.46 (s)], 1.55 (3H, s) [1.53 (s)], 1.58 (3H, d, J = 6.8 Hz) [1.60 (d, J = 6.4 Hz)], 1.61 (2H, m), 2.60 (1H, m), 2.86 (2H, m), 3.22 (1H, t, J = 5.2 Hz), 3.67 (1H, d, J = 2.8 Hz) [3.53 (d, J = 2.8 Hz)], 3.85 (1H, m), 4.47 (1H, dq, J = 6.3, 10.0 Hz) [4.37 (dq, J = 6.4, 9.9 Hz)], 5.05 (1H, s), 6.05 (1H, s); ^{13}C NMR ($CDCl_3$) δ [with the minor isomer listed in brackets] 10.1 [10.5], 13.7 [14.0], 19.7 [19.6], 22.0 [21.6], 23.0 [22.5], 24.6 [24.9], 31.5, 39.0, 47.0 [47.1], 53.4, 61.7, 70.0 [70.2], 80.3 [80.4], 103.6, 104.7, 106.3, 106.6, 112.4, 156.0 [155.8], 157.8, 162.9 [163.2], 189.5; IR (film) 3461, 2972–2874, 1732, 1717, 1701, 1609, 1583, 1559, 1208, 1115 cm^{-1}; MS m/e 403 (M+1), 385 (M-OH); analysis calculated for $C_{22}H_{26}O_7$: C, 65.66; H, 6.51. Found C, 65.50; H, 6.56.

9-hydroxy-4-propyl-6,6-dimethyl-10-(1-oxo-2,3-dihydroxy-2-methyl-butyl)-2H,6H-benzo[1,2-b:3,4-b^2]dipyran-2-one (25). To a solution of trans-(±)-ketone 22 (0.50 g, 1.3 mmol) in CH_2Cl_2 (5 ml) and MeOH (15 ml) was added H_2O_2 (0.25 ml, 50% aqueous solution, 0.30 g, 4.1 mmol) at 15°C, followed by a dropwise addition of NaOH solution (2.5 ml, 0.275 M aqueous solution, 0.68 mmol). After the yellow solution was stirred at 15°C for 24 h, water (20 ml) and ether (15 ml) were added. The two layers were separated and the aqueous layer was further extracted with ether (3×15 ml). The unreacted starting ketone **22** (320 mg, 64%) was recovered from the combined ethereal solution. The aqueous phase was then acidified with concentrated HCl solution until the pH 3 and extracted again with ether (3×10 ml) extractions. The combined ethereal solution was concentrated to afford compound **25** (67 mg, 13% yield) as an orange solid with the following profile: ^{1}H NMR ($CDCl_3$) δ 1.03 (3H, t, J = 7.2 Hz), 1.23 (3H, d, J = 6.0 Hz), 1.32 (3H, d, J = 7.2 Hz), 1.44 (3H, s), 1.46 (3H, s), 1.66 (2H, sextet, J = 7.5 Hz), 2.91 (2H, m), 4.89 (1H, dq, J = 7.5, 7.8 Hz), 5.61 (1H, d, J = 9.9 Hz), 6.04 (1H, s), 6.62 (1H, d, J = 10.2 Hz); ^{13}C NMR ($CDCl_3$) δ 13.1, 13.8, 16.2, 23.0, 27.2, 27.3, 38.4, 45.5, 76.7, 78.8, 106.6, 111.6, 112.6, 117.6, 129.1, 131.7, 142.3, 143.2, 143.5, 159.6, 161.7, 179.3; IR (film) 3192–2872, 1717, 1593, 1458, 1136 cm^{-1}; MS m/e 403 (M+1); analysis calculated for $C_{22}H_{26}O_7 \cdot 1.5H_2O$: C, 61.53; H, 6.80. Found C, 60.97; H, 6.51.

Evaluation of coagulation inhibitory activity

Compounds **7**, **9** and **18** were evaluated for anticoagulant activity in prothrombin timing and partial thromboplastin timing (aPTT) assays. Two dilutions of each compound were added to human plasma from normal individuals with no known coagulopathies to a final concentration of 10 μg/ml or 1 μg/ml. DMSO, the compound solvent, was also evaluated using the same concentrations of DMSO found in the two compound dilutions. The assays were done with a Dade Behring Sysmex CA 500 coagulation analyser (Dade Behring, Chicago, Ill., USA) using the protocol and reagents specified for that instrument.

Materials and methods: virology

Solubilization of Compounds

The coumarin analogues were dissolved in DMSO prior to dilution in culture medium for antiviral assays. Ribavirin was soluble in aqueous media.

Cells and virus

African green monkey kidney cells (CV-1 cells) were obtained from American Type Culture Collection (ATCC, Manassas, Va., USA). The cells were grown in minimal essential medium (MEM, Gibco-BRL, Gaithersburg, Md., USA) supplemented with 0.1% $NaHCO_3$ and 10% fetal bovine serum (FBS, Hyclone Laboratories, Logan, UT, USA). When performing antiviral assays, serum was reduced to 2%, and 50 μg/ml gentamicin (Sigma Chemical Company, St. Louis, Mo., USA) was added to the medium.

Measles virus strain CC was obtained from Pennsylvania State University (Beavertown, Pa., USA) and the Edmonston wild-type strain purchased from ATCC. Strains MO2 and MO6 were kindly provided by P Wyde (Baylor College of Medicine, College Station, Tex., USA). All other strains were acquired from PA Rota and JS Rota (Centers for Disease Control and Prevention, Atlanta, Ga., USA).

For all studies, except for the multiplicity of infection (MOI) study, the viral MOI used ranged from 0.0005–0.001 for each virus tested.

Cytopathic effect inhibition assay

The protocol of Barnard *et al.* (1997a) was used. Compounds were tested at varying concentrations (seven 0.5 log_{10} dilutions) one time with this assay and the activity was then verified spectrophotometrically by neutral red (NR) uptake assay on the same plate (see below). Virus and compound were added in equal volumes to near-confluent cell monolayers in 96-well tissue culture plates. The MOI

used was virus-dependent and selected for each strain such that 100% of the cells in the virus controls showed cytopathic effects (CPE) within 5–10 days for most MV strains. The plates were incubated at 37°C until the cells in the virus control wells showed complete viral CPE as observed by light microscopy. Each concentration of drug was assayed for virus inhibition of viral CPE in quadruplicate and for cytotoxicity in duplicate. Four wells were set aside as uninfected, untreated cell controls per test and four wells per test compound received virus in medium only and represented controls for virus replication. Ribavirin was included as a positive control drug for each set of compounds tested. For all CPE-based assays, the 50% effective concentrations (EC_{50}) were calculated by linear regression analysis of the means of the CPE ratings expressed as percentages of untreated, uninfected controls for each concentration.

Morphological changes resulting from cytotoxicity of a compound were graded on a scale of 0–5; 5 being defined as complete cytotoxicity. The 50% cytotoxic doses (IC_{50}) were calculated by regression analysis and a selectivity index (SI) was calculated using the formula: $SI = IC_{50}/EC_{50}$.

Neutral red uptake assay of CPE inhibition and compound cytotoxicity

This assay was done on the same CPE inhibition test plates described above to verify the inhibitory activity and the cytotoxicity observed by visual observation. The usual correlation between visual and NR assays in our hands has been greater than 95% (Barnard, 1997a). The NR assay was performed using a modified method of Cavenaugh *et al.* (1990) as described by Barnard *et al.* (1999). Briefly, medium was removed from each well of a plate, 0.034% NR added to each well of the plate and the plate incubated for 2 h at 37°C in the dark. The NR solution was removed from the wells, rinsed and the remaining dye extracted using ethanol buffered with Sörenson's citrate buffer. Absorbances at 540/450 nm were read with a microplate reader (Bio-Tek EL 1309; Bio-Tek Instruments, Inc., Winooski, Vt., USA). Absorbance values were expressed as percents of untreated controls and EC_{50}, IC_{50} and SI values were calculated as described above.

Virus Yield Reduction Assay

All compounds with a SI greater than 10 were evaluated in a more sensitive assay to confirm the results of the CPE inhibition/NR uptake assays. Infectious virus yields from each well from a second CPE inhibition assay were determined as previously described (Barnard, *et al.*, 1997b). After CPE was scored as described above, each plate was frozen at −80°C and thawed. Sample wells at each compound concentration tested were pooled and titered in CV-1 cells for infectious virus by CPE assay as described previously by Barnard *et al.* (1997b).

A 90% reduction in virus yield was then calculated by linear regression analysis. This represented a 1 $\log_{10}$ inhibition in titre when compared to untreated virus controls.

Virucidal assay

For compounds showing good antiviral inhibitory activity, a virucidal test was done to exclude the possibility that the compounds inhibited the virus by physically inactivating or disrupting the virion. The method of Barnard *et al.* (1997b) was used. Equal volumes of undiluted virus stock and a single concentration of compound were incubated at 37°C for 1 h. Surviving virus was quantified by CPE assay and titres were calculated as described previously by Barnard *et al.* (1997b). Concentrations of compound tested bracketed the concentration determined to represent the EC_{50}; each concentration of test compound was assayed in quadruplicate.

Evaluation of cytotoxicity in rapidly dividing cells

Cytotoxicity in rapidly dividing cells was evaluated by determining the total number of cells as reflected by a NR uptake assay after a 3-day exposure to several concentrations of compound. The method of Barnard *et al.* (1997b) was used. To quantitate cell growth after 72 h in the presence or absence of drug, the plates were then treated as described above for the NR assay. Absorbance values were expressed as percent of untreated controls and IC_{50} values were calculated by regression analysis.

Determining the influence of multiplicity of infection

This assay was done as described previously by Barnard *et al.* (1997a). Virus (strain Chicago) was used for the study of the inhibition by selected compounds at different MOIs ranging from 0.0004–0.04 μg/ml (see Table 5). Compounds at varying concentrations were evaluated as described above for the CPE inhibition and NR assays in the presence of virus at a selected MOI. EC_{50} and IC_{50} values were determined as previously described.

Evaluation of the timing of treatment initiation

The time that the compound was added to the plates was varied relative to the time of virus exposure. Varying dilutions of test compounds were added to the cells without removal of the virus (MOI = 0.04) at 1, 2, 4, 8 and 24 h post virus exposure. When a treatment was done 1 h before virus was added, compound was added to the wells and incubated at 37°C for 1 h, after which virus was added to each well in a small volume. At '0' time, the virus was added within 5 min of drug addition as described previously for the CPE inhibition assay (Barnard *et al.*, 1997a). The EC_{50},

IC_{50} and SI values were derived from visual and NR assays as described above. The Chicago strain of MV was used for this study.

Evaluation of drug combinations

Concentrations of test compound (0.0032–10 μg/ml) were evaluated alone and in combination with varying concentrations of ribavirin (0.032–32 μg/ml) against the Chicago strain of MV. All combinations, as well as each drug concentration used alone, were done in quadruplicate in 96-well microplates. Toxicity controls for each combination were also run in parallel on the same plate; these consisted of uninfected cells similarly treated with each drug combination as well as each concentration of either drug used alone. Ribavirin was tested from 0.32–320 μg/ml and **7** from 0.01–32 μg/ml. Visual and NR assays were used to generate EC_{50} or an IC_{50} values for each compound used alone or in combination.

The evaluation of synergism was done utilizing the 3-dimensional computerized system of Prichard & Shipman (1990) with the MacSynergy™ computer program.

Results: chemistry

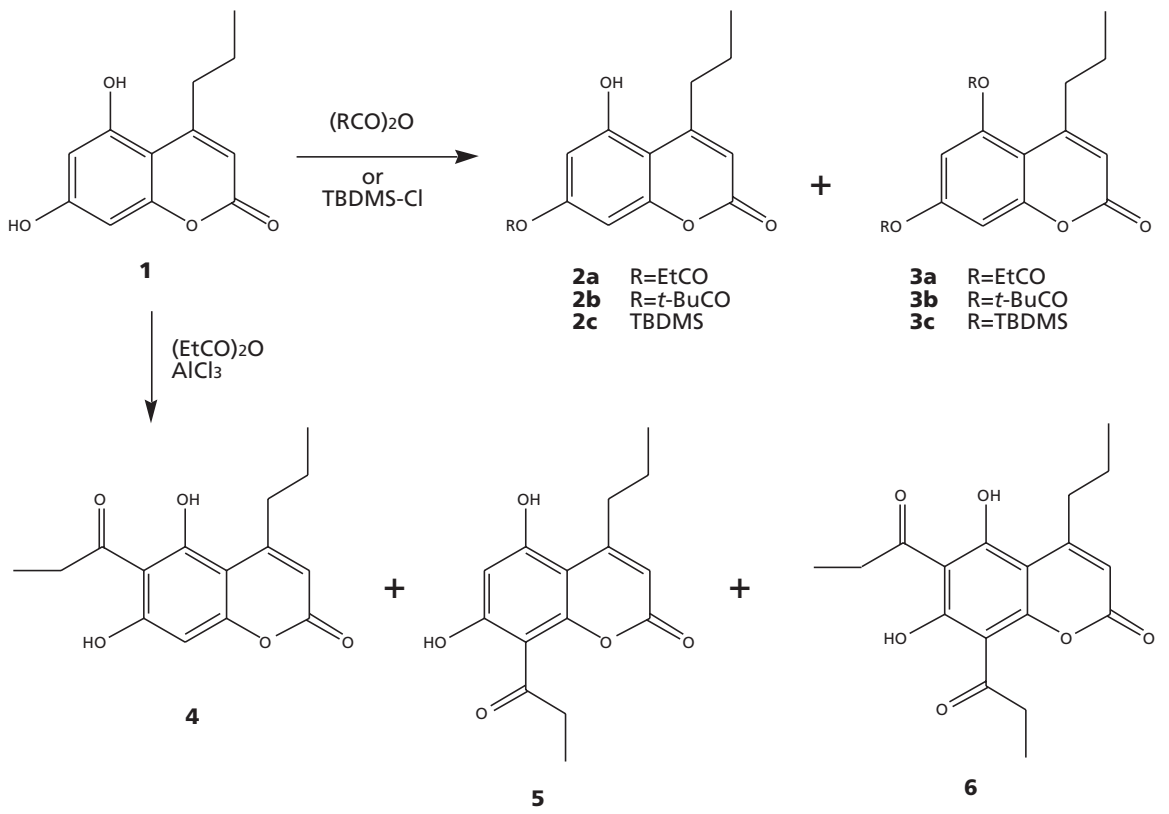
It has been reported (Flavin *et al.*, 1996) that 5,7-dihydroxy-4-propylcoumarin (**1**) was selectively acylated under the modified Friedel-Crafts reaction conditions to form 5-hydroxy-7-propionyloxy-4-propylcoumarin (**2a**) in a 10% isolated yield. Due to the low yield from that synthesis, a more practical procedure needed to be developed. Thus, a variety of reaction conditions were investigated and are summarized in Table 1. Without a Lewis acid such as $AlCl_3$ or bases such as pyridine and 4-dimethylaminopyridine (DMAP), no reaction took place between coumarin **1** and propionyl anhydride (entries 1 and 2 in Table 1). The reported conditions using $AlCl_3$ (Flavin *et al.*, 1996) were repeated and led to 20% conversion to **2a**, as indicated by HPLC analysis (entry 3). The best results were obtained when a catalytic amount of pyridine was used (entry 4), resulting in a 47% conversion to **2a** along with a small amount of the corresponding diester **3a** (Scheme 1) and some unreacted starting material **1**. The formation of the diester **3a** could be minimized by shortening the reaction time or lowering the reaction temperature, which, however, also decreased the yield of the monoester **2a** with increased recovery of the unreacted starting **1** (entry 5). On the other hand, prolonged reaction time or increasing the reaction temperature increased the conversion of the starting material **1** to **2a**, which was accompanied by an increase in the formation of undesired **3a** and led to more difficulty in purification of **2a**. It appeared that DMAP might have been too strong a base (entries 6 and 7) and proprionyl chloride too reactive an acylating agent (entry 8) for the selective acylation. Therefore, the pyridine-promoted acylation was scaled up in a 50 g scale reaction, affording a 36% isolated yield of **2a**. As reported by Flavin *et al.* (1996), **2a** was positive in a Gibbs colour test, resulting in a greenish blue colour characteristic of *p*-unsubstituted phenol (Josephy & Damme, 1984). Furthermore, a COSY/NOSY NMR spectrum showed that the free hydroxyl group (5-OH) at 11.10 ppm only correlated with one aromatic proton (H_6) at 6.57 ppm, which was supported by NOE enhancements (20%) observed between these two protons. The results further confirmed the structural assignment, since the hydroxyl group (7-OH) would be expected to correlate with both aromatic protons (H_6 and H_8) should the propionyl group be attached to the 5-OH.

For the introduction of a more bulky group at the 7-position of **1**, a more reactive acylating agent such as acyl chloride was used. For example, the reaction between **1** and pivaloyl chloride at room temperature for 4 days in the presence of pyridine yielded 7-monosubstituted **2b** and 5,7-disubstituted **3b** in isolated yields of 36% and 18%, respectively. The bispivaloate **3b** was exclusively formed when **1** was reacted with 2 equivalents of pivaloyl chloride. It is worthwhile noting that reaction of **1** with 1.0 equivalent TBDMS-Cl in the presence of imidazole in DMF afforded 31% of 7-TBDMS substituted **2c**, along with 24% of 5,7-bis(TBDMS) substituted **3c** (Scheme 1). The

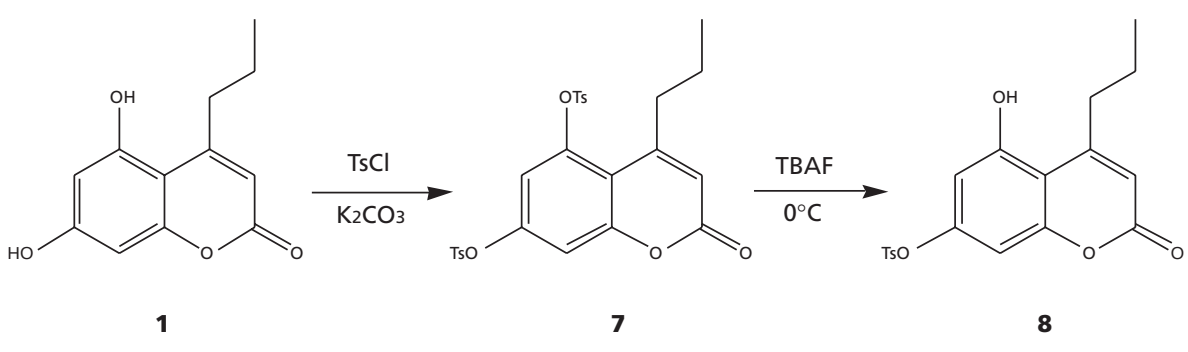
Table 1. Acylation of coumarin 1 with propionyl anhydride to form 7-monoester **2a**

Entry	Reaction conditions	HPLC Yield of **2a**
1	In THF at 0°C for 2 h	No reaction
2	In THF at 30°C for 2 h	No reaction
3	$AlCl_3$ (2 equivalents) in 1,2-dicholorethane at room temperature for 24 h	20%
4	Pyridine (3 drops) in THF at room temperature for 1.5 h	47%
5	Pyridine (3 drops) in THF at room temperature for 1 h	26%
6	4-dimethylaminopyridine (0.1 equivalents) in 1,2-dicholorethane at room temperature for 4 h	Complicated mixture
7	4-dimethylaminopyridine (0.1 equivalents) in 1,2-dicholorethane at 0°C for 45 min	47% isolated yield of **3a**
8	Propionyl chloride in pyridine at 0°C for 4 h	Complicated mixture

Scheme 1.



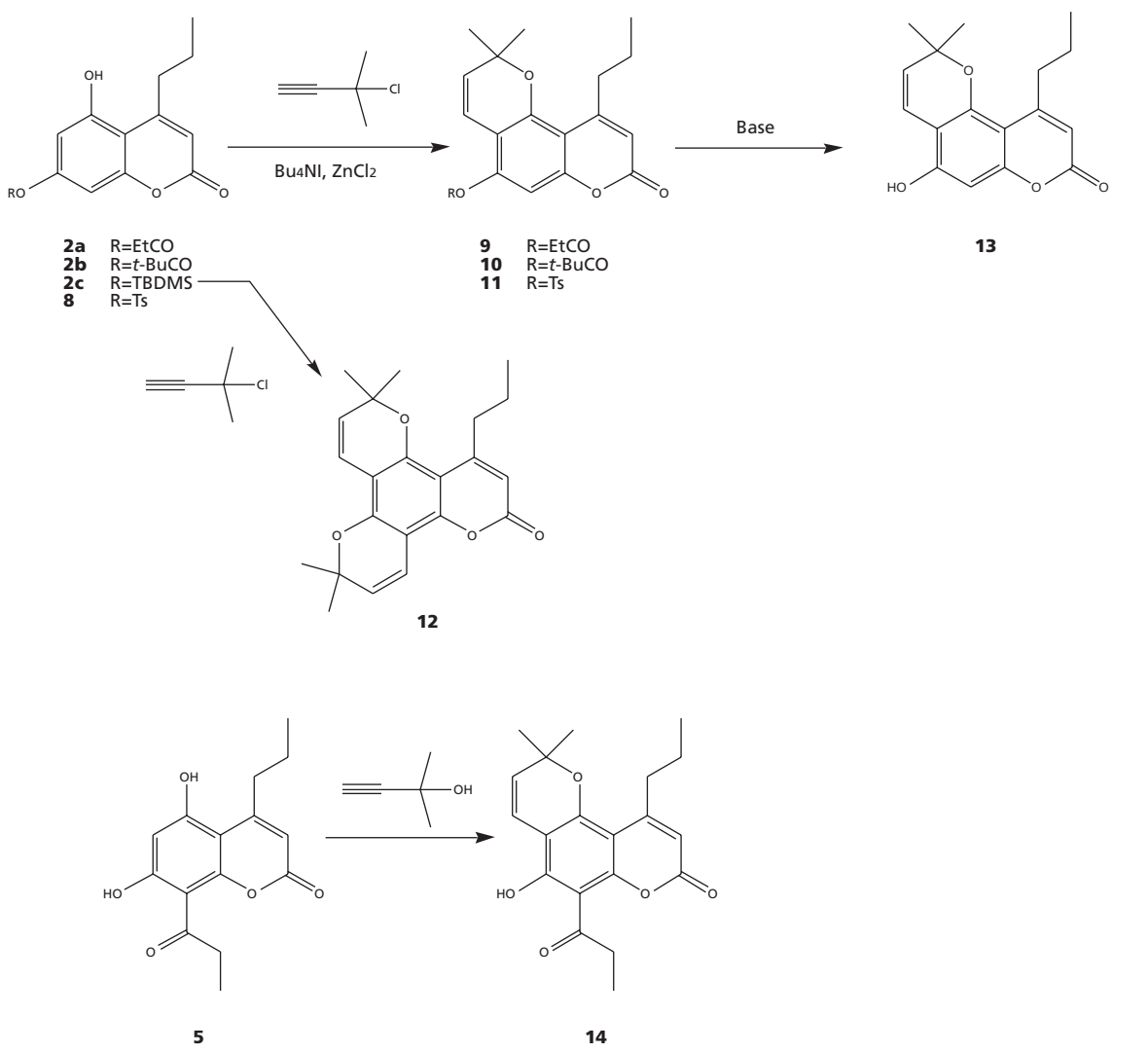
Scheme 2.



C-acylated coumarins **4**, **5**, and **6** were prepared using the method of Flavin *et al.* (1996) and shown in Scheme 1.

The tosylated compounds **7** and **8** were prepared according to the methods of Desai *et al.* (1956) and Fox *et al.* (2000) with some modifications. Thus, tosylation of **1** with tosyl chloride and potassium carbonate led to bistosylate **7** with a 90% yield. Treatment of **7** with 1.0 equivalent of TBAF under mild conditions afforded

Scheme 3.

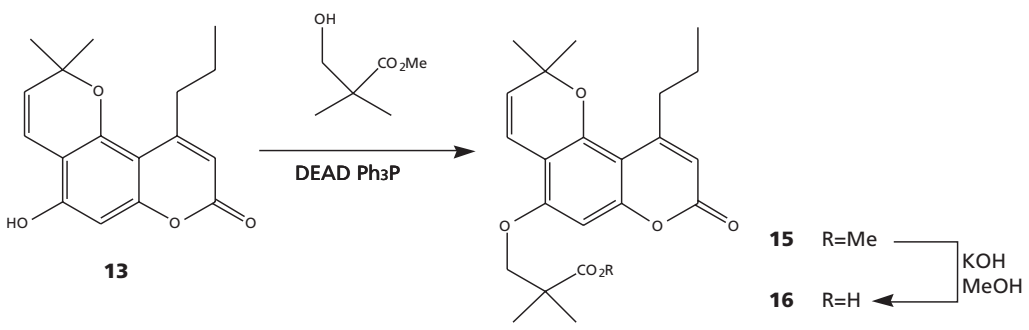


the corresponding monotosylate **8** with a 43% yield (Scheme 2).

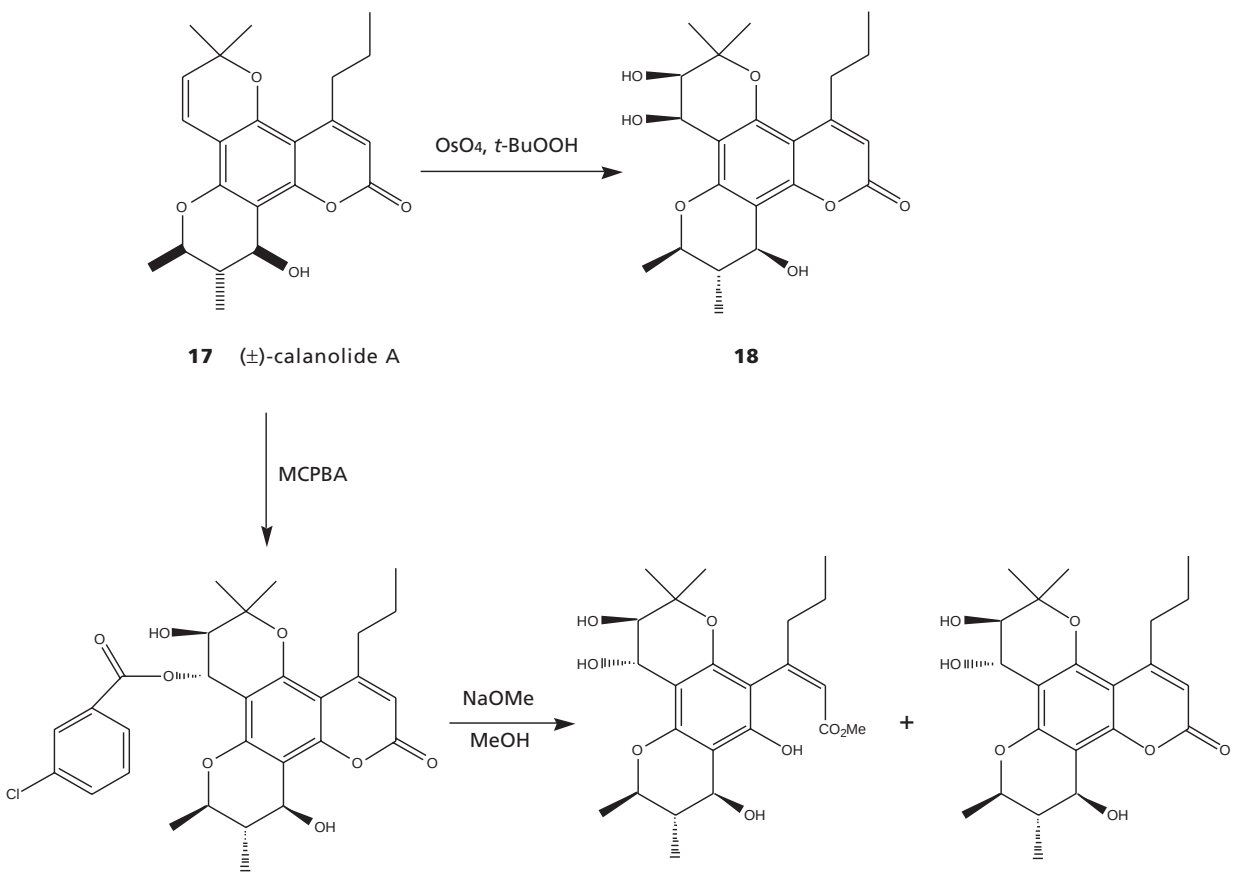
Chromenylation of **2a** was initially attempted employing 4,4-dimethyoxy-2-methylbutan-2-ol (Flavin *et al.*, 1996), but only approximately 5% of **9** was detected by ^{1}H NMR. However, when 3-chloro-3-methyl-1-butyne was used in the presence of zinc chloride and phase transfer catalyst tetrabutylammonium iodide (Chenera *et al.*, 1993; Bell *et al.*, 1995), **9** was obtained in a 27% isolated yield (Scheme 3). The same procedure on **2b** afforded **10** in an impressive 73% yield. Similarly, compound **11** (Fox *et al.*, 2000) was successfully synthesized from **8** by using 3-chloro-3-methyl-1-butyne and zinc chloride with a 50% yield. In contrast, when **2c** was reacted with 3-chloro-3-methyl-1-butyne under the same conditions, a tripyranone derivative **12** (Games *et al.*, 1971) was formed without detection of the corresponding dipyranone. The structure assignment of **12** was based on ^{1}H NMR and MS. This indicated that the TBMDS-protecting group was lost during the course of chromenylation. Compound **14** was prepared by reacting **5** with 4,4-dimethyoxy-2-methylbutan-2-ol as described by Flavin *et al.* (1996) (Scheme 3).

Compound **13** was obtained through hydrolysis of **9–11** under basic conditions. For example, conversion of **9** to **13** was uneventful with sodium bicarbonate in aqueous MeOH and **13** was obtained in a 44% yield (Scheme 3). Similarly, treatment of **11** with TBAF at 0°C afforded **13**

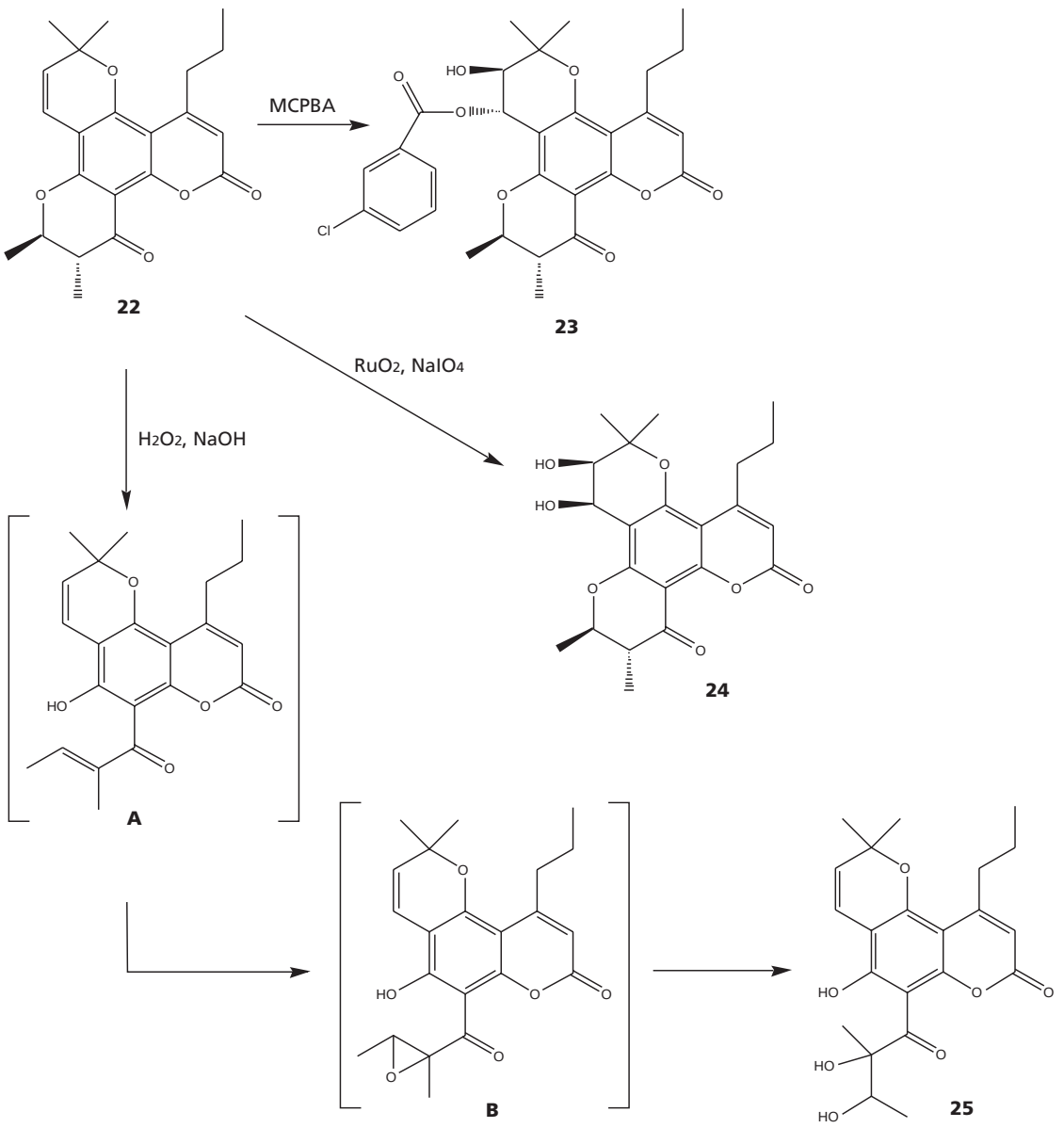
Scheme 4.



Scheme 5.



Scheme 6.



with a 43% yield. It is worthwhile to note that direct chromenylation of **1** with 4,4-dimethyoxy-2-methylbutan-2-ol resulted in a mixed product, with **13** being isolated in less than a 10% yield (Bell *et al.*, 1995).

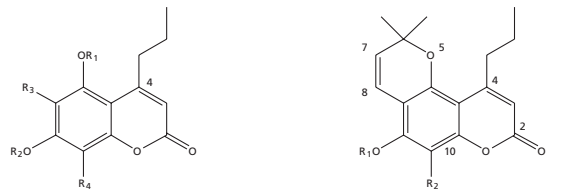
Alkylation of **13** with commercially available methyl 2,2-dimethyl-3-hydroxy propionate under Mitsunobu conditions furnished **15** with a 69% yield, which was then hydrolyzed to acid **16** under basic conditions with a 78% yield (Scheme 4).

The preparation of *cis*-isomer of 7,8-dihydroxy calanolide A (**18**) from (±)-canalolide A (**17**) was straightforward using OsO_4/tBuOOH. Separation of the diastereomeric mixture by chromatographic technique was unsuccessful. Treatment of (±)-canalolide A (**17**) with MCPBA afforded

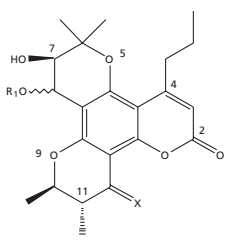
the hydroxyl benzoate **19** (Scheme 5). Conceivably, **19** was formed via the epoxide intermediate. Under the reaction conditions, exclusive opening of the epoxide at the benzylic position was observed. It was assumed that the hydroxyl group and the benzoate were *trans* to each other. Further evidence for this was obtained by the hydrolysis of **19** to give the 7,8-dihydroxy compound. Thus, hydrolysis of **19** with NaOMe in MeOH yielded the lactone-opened derivative **20** and the corresponding *trans*-7,8-dihydroxy calanolide A (**21**). Spectroscopic data of **21** were consistent with the 7,8-diol structure. The relative stereochemistry of the diol was assigned as *trans* as compared with the *cis*-isomer **18** obtained from stereospecific dihydroxylation of **17** using OsO_4 (*vide infra*).

Similarly, the MCPBA oxidation of racemic **22** led to the formation of *m*-chlorobenzoate **23** (Scheme 6). The preparation of *cis*-isomers of 7,8-dihydroxy *trans*-ketone **24** was successful using $RuO_2/NaIO_4$. Both products were fully characterized by ^1H and ^{13}C NMR, IR, mass spectroscopy, and elemental analysis. The assignment of *cis*-configuration of the two hydroxyl groups in **24** was based on the stereospecificity of the dihydroxylation by $RuO_2/NaIO_4$. Treatment of **22** with H_2O_2 in the presence of NaOH in methanol and methylene chloride produced a triol compound **25** with a low yield (13%) and a 65% recovery of the unreacted starting material **22**. The formation of **25** might have been involved with a retro-Michael addition of **22** under the basic conditions to form the intermediate **A** which was then epoxided by H_2O_2 followed by opening of the epoxide by NaOH (Scheme 6).

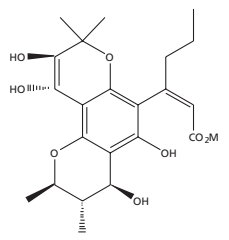
Figure 1. Structures of coumarin and pyranocoumarin analogues tested against measles virus



1 $R_1=R_2=R_3=R_4=H$
2a $R_1=R_3=R_4=H$, $R_2=CH_3CH_2CO$
3b $R_1=R_2=(CH_3)_3CCO$, $R_3=R_4=H$
4 $R_1=R_2=R_4=H$, $R_3=CH_3CH_2CO$
5 $R_1=R_2=R_3=H$, $R_4=CH_3CH_2CO$
6 $R_1=R_3=H$, $R_2=R_4=CH_3CH_2CO$
7 $R_1=R_2=p$-CH_3-$C_6H_4SO_2$, $R_3=R_4=H$
8 $R_1=R_3=R_4=H$, $R_2=p$-CH_3-$C_6H_4SO_2$
9 $R_1=CH_3CH_2CO$, $R_2=H$
10 $R_1=(CH_3)_3CCO$, $R_2=H$
11 $R_1=p$-CH_3-$C_6H_4SO_2$, $R_2=H$
13 $R_1=R_2=H$
14 $R_1=H$, $R_2=CH_3CH_2CO$
15 $R_1=CH_2C(CH_3)_2CO_2CH_3$, $R_2=H$
16 $R_1=CH_2C(CH_3)_2CO_2H$, $R_2=H$
25 $R_1=H$, $R_2=CH_3CH(OH)C(OH)(CH_3)CO$



18 7,8-*cis*-11,12-*trans*, $R_1=H$, X=OH
21 7,8-*trans*-11,12-*trans*, $R_1=H$, X=OH
23 7,8-*trans*, $R_1=m$-Cl-C_6H_4CO, X=O



20

Evaluation of coagulation inhibitory activity

Compounds **7**, **9** and **18**, at 10 or 1 μg/ml, were evaluated in two assays to measure the anticoagulant properties of these compounds in normal human plasma. These concentrations bracketed the EC_{50} values for these compounds. Sera from five patients were pooled and aliquots were mixed with each compound and tested in a prothrombin timing test or in a partial thrombin timing test (aPTT). The baseline values for the normal plasma was 12.4 s and for each compound the values ranged from 12.3–12.5 s, regardless of the dilution of compound tested. In the aPTT test, the baseline level for the normal plasma was 26.4 s and the values for the compounds ranged from 26.0 to 26.4 s, regardless of dilution. Thus, none of the compounds tested were found to inhibit *in vitro* coagulation at the concentrations tested.

Results: virology

Primary characterization of antiviral activity

Twenty-two coumarin and pyranocoumarin derivatives (Figure 1), including the enantiomerically pure (+)-calanolide A [(+)-**17**] and calanolide ketone (+)-**22** (Flavin *et al.*, 1996; Khilevich *et al.*, 1996), were tested against MV strain Chicago. Of the compounds evaluated, six were considered active as determined by visual assay and verified by neutral red assay of the same test plate (Table 2). The EC_{50} values ranged from 0.2–10 μg/ml by visual assay and 0.3–5 μg/ml by neutral red assay. Three of those were propyl coumarin analogues (**2a**, **7** and **8**), two were benzodipyranone with a coumarin nucleus (**9** and **10**) and one was a benzotripyranone with a coumarin nucleus (**18**). Sixteen of the 22 compounds tested were not active (Table 3), because their selective indices, using both assay methods, were less than 10. Further characterization of the MV-inhibitory compounds, by virus yield reduction assay, revealed that compounds **10** and **18**, as measured in virus yield reduction assay, were the most potent inhibitors of MV replication (Table 2). This antiviral activity was also independent of virucidal activity, which was seen only at high concentrations of compound **10**. Of the active drugs tested, **7** and **8** appeared to be more toxic in actively growing cells; whereas compounds **9** and **18** appeared to be less

Table 2. Inhibition of strain Chicago-1 measles virus replication in African green monkey kidney (CV-1) cells by coumarin analogues

	Visual assay			Neutral red assay			Virus yield reduction assay	Virucidal assay	Cell cytotoxicity*
Compound	EC_{50} (μg/ml)	IC_{50} (μg/ml)	SI	EC_{50} (μg/ml)	IC_{50} (μg/ml)	SI	EC_{90} (μg/ml)	Lowest virucidal concentration (μg/ml)	IC_{50} (μg/ml)
2a	2	90	45	1	100	100	40	>100†	32
7	0.5	10	20	1	10	10	1	>10	1
8	1	10	10	1	10	10	3	>100	3
9	0.4	6	15	0.3	10	33	2	ND	>100
10	0.2	3	15	0.6	2	3	0.3	40	10
18	10	>100	>10	5	50	10	0.3	>10	41
Ribavirin	25	270	11	20	196	10	2	>1000	33

*Cytotoxicity was determined in actively growing cells by neutral red assay.
†Concentrations higher than those shown were not tested.
ND ; not determined.

Table 3. Coumarin analogues weakly selective* or not inhibiting strain Chicago-1 measles virus replication in African green monkey kidney cells (CV-1)

	Visual assay			Neutral red assay		
Compound	EC_{50} (μg/ml)	IC_{50} (μg/ml)	SI	EC_{50} (μg/ml)	IC_{50} (μg/ml)	SI
1	>100†	10	0	>100	>100	0
3b	2	12	6	3	10	3
4	3	7	2	1	6	6
5	20	10	0	>100	20	0
6	>100	80	0	>100	100	0
11	10	20	2	>100	>100	0
13	0.5	3	6	0.6	2	3
14	20	80	4	>100	100	0
15	40	20	0	30	25	0
16	>100	10	0	>100	20	0
(+)-**17**	>100	>100	0	>100	>100	0
20	10	15	1	20	20	1
21	10	> 100	>10	100	5	0
(+)-**22**	3	10	3	9	14	1
23	3	7	2	0.4	2	5
25	20	20	1	40	20	0

*Defined as a compound with a selective index of ≤10 μg/ml.
†Concentrations higher than those values shown were not tested.

toxic in these cells (Table 2). Ribavirin inhibited virus as expected with no virucidal effects seen at the concentrations tested.

Other measles virus strains

Some of the more readily available, active drugs were tested against other measles virus strains (Table 4). Compounds **7** and **8** appeared to inhibit all strains equally effectively. However, **10** did not appear to inhibit strain MO6, an African isolate, although it did inhibit all other strains with similar potency. Interestingly, compound **18** was not a potent inhibitor

Table 4. Inhibition of the replication of various strains of measles virus in African green monkey kidney (CV-1) cells by selected active compounds

	7		**8**		**10**		**18**		Ribavirin	
	Visual assay	Neutral red assay	Visual assay	Neutral red assay	Visual assay	Neutral red assay	Visual assay	Neutral red assay	Visual assay	Neutral red assay
Virus Strain	EC_{50} (μg/ml)	EC_{50} (μg/ml)	EC_{50} (μg/ml)	EC_{50} (μg/ml)	EC_{50} (μg/ml)	EC_{50} (μg/ml)	EC_{50} (μg/ml)	EC_{50} (μg/ml)	EC_{50} (μg/ml)	EC_{50} (μg/ml)
Bil	1	0.4	0.4	0.1	0.2	0.1	1	0.9	10	2.7
CC	1	2	0.3	1	1	2	15	4	80	250
Chicago	3	1	1	1	0.2	0.6	10	5	20	25
Edmonston	3	5	1	0.4	2	0.3	20	20	20	10
MO2	4	3	0.6	7	4	1	20	50	20	26
MO6	2	0.5	0.6	0.6	>100*	>100	10	5	100	100
NS2	3	1	0.6	0.5	3	0.2	5	0.5	10	1
Sa	0.3	0.5	0.1	0.2	4	0.6	1	5	20	60
TN 1994	3	ND	0.1	ND	0.1	ND	3	ND	30	ND
X-1108	2	6	0.6	0.3	3	3.6	12	10	100	130

*Concentrations higher than those shown were not tested.
ND, not determined.

of most of the virus strains used when compared to the other compounds in Table 4, which correlated with the results described above using the Chicago strain.

Effects of multiplicity of infection

The effects of various MOIs were determined with **7** (most readily available) against strain Chicago. The potency of this compound was slightly MOI-dependent by both visual and NR methods (Table 5). The EC_{50} concentration rose slightly as the concentration of virus increased, although by visual assay a titre was not calculable at the higher concentrations tested. Ribavirin also showed a similar pattern, a slight increase in the inhibition concentration as the MOI increased, using both visual and NR assays.

Preliminary mode of action studies

To better understand at which part of the virus replication cycle these compounds were most efficacious, a timing assay was done using an MOI as high as possible (MOI = 0.04) with strain Chicago to approach a scenario in which there is a simultaneous infection of as many cells as possible. Using such a format, two of the drugs (**7** and **9**) were evaluated along with ribavirin in a timing assay. The compounds were added at various times before or after virus infection or near simultaneously as in the typical antiviral assay. The EC_{50} values derived from this experiment are shown in Table 6. For both coumarin analogues, the time of addition did not appear to affect their potency. However, ribavirin was somewhat less inhibitory when added at 24 h post virus exposure than when added simultaneously or at early times after virus exposure.

Drug combination studies

It was interesting to determine how one of the coumarin analogues would interact with ribavirin when used in combination with it in antiviral experiment versus MV. Varying concentrations of **7** were tested alone and in combination with varying concentrations of ribavirin. The peaks of synergism or antagonism, as ascertained by the MacSynergy (computer program), were used as indicators of drug interactions. The anti-MV activity of ribavirin was not augmented by any significant degree when used in

Table 5. Effects of the multiplicity of infection on the inhibition of measles virus (strain CC) replication in African green monkey kidney cells (CV-1) by **7** and ribavirin

	7		Ribavirin	
	EC_{50}		EC_{50}	
MOI	Visual assay (μg/ml)	Neutral red assay (μg/ml)	Visual assay (μg/ml)	Neutral red assay (μg/ml)
0.04	>10*	10	25	25
0.02	>10	10	20	20
0.01	10	6	25	25
0.005	10	10	20	20
0.002	6	10	20	15
0.001†	1	6	10	10
0.0004	1	6	15	15

*Denotes that a concentration greater than indicated was not evaluated.
†Multiplicity of infection (MOI) used in normal antiviral test.

Table 6. The effects of time of addition of **7** and **9** on measles virus (Chicago) replication in (CV-1) cells

Time of addition of compound	**7**		**9**		Ribavirin	
	Visual assay	Neutral red assay	Visual assay	Neutral red assay	Visual assay	Neutral red assay
–1*	0.5	3	0.7	2	10	5
0†	0.1	0.1	1	1	5	2
1‡	0.2	<0.1§	0.4	1	3	10
2	0.5	1	1	0.5	10	10
4	0.5	3	1	1	10	5
6	0.5	3	1	0.5	5	5
8	0.5	1	1	0.5	4	10
24	0.5	3	2	2	20	20

*Compound was added to the cells 1 h prior to exposure to virus. At time 0, virus was added at a multiplicity of infection=0.04 to the compound-treated cells and incubated with the cells until the assay was ended.
†An equal volume of virus was added to dilutions of compound on cells within 5 min of compound addition to cells.
‡After exposure of cells to virus for one of the time periods (1–24 h) indicated, an appropriate concentration of was added to the virus-infected cells and incubated with those cells until the assay ended.
§Concentrations lower than shown were not tested.

combination with **7** (Figure 2a). This was also verified by NR assay (data not shown). In contrast, there was some apparent antagonism detected at the highest concentration of ribavirin used in combination with most concentrations of **7** used. The effects on cytotoxicity followed a similar pattern of antagonism, although this amelioration of ribavirin toxicity was relatively minor (Figure 2b).

Discussion

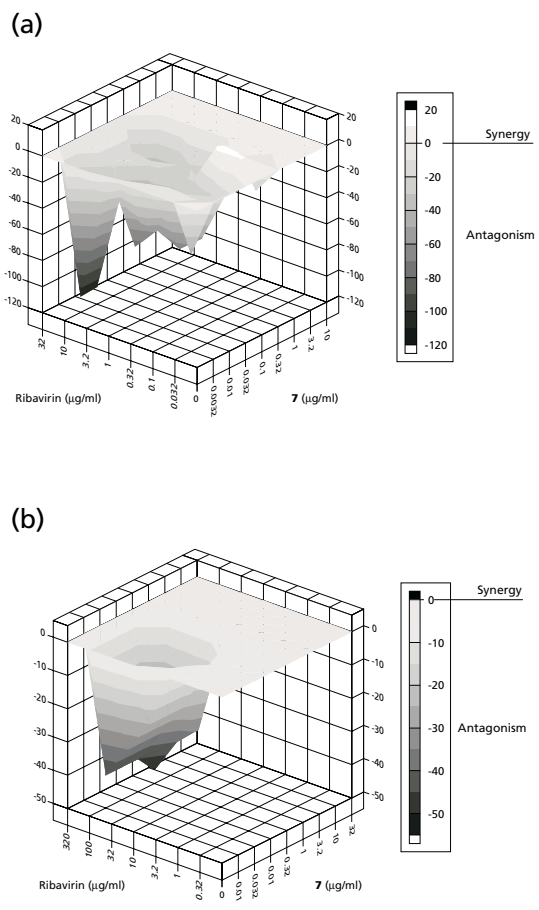
Coumarin-type compounds have previously been shown to inhibit tumours and viruses (McKee *et al.*, 1996; Pengsuparp *et al.*, 1999; Semple *et al.*, 1999). Thus, these lead compounds have stimulated interest in the synthesis of more potent, less toxic analogues. This study was part of a larger project to determine the virus spectrum inhibited by this set of coumarin analogues. In addition to the evaluation of MV inhibition shown in Tables 2 and 3, the 22 coumarins were also screened against other viruses selected from the following families: *Arenaviridae*, *Adenoviridae*, *Bunyaviridae*, *Flaviviridae*, *Orthomyxoviridae* (influenza viruses A and B), *Paramyxoviridae* (parainfluenza 3 virus and respiratory syncytial virus) and the *Togaviridae*. None of the compounds were active against these viruses, with exception of **7** which exhibited some activity against rhinoviruses (data not shown). Thus, the results suggest that most of the compounds shown to be active against MV appear to be specific inhibitors of MV replication.

When the active analogues were evaluated against other MV strains, all other strains were also potently inhibited with the exception of one isolate from Africa, MO6, which was not sensitive to inhibition by **10** (Table 4). Thus, the compounds have inhibited all measles viruses tested to date. The spectrum of viruses strains inhibited and the potency of the inhibition appeared to be independent of any particular clade system or genetic grouping system developed for MV (Bellini & Rota, 1998; Rota *et al.*, 1998; Takada *et al.*, 1999).

The mode of action of these compounds remains unclear. The virucidal studies strongly suggest that the compounds do not simply degrade the virus when it is exposed to the analogues. In timing studies, virus was inhibited at any time the compound (**7** or **9**) was added, pre- or postvirus exposure (Table 5). However, because the MOI (0.01) used may not have statistically achieved a condition in which every cell was exposed to virus simultaneously, it may be that the compounds could have simply prevented the spread of virus by blocking attachment or penetration of any infectious virus released through multiple cycles of virus replication conducive to a low MOI experiment. The coumarin analogues, as represented by **7**, did not appear to act synergistically when combined with ribavirin against MV replication; at the highest concentration evaluated, ribavirin seemed to antagonize the inhibition of MV replication by coumarin **7** when they were used together in a combination therapy. Taken all together, the results could suggest that the compounds did not inhibit the same part of the replication cycle of MV that ribavirin does; for example, perhaps they do not act as mutagens for viral RNA (Crotty *et al.* 2001).

Preliminary analysis of structural anti-MV activity relationship on the 22 compounds tested revealed at least four characteristics (Figure 1). (1) The unsubstituted or C-substituted 5,7-dihydroxycoumarins such as **1**, **4**, **5** and **6** were generally inactive against MV, with compound **4** being somewhat active (EC_{50} of 1 mg/ml in neutral red assay). (2) The O-substituted 5,7-dihydroxycoumarins such as **2a**, **7** and **8** were generally active against MV. Compound **2a** was relatively less active but also less toxic. (3) In the benzodipyranone (or chromeno-coumarin) system, 10-C-substitution was detrimental to the anti-MV activity (**14** and

Figure 2. (a) The effects of the combination of (**7**) and ribavirin on the replication of MV in African green monkey kidney cells (CV-1). (b) The cytotoxic effects of the combination of (**7**) and ribavirin on African green monkey kidney cells (CV-1)



25 versus **13**). The 9-O-substitution had a critical impact on the anti-MV activity of benzodipyranone compounds. For example, the 9-O-unsubstituted compound **13**, with a free OH group, was active against MV but was relatively toxic as well, with an SI ranging from 3 to 6. The 9-O-acylated derivatives (**9** and **10**) maintained the anti-MV of **13** but with decreased toxicity. The SI of **9** was increased from 15 to 33. However, the 9-O-sulfonate **11** and the alkylated analogues **15** and **16** appeared to be detrimental. One may consider that **13** was the actual biologically active agent responsible for both the anti-MV activity as well as the toxicity seen. The 9-O-acylated **9** and **10** may have exerted their anti-MV activity after being hydrolyzed to the active form **13** during the assay process and these esters may be less toxic than the parent **13**. This hypothesis may be supported by the following observations. First, compound **10** is more toxic than **9**. It may not be surprising considered that the pivaloyl group in **10** may be easier, compared with the acetyl group in **9**, to be hydrolyzed chemically or enzymatically, resulting in a quicker release of **13** and the toxicity observed, while release of **13** from **9** may be in a slower and more controlled fashion. Second, when the 9-O-position of **13** is substituted by a non-cleavable group (**15** and **16**) or by a sulfonyl group which is relatively difficult in hydrolysis, its anti-MV activity was greatly reduced. (4) For the benzotripyranone compounds, the hydroxyl group(s) at the 7- and/or 8- position is essential for anti-MV activity [**18** versus (+)-**17**, and **23** versus (+)-**22**]. Furthermore, the stereochemistry of the 7,8-dihydroxyl groups in the hydroxylated benzotripyranones may play an important role in the anti-MV activity. For instance, the *cis*-dihydroxyl compound **18** is active while the corresponding *trans*-isomer **21** is not and neither is **20** with the two hydroxyl groups being *trans* to each other and the coumarin ring open. It is interesting to note that compound **23** is active against MV even though the hydroxyl and benzoate groups are *trans* to each other. However, **23** is relatively toxic resulting in an SI of only 2 to 5. It is also worth noting that both **18** and **23** are a diastereomeric mixture containing a total of four different stereoisomers. One may assume that the pure form of a stereoisomer should be at least fourfold more active than **18** or **23** if only one isomer is accounted for the activity observed. Confirmation of this hypothesis awaits the separation and test of these stereoisomers, which is currently under way.

In summary, the coumarin and pyranocoumarin analogues evaluated in this study represent a new class of compounds that have been found to be active against MV. They appear to be rather specific inhibitors of MV and inhibit all strains of MV tested to date. Thus, these compounds may be valuable leads in the development of a potent and selective class of MV inhibitors that could be used in future in the clinic.

Acknowledgements

This work was supported in part by contract NO1-AI-85348, Virology Branch (Program Officer: Dr Christopher KH Tseng), National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH), Bethesda, Maryland; and by Sarawak MediChem Pharmaceuticals Inc, Woodridge, Illinois. We also thank Dr Kyaw Htin at the Analytical Department of MediChem Research for his excellent assistance with NMR spectrum analysis.

References

Afzal MA, Minor PD & Schild GC (2000) Clinical safety issues of measles, mumps and rubella vaccines. *Bulletin of the World*

Health Organisation **78:**199–204.

Barnard DL, Bischofberger N, Kim CU, Huffman JH, Sidwell RW, Dougherty JP, Lew W, Williams MA & Yang W (1997a) Acyclic phosphonomethylether nucleoside inhibitors of respiratory viruses. *Antiviral Chemistry & Chemotherapy* **8:**223–233.

Barnard DL, Hill CL, Gage TL, Matheson JL, Huffman JH, Sidwell RW, Otto MJ & Schinazi RF (1997b) Potent inhibition of respiratory syncytial virus by polyoxometalates of several structural classes. *Antiviral Research* **34:**27–37.

Barnard DL, Sidwell RW, Xiao W, Player MR, Adah S & Torrence PF (1999) 2-5A-antisense inhibition of respiratory syncytial virus replication: Effects of oligonucleotide structure modifications and RNA target site selection. *Antiviral Research* **41:**119–134.

Barnard DL, Stowell VD, Seley KL, Hegde VR, Das SR, Rajappan VP, Schneller SW, Smee DF & Sidwell RW (2001) Inhibition of measles virus replication by 5'-nor carbocyclic adenosine analogues. *Antiviral Chemistry & Chemotherapy* **12:**221–230.

Bell D, Davies MR, Geen GR & Mann IS (1995) Copper(I) iodide: a catalyst for the improved synthesis of aryl propargyl ethers. *Synthesis* 707–712.

Bellini WJ & Rota PA (1998) Genetic diversity of wild-type measles viruses: implications for global measles elimination programs. *Emerging Infectious Diseases* **4:**29–35.

Bourinbaiar AS, Tan X & Nagorny R (1993) Inhibitory effect of coumarins on HIV-1 replication and cell-mediated or cell-free viral transmission. *Acta Virologica* **37:**241–250.

Cavenaugh PR Jr, Moskwa PS, Donish WH, Pera PJ, Richardson D & Andrese AP (1990) A semi-automated NR red based chemosensitivity assay for drug screening. *Investigational New Drugs* **8:**347–354.

Chenera B, West ML, Finkelstein JA & Dreyer GB (1993) Total synthesis of (±)-calanolide A, a non-nucleoside inhibitor of hiv-1 reverse transcriptase. *Journal of Organic Chemistry* **58:**5605–5606.

Clements CJ & Cutts FT (1995) The epidemiology of measles: thirty years of vaccination. *Current Topics in Microbiology & Immunology* **191:**13–33.

Creagh T, Ruckle JL, Tolbert DT, Giltner J, Eizenhamer DA, Dutta B, Flavin MT & Xu ZQ (2001) Safety and pharmacokinetics of single doses of (+)-calanolide a, a novel, naturally occurring nonnucleoside reverse transcriptase inhibitor, in healthy, human immunodeficiency virus-negative human subjects. *Antimicrobial Agents & Chemotherapy* **45:**1379–1386.

Crotty S, Cameron CE & Andino R (2001) RNA virus error catastrophe: direct molecular test by using ribavirin. *Proceedings of the National Academy of Sciences USA* **98:**6895–6900.

Desai RD & Parghi JV (1956) Heterocyclic compounds. Part XXXI. The synthesis of partially methylated ethers of polyhydroxy coumarins. *Journal of the Indian Chemical Society* **33:**661–664.

De Serres G, Gay NJ & Farrington CP (2000) Epidemiology of transmissible diseases after elimination. *American Journal of Epidemiology* **151:**1039–1048.

Flavin MT, Rizzo JD, Khilevich A, Kucherenko A, Sheinkman AK, Vilaychack V, Lin L, Chen W, Greenwood EM, Pengsuparp T, Pezzuto JM, Hughes SH, Flavin TM, Cibulski M, Boulanger WA, Shone RL & Xu ZQ (1996) Synthesis, chromatographic resolution and anti-HIV activity of (±)-calanolide A and its enantiomers. *Journal of Medicinal Chemistry* **39:**1303–1313.

Fox ME & Meek GA (2000) Process for the preparation of calanolide precursors. WO0064902.

Games DE & Haskins NJ (1971) Synthesis of some dimethylpyrano- and 3-methylbut-2-enyl-4-phenyl- and –4-n-propyl-coumarins. *Journal of the Chemical Society, Chemical Communications* 1005–1006.

Gay NJ (2000) Eliminating measles-no quick fix. *Bulletin of the World Health Organization* **78:**949.

Griffin, DE & Bellini WJ (1996) Measles virus. In *Virology*, vol. 3; pp. 1267–1312. Edited BN Fields, DM Knipe & PM Howley. Philadelphia: Lippincott-Raven.

Gururangan S, Stevens RF & Morris DJ (1990) Ribavirin response in measles pneumonia. *Journal of Infection* **20:**219–221.

Josephy PD & Damme AV (1984) Reaction of Gibbs reagent with para-substituted phenols. *Analytical Chemistry* **56:**813–814.

Kawashima H, Mori T, Kashiwagi Y, Takekuma K, Hoshika A & Wakefield A (2000) Detection and sequencing of measles virus from peripheral mononuclear cells from patients with inflammatory bowel disease and autism. *Digestive Diseases & Sciences* **45:**723–729.

Khilevich A, Mar A, Flavin MT, Rizzo JD, Dzekhtser S, Brankovic D, Lin L, Zhang H, Chen W, Liao S, Zembower DE & Xu ZQ (1996) Synthesis of (+)-calanolide A, An anti-HIV agent, *via* enzyme-catalyzed resolution of the aldol products. Tetrahedron: *Asymmetry* **7:**3315–3326.

Manchester M, Naniche D & Stehle T (2000) CD46 as a measles receptor: form follows function. *Virology* **274:**5–10.

Marttila J, Hinkkanen A, Ziegler T, Vainionpaa R, Salmi A & Ilonen J (2001) Cell membrane-associated measles virus components inhibit antigen processing. *Virology* **279:**422–428.

Martyanov IV, Zakharova OD, Sottofattori E, Pyshnyi DV, Yurchenko EY, Babbi P, Mazzei M, Balbi A, Andreola ML, Litvak S, Tarrago-Litvak L & Nevinsky GA (1999) Interaction of oligonucleotides conjugated to substituted chromones and coumarins with HIV-1 reverse transcriptase. *Antisense & Nucleic Acid Drug Development* **9:**473–480.

McKee TC, Fuller RW, Covington CD, Cardellina JH 2nd, Gulakowski RJ, Krepps BL, McMahon JB & Boyd MR (1996) New pyranocoumarins isolated from Calophyllum lanigerum and Calophyllum teysmannii. *Journal of Natural Products* **59:**754–758.

Pengsuparp T, Serit M, Hughes SH, Soejarto DD & Pezzuto JM (1999) Specific inhibition of human immunodeficiency virus type 1 reverse transcriptase mediated by soulattrolide, a coumarin isolated from the latex of *Calophyllum teysmannii*. *Journal of Natural Products* **59:**839–842.

Prichard MN & Shipman C Jr (1990) A three-dimensional model to analyze drug-drug interactions. *Antiviral Research* **14:**181–206.

Rota JS, Rota PA, Redd SB, Redd SC, Pattamadilok S & Bellini WJ (1998) Genetic analysis of measles viruses isolated in the United States, 1995-1996. *Journal of Infectious Diseases* **177:**204–208.

Sekiguchi J, Stivers JT, Mildvan AS & Shuman S (1996) Mechanism of inhibition of vaccinia DNA topoisomerase by novobiocin and coumermycin. *Journal of Biological Chemistry* **271:**2313–2322.

Semple SJ, Nobbs SF, Pyke SM, Reynolds GD & Flower RL (1999) Antiviral flavonoid from *Pterocaulon sphacelatum*, an Australian Aboriginal medicine. *Journal of Ethnopharmacology* **68:**283–288.

Skulnick HI, Johnson PD, Aristoff PA, Morris JK, Lovasz KD, Howe WJ, Watenpaugh KD, Janakiraman MN, Anderson DJ, Reischer RJ, Schwartz TM, Banitt LS, Tomich PK, Lynn JC, Horng M-M, Chong K-T, Hinshaw RR, Dolak LA, Seest EP, Schwende FJ, Rush BD, Howard GM, Toth LN, Wilkinson KR, Kakuk TJ, Johnson CW, Cole SL, Zaya RM, Zipp GL, Possert PL, Dalga RJ, Zhong W-Z, Williams MG & Romines KR (1997) Structure-based design of nonpeptidic HIV protease inhibitors: the sulfonamide-substituted cyclooctylpyramones. *Journal of Medicinal Chemistry* **40:**1149–1164.

Steingart KR, Thomas AR, Dykewicz CA & Redd SC (1999) Transmission of measles virus in healthcare settings during a community wide outbreak. *Infection Control & Hospital Epidemiology* **20:**1195–1199.

Stogner SW, King JW, Black-Payne C & Bocchini J (1993) Ribavirin and intravenous immune globulin therapy for measles pneumonia in HIV infection. *Southern Medical Journal* **86:**1415–1418.

Takeda M, Sakaguchi T, Li Y, Kobune F, Kato A & Nagai Y (1999) The genome nucleotide sequence of a contemporary wild strain of measles virus and its comparison with the classical Edmonston strain genome. *Virology* **256:**340–350.

Wyde PR, Moore-Poveda DK, De Clercq E, Neyts J, Matsuda A, Minakawa N, Guzman E & Gilbert BE (2000) Use of cotton rats to evaluate the efficacy of antivirals in treatment of measles virus infections. *Antimicrobial Agents and Chemotherapy* **44:**1146–1152.

Xie L, Takeuchi Y, Cosentino LM & Lee KH (1999) Anti-AIDS agents. 37. Synthesis and structure-activity relationships of (3′R,4′R)-(+)-cis-khellactone derivatives as novel potent anti-HIV agents. *Journal of Medicinal Chemistry* **42:**2662–2672.

Xie L, Takeuchi Y, Cosentino LM, McPhail A & Lee KH (2001) Anti-AIDS agents. 42. Synthesis and anti-HIV activity of disubstituted (3′R,4′R)-3′,4′-di-O-(S)-camphanoyl-(+)-cis-khellactone analogues. *Journal of Medicinal Chemistry* **44:**664–671.

Yang EB, Zhao YN, Zhang K & Mack P (1999) Daphnetin, one of coumarin derivatives, is a protein kinase inhibitor. *Biochemical and Biophysical Research Communications* **260:**682–685.

Zhao H, Neamati N, Hong H, Mazumder A, Wang S, Sunder S, Milne GW, Pommier Y & Burke TR Jr (1997) Coumarin-based inhibitors of HIV integrase. *Journal of Medicinal Chemistry* **40:**242–249.

Received 21 January 2002; accepted 4 April 2002