JPP Journal of Pharmacy and Pharmacology

JPP 2004, 56: 1163–1170

Received May 4, 2004
Accepted June 15, 2004
DOI 10.1211/0022357044201
ISSN 0022-3573

Cytotoxicity of 3,4-dihalogenated 2(5H)-furanones

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Abstract

Mucohalogen acids have been used for the preparation of a variety of 3,4-dihalogenated 2(5H)-furanones. In one synthetic step the carbamates **2a–c** and the pseudoanhydrides **4a–e** were prepared using isocyanates and acid anhydrides. A series of 5-alkoxylated 3,4-dichloro-2(5H)-furanones **5a–o** have been synthesized with a wide range of lipophilicity, using the hydroxy-form of mucohalogen acids **1a** and **1b**. The 5-allyl-3,4-dichloro-2(5H)-furanone **5f** was derived into the dihydro-isoxazol **6** and the oxirane **7**. The methyl ester **5a** was converted with ammonia into the tetramic acid chloride **11**. The pseudo acid chloride **3** was reacted further into the bis aziridine **8**. Reduction of the mucochloric acid **1a** furnished the trichlorofuranone **3**. The cytotoxicity of these simple and bis-cyclic butenolides have been evaluated in tissue culture on MAC13 and MAC16 cancer cell lines using the MTT cytotoxicity assay. The ester **5g**, the acetate **4b** and the carbamate **2b** displayed a cytotoxicity in the low micromolar range. Further, an IC50 (50% inhibitory concentration) of 50 nM and 30 nM was determined for the epoxide **7** and the aziridine **8**.

Introduction

Many cancer patients have metastatic disease at diagnosis and cannot be cured by modern cancer treatment, though there are tumours (e.g., tumour of the testes, choriocarcinomas and Hodgkin's disease) that are now curable even at an advanced stage. Some other tumours (e.g., in the lung, breast and prostate) may show considerable benefit from chemotherapy or hormonal manipulation.

There are highly potent anti-cancer drugs in clinical use, such as docetaxel (Querolle et al 2003), epirubicin (Ceruti et al 1999) and the Vinca alkaloids (Fathy et al 2002) just to name a few, which are all derived from natural products. On the other side, there are established purely synthetic antineoplastic agents (Figure 1) such as chlorambucil and cyclophosphamide (Rang & Dale 1994), which contain chemically reactive chlorine (Mutschler 1996). TEM (Sosnovsky et al 1986), a reactive aziridine, has structural similarity to the biscyclic furanones reported here and they are of a higher therapeutic value than N-nitroso derivatives (Reynolds et al 2000). Platinum compounds, which are in clinical use for first-line treatment of certain forms of cancer, also contain 2 chlorine atoms and have a structural similarity to our novel dihalogenated furanones (Ayuko & Lattmann 1999).

Penicillic acid (Black 1966) and basidalin (Hiyama et al 1987) are butenolide natural products (Coombs et al 1998) and exhibit anti-tumour activity in the millimolar range. Butalactin is an antibiotic and antineoplastic agent containing an epoxide side chain (Franco et al 1991).

Here, we wish to report our findings on the synthesis of 3,4-dihalogenated 5-substituted 2(5H)-furanones (Lattmann & Hoffmann 1996; Hoffmann et al 1996), and their in-vitro evaluation. The cytotoxicity of dihalogenated butenolides (Harcken & Brückner 1997) was determined in the Mossmann cytotoxicity assay.

Materials and Methods

Chemistry

Spectra were obtained as followed. Atmospheric pressure chemical ionisation mass spectrometry (APCI-MS) was carried out on a Hewlett-Packard 5989B quadrupole

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Funding: The authors would like to thank Kanvir Research LTD for financial support.

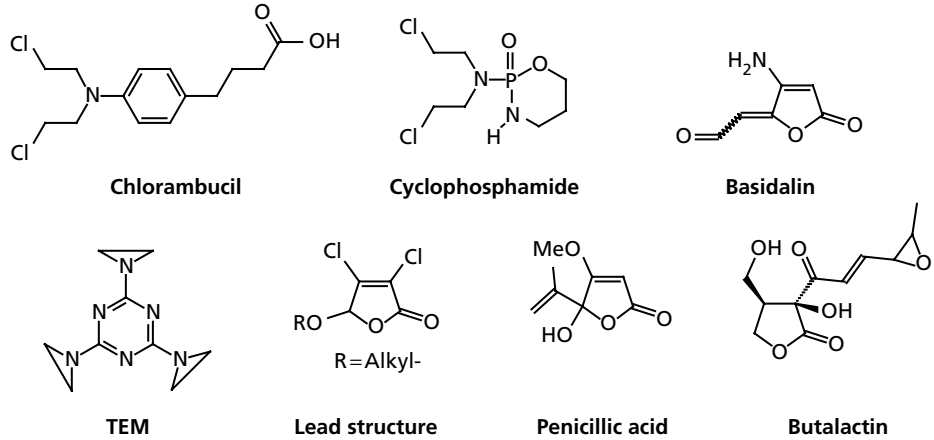

Figure 1 Selected anti-cancer agents.

instrument connected to an APCI accessory. IR spectra were recorded as KBr discs on a Mattson 3000 FT-IR spectrophotometer. Resonance spectra were obtained on a Bruker AC 250 instrument operating at 250 MHz (^{1}H NMR) and 62.5 MHz (^{13}C NMR), with TMS as internal standard.

Synthesis of carbamates

5-a-Naphthylcarbamyl-3,4-dichloro-2(5H)-furanone ***2b.*** 1-Naphthylisocyanate (5 g, 29 mmol) and mucochloric acid (Jähnisch & Duczek 1990) were refluxed in benzene (10 mL) for 48 h. The hot solution was diluted with two volumes of hexane and cooled to room temperature to give yellow crystals. The crude product was recrystallised from ethanol to furnish the desired carbamate (mp 127°C) as a white powder (6.39 g, 65%)

MW: 338, APCI$^+$: 339, 323; ^{1}H NMR ($CDCl_3$): $\delta = 8.80$ (s, 1H, N*H*), 7.80–7.30 (m, 7H, Ar-H), 5.90 (s, 1H, C5H) ppm; ^{13}C NMR ($CDCl_3$): $\delta = 163.24$ (C2), 149.48 (NCO), 133.42 (C4), 131.54, 129.11, 128.49, 128.24, 126.59, 126.31, 125.48, 124.12, 123.01, 121.32 (C3), 99.95 (C5) ppm; IR: $\nu = 3368$, 3270, 3065, 2958, 2873, 2335, 1760, 1644, 1550, 1332, 1150, 985 cm^{-1}; Anal. $C_{15}H_9O_4NCl_2$; C, H, N, Cl

3,4,5-Trichloro-2(5H)-furanone ***3.*** Mucochloric acid (10 g, 59 mmol), thionyl chloride (21.1 g, 178 mmol) and 0.1 g of anhydrous zinc chloride were refluxed vigorously over a period of 48 h until the evolution of hydrogen chloride and sulfur dioxide had ceased. Vacuum distillation afforded the compound (6.2 g, 56%) as a clear liquid (109°C, 21 mm)

MW: 225.07; ^{1}H NMR ($CDCl_3$): $\delta = 6.50$ (s, 1H, C5H) ppm; ^{13}C NMR ($CDCl_3$): $\delta = 162.24$ (C2), 150.24 (C4), 123.50 (C3), 85.78 (C5) ppm; IR: $\nu = 2967$, 2335, 1809, 1739, 1634, 1455, 981 cm^{-1}.

Benzoate ***4a****: benzoic acid 3,4-dichloro-5-oxo-2,5-dihydrofuran-2-yl ester*

Mucochloric acid (16.8 g, 59 mmol) and benzoyl chloride (24 g, 0.1 mol) were heated at 100–110°C for 3 h while hydrogen chloride was evolved. The product was poured into cold hexane, triturated and filtered. The crude product was recrystallised from methanol to give a white solid (mp 111°C, 24.6 g, 90%).

MW: 225.07; ^{1}H NMR ($CDCl_3$): $\delta = 7.60$ (m, 5H, Ar-H), 5.80 (s, 1H, C5H) ppm; ^{13}C NMR ($CDCl_3$): $\delta = 193.07$ (COPh), 171.64 (C2), 147.02 (C4), 134.59 (C3), 128.30, 129.47, 127.17, 124.21, 101.39 (C5) ppm; IR: $\nu = 3069$, 2967, 1803, 1739, 1634, 1453, 1249, 935 cm^{-1} Anal. $C_{11}H_6O_4Cl_2$; C, H, N, Cl.

Acetate ***4b****: 5-Acetoxy-3,4-dichloro-2(5H)-furanone*

Mucochloric acid (10 g, 59 mmol) and acetic anhydride (24.1 g, 24 mmol) were refluxed for 24 h. Excess of acetic anhydride was removed in vacuum and the desired acetate was distilled under reduced pressure to yield a colourless liquid (10.6 g, 91%, bp 135–139°C, 15 mm).

MW: 225.07; ^{1}H NMR ($CDCl_3$): $\delta = 6.84$ (s, 1H, C5H), 2.16 (s, 3H, Me) ppm; ^{13}C NMR ($CDCl_3$): $\delta = 168.42$ (AcO), 162.57 (C2), 147.02 (C4), 124.21(C3), 96.39 (C5) 20.57 (Me) ppm; IR: $\nu = 2967$, 1805, 1741, 1634, 1455, 1249, 981 cm^{-1}.

Synthesis of pseudoesters

To a solution of the mucochloric acid **1a** (10 g, 0.059 mol) in 50 mL of the parent alcohol, a catalytic amount of concentrated sulfuric acid (0.7 mL) was added. The reaction mixture was refluxed for 48 h and was quenched with 250 mL of a saturated solution of sodium hydrogen carbonate. The mixture was extracted with petrolether (3×50 mL) and the combined organic layers were dried with magnesium sulfate. The solvent was removed in vacuum and the crude compounds were distilled under reduced pressure (20 mm) to give the pseudo-esters as pure compounds.

5-Butoxy-3,4-dichloro-2(5H)-furanone ***5i.*** Yield: 85%. MW: 225.07; ^{1}H NMR ($CDCl_3$): $\delta = 5.80$ (s, 1H, C5H), 3.86–3.59 (m, 2H, OCH_2), 1.66–1.35 (m, 4H, OCH_2), 0.92 (t, $J = 7.3$ Hz, 3H, Me) ppm; ^{13}C NMR ($CDCl_3$):

$\delta = 163.24$ (C2), 147.48 (C4), 124.12 (C3), 100.94 (C5), 70.05 (OCH_2), 31.15 (OCH_2CH_2), 18.84 (CH_2CH_3), 13.59 (CH_3) ppm; IR: $\nu = 2958$, 2873, 2335, 1809, 1637 cm^{-1}.

3,4-Dichloro-5-(hexyloxy)-2(5H)-furanone ***5j.*** Yield: 82%. MW: 253.12; ^{1}H NMR ($CDCl_3$): $\delta = 5.80$ (s, 1H, C5H), 3.87–3.59 (m, 2H, OCH_2), 1.67–1.29 (m, 8H, $OCH_2(CH_2)_4$), 0.87 (t, $J = 6.7$ Hz, 3H, CH_3) ppm; ^{13}C NMR ($CDCl_3$): $\delta = 163.22$ (C2), 147.47 (C4), 124.13 (C3), 100.93 (C5), 70.34 (OCH_2), 31.29 (OCH_2CH_2), 29.10 ($OCH_2CH_2CH_2$), 25.28 ($CH_3CH_2CH_2$), 22.41 (CH_3CH_2), 13.89 (Me) ppm; IR: $\nu = 2860$, 2335, 1801, 1637 cm^{-1}.

3,4-Dichloro-5-(nonyloxy)-2(5H)-furanone ***5l.*** Yield: 52%. MW: 295.20; ^{1}H NMR ($CDCl_3$): $\delta = 5.80$ (s, 1H, C5H), 3.87–3.61 (m, 2H, OCH_2), 1.71–1.49 (m, 2H, OCH_2CH_2), 1.25 (m, 12H, $OCH_2CH_2(CH_2)_6$), 0.86 (t,$J = 6.5$ Hz, 3H, Me) ppm; ^{13}C NMR ($CDCl_3$): $\delta = 163.17$ (C2), 147.45 (C4), 124.13 (C3), 100.93 (C5), 70.35 (OCH_2), 31.75 (OCH_2CH_2), 29.35 ($OCH_2CH_2CH_2$), 29.15 ($OCH_2CH_2CH_2CH_2$), 29.12 ($OCH_2CH_2CH_2CH_2CH_2$), 26.11 ($CH_3CH_2CH_2CH_2$), 25.61 ($CH_3CH_2CH_2$), 22.57 (CH_3CH_2), 14.00 (CH_3) ppm, IR: $\nu = 2927$, 2860, 2335, 1793, 1641 cm^{-1}.

3,4-Dichloro-5-(dodecyloxy)-2(5H)-furanone ***5m.*** Yield: 49%. MW: 337.28; ^{1}H NMR ($CDCl_3$): $\delta = 5.81$ (s, 1H, C5H), 3.86–3.70 (m, 2H, OCH_2), 1.69–1.56 (m, 2H, OCH_2CH_2), 1.26 (m, 18H, $OCH_2CH_2(CH_2)_9$), 0.88 (t, $J = 6.6$ Hz, 3H, CH_3) ppm; ^{13}C NMR ($CDCl_3$): $\delta = 163.22$ (C2), 147.42 (C4), 124.22 (C3), 100.91 (C5), 70.37 (OCH_2), 31.84 (OCH_2CH_2), 29.70 ($OCH_2CH_2CH_2$), 29.60 ($OCH_2CH_2CH_2CH_2$), 29.55 ($OCH_2CH_2CH_2CH_2CH_2$), 29.40 ($OCH_2CH_2CH_2CH_2CH_2CH_2$), 29.27 ($CH_3CH_2CH_2CH_2CH_2CH_2$), 29.16 ($CH_3CH_2CH_2CH_2CH_2$), 26.12 ($CH_3CH_2CH_2CH_2$), 25.64 ($CH_3CH_2CH_2$), 22.62 ($CH_3CH_2$), 14.05 ($CH_3$) ppm; IR: $\nu = 2925$, 2858, 2335, 1787, 1641 cm^{-1}.

Isoxazolyl-furanone ***6****: 3,4-dichloro-5-[(3-phenyl-4,5-dihydroisoxazol-5-yl)methoxy]-2(5H)-furanone*

5-Allyloxy-2(5H)-furanone (0.36 g, 173 mmol) was added to a stirring solution of sodium hydrogencarbonate (0.52 g, 6.2 mmol) and benzaldehyde oxime (0.315 g, 2.6 mmol in 2 mL of ethyl acetate at room temperature. N-chlorosuccinimide was added to this solution (0.347 g, 2.6 mmol), which was left to react overnight under argon atmosphere. For work up, bicarbonate was added, the mixture was extracted with ether and dried with magnesium sulfate. The solvent was removed in vacuum to give the cyclo adduct as pale yellow crystals that were recrystallised from ethyl acetate (mp: 89°C).

Yield: 62%. MW: 328.15; MS (APCI+): 328/330 M+H m/z; ^{1}H NMR ($CDCl_3$): 7.60–7.31 (m, 5H, Ar), 5.93 (s, 1H, C5H), 4.89–3.84 (m, 4H), 3.45–3.13 (m, 1H) ppm; ^{13}C NMR ($CDCl_3$): δ 162.8 (C2), 158.6 (C=N), 148.2 (C4), 129.8, 129.7, 126.7, 125.9 (Ar-C), 124.1 (C3), 101.4 (C5), 73.0 (CH-O-N=), 63.3 (CH_2-O-), 39.6 (CH-C=N) ppm; IR (KBr) cm^{-1}: 3052, 2883, 1787, 1690, 1271, 1149, 970. Anal. $C_{14}H_{11}O_4NCl_2$; C, H, N, Cl.

Epoxide 7: 3,4-dichloro-5-(oxiran-2-ylmethoxy)-2(5H)-furanone

5-Allyloxy-2(5H)-furanone **5f** (5.0 g, 0.024 mol) was added drop-wise to a solution of m-CPBA (9.9 g, 0.058 mol) in 20 mL of dichloromethane (DCM) at ambient temperature. The reaction was monitored by TLC and after 3 h a further 1.0 equiv. of m-CPBA was added. After complete reaction the m-chlorobenzoic acid was filtered off and 3 mL of a saturated solution of sodium thiosulfite was added. The mixture was extracted with ether (3×30 mL) and the combined organic phases were dried with magnesium sulfate. The solvent was removed in vacuum and the crude epoxide was purified further by column chromatography with ether–petrolether (PE) (1:1).

Yield: 27%. MW: 225.03; MS (APCI+): 225/227 M+H m/z; ^{1}H NMR ($CDCl_3$): δ 5.92 (s, 1H, C5H), 4.18–3.60 (m, 2H, -CH_2), 3.28–3.23 (m, 2H, -CH_2), 2.91–2.64 (m, 1H, CH) ppm; ^{13}C NMR ($CDCl_3$): δ 166.3 (C2), 148.5 (C4), 132.9 (C3), 100.7 (C5), 71.3 (CH_2-O-), 49.9 (COH), 40.4 ($COCH_2$) ppm; IR (KBr) cm^{-1}: 3050, 2853, 1750, 1632, 1255, 1030, 703. Anal. $C_7H_6O_4Cl_2$ C, H, N, Cl.

Aziridine ***8****: 4,5-diaziridin-1-yl-3-chloro-2(5H)-furanone*

Trichloro-2(5H)-furanone **3** (0.30 g, 1.63 mmol) in 3 mL of tetrahydrofuran (THF) was added drop-wise to a stirring solution of aziridine (0.10 g, 2.39 mmol) and triethylamine (0.16 g, 1.60 mmol) in 3 mL of THF at 0°C. The reaction was stirred overnight at room temperature and filtered. The solvent was removed at room temperature in vacuum and the crude aziridine was purified by column chromatography with ether.

Yield: 36%. MW: 200.62; MS (APCI+): 201/203 M+H m/z; ^{1}H NMR ($CDCl_3$): δ 5.21 (s, 1H, C5H), 2.24–2.13 (m, 4H, -CH_2), 1.11–1.06 (m, 4H, -CH_2) ppm; ^{13}C NMR ($CDCl_3$): δ 169.5 (C2), 150.1 (C4), 104.9 (C3), 94.2 (C5), 37.1 ($2 \times CH_2$), 31.1 ($2 \times CH_2$) ppm; IR (KBr) cm^{-1}: 2895, 2822, 1770, 1667, 1342, 1267, 974. Anal. $C_8H_9O_2N_2Cl$; C, H, N, Cl.

Synthesis of amides

N-Methyl-(3,4-dichloro-5-oxo-2,5-dihydrofuran-yl)formamide ***9a.*** Mucochloric acid (15.0 g, 88.8 mmol) and N-methylformamide (4.73 g, 90 mmol) were reacted at 50°C in toluene (180 mL) with 8–10 drops of conc. H_2SO_4. After 50–55 h the mixture was cooled to room temperature. Chloroform and water was added, and the organic layer separated and washed with a further portion of water. The organic layer was dried over magnesium sulfate and removed in vacuum. A viscous crude liquid was obtained. Column chromatography (10% methanol in ether) gave a yellow oil.

Yield: 10.5%. R_f(10% MeOH–ether) = 0.53. MW: 210. MS (APCI(+)): 210 (M+1) m/z. ^{1}H NMR (DMSO-d_6) 300 K δ: (isomers) 8.52 (s, 1H, CHO), 6.22 (s, 1H, C5H), 2.84 (s, 3H, CH_3) ppm. ^{13}C NMR (DMSO-d_6) 300 K δ: (isomers) 167.4 (C=O), 161.8 (C2), 146.3 (C4), 124.0 (C3), 88.6 (C5), 24.4 (CH_3) ppm. IR (KBr-disc) ν max: 2961, 1806, 1701, 1408, 1299, 1030, 913, 747 cm^{-1}.

N-(3,4-Dichloro-5-oxo-2,5-dihydrofuran-2-yl)-N-methylacetamide ***9b.*** Yield: 6.0%. R_f (10% MeOH–ether) = 0.73. MW: 224.0. MS (APCI(+)): 224 (M+1), 182, 183, 184 (M+) m/z. ^{1}H NMR (DMSO-d_6) 300 K δ: (isomers) 2.32 (s, 3H, Me), 2.97 (s, 3H, N-CH_3), 6.23 (s, 1H, C5H) ppm. ^{13}C NMR ($CDCl_3$) 300 K δ: (isomers) 172.3 (C=O), 163.5 (C2), 148.0 (C4), 124.2 (C3), 83.3 (C5), 28.9 (N-CH_3), 22.0 (CH_3) ppm. IR (KBr-disc) ν max: 3372, 2963, 1769, 1640, 1447, 1233, 1150, 1023, 946, 886, 748 cm^{-1}.

N-t-butyl(3,4-dichloro-5-oxo-2,5-dihydrofuran-2-yl)formamide ***9c.*** Yield: 11.9%. R_f (10% MeOH–ether) = 0.61. MW: 252.1. MS (APCI(+)): 253 (M+1), 162, 163, 164 (M+) m/z. ^{1}H NMR ($CDCl_3$) 300 K δ: (isomers) 8.89 (s, 1H, CHO) 6.27 (C5H), 1.25–1.32 (m, 9H, CH_3) ppm. ^{13}C NMR ($CDCl_3$) 300 K δ: (isomers) 180.4 (CHO), 160.8 (C2), 148.8 (C4), 116.4 (C3), 98.3 (C5), 63.1 ($C(CH_3)_3$), 29.9 (Me) ppm. IR (KBr-disc) ν max: 3279, 2971, 1679, 1614, 1392, 1346, 1266, 1195, 1006 cm^{-1}.

N-Benzyl(3,4-dichloro-5-oxo-2,5-dihydrofuran-2-yl) formamide ***9e.*** Yield: 15.0%. R_f (10% MeOH–ether) = 0.71. MW: 286.1. MS (APCI(+)): 287 (M+1), 196, 197, 198 (M+) m/z. ^{1}H NMR (DMSO-d_6) 300 K δ: (isomers) 8.52, (s, 1H, CHO) 7.22–7.41 (m, 5H, phenyl), 6.85 (s, 1H, C5H), 4.32–4.34 (m, 2H, CH_2O), ppm. ^{13}C NMR (DMSO-d_6) 300 K δ: (isomers) 185.7, (CHO), 165.5 (C2), 140.1 (C4), 129.3 (C2), 128.8, 127.8, 127.3, 127.4 (Ar-C), 100.4 (C5), 41.3, (m, 2H, OCH_2), ppm. IR (KBr-disc) ν max: 3281, 3052, 2882, 2358, 1648, 1530, 1451, 1386, 1241, 753, 695 cm^{-1}.

3,4-Dichloro-2(5H)-furanone ***10.*** Mucochloric acid (33.8 g, 0.2 mol) and aluminium isopropoxide (50.0 g, 0.25 mol) was dissolved in isopropanol (200 mL) and refluxed using a vigreux column, until acetone ceased distilling. The excess isopropanol was removed by distillation and the mixture poured into a mixture of ice (300 g) and conc. HCl (100 mL). The resulting slurry was heated to 50°C and extracted with chloroform. After washing with water, sodium carbonate and HCl solutions twice, the extract was distilled to give a crude product. Recrystallisation from dilute ethanol gave a white solid (mp 51°C)

Yield: 33.1%. MW: 152.9. MS (APCI(+)): 153, 155 (M+1) m/z. ^{1}H NMR ($CDCl_3$) 300K δ: 4.86 (s, 2H, C5H) ppm. ^{13}C NMR ($CDCl_3$) 300K δ: 165.9 (C2), 149.3 (C4), 120.6 (C3), 72.0 (C5) ppm. IR (KBr-disc) ν: 1781, 1631, 1442, 1351, 1243, 1013, 913, 747 cm^{-1}.

Tetramic acid chloride ***11.*** An ammonia solution was added drop-wise to a solution of the methoxy-furanone **5a** (1 g, 0.7 mmol) in 9 mL of ether–ethanol (2:1). The solution turned yellow after some hours and the desired compound precipitated out overnight as slightly brown solid which was purified further by column chromatography with ether–PE (1:1)

Yield: 37%. MW: 196, 198. APCI+ MS: m/z 197/199; ^{1}H NMR ($CDCl_3$): δ = 9.21(s, 1H, NH), 7.10 (bs, 1H, OH), 6.2 (s, 1H, C5H); ^{13}C NMR ($CDCl_3$): δ = 163.9 (C2), 146.2 (C4), 125.4 (C3), 79.5 (C5), IR: ν = 3098m, 2925m, 2855m, 1786s, 1749vs, 1599, 1585, 14441, 1342, 1262, 1157, 1045, 883 cm^{-1}.

Pharmacology

Cytotoxicity assays using murine carcinoma cell lines (MAC13 and MAC16)

The culture media used was RPMI 1640 containing hepes, glutamine, antibiotics and 5% fetal calf serum for MAC13 cells or 10% fetal calf serum for MAC16 cells. On day 0, media from 250-mL flasks, containing 70% confluent MAC13 or MAC16 cells, were poured off and the cells were washed with 10 mL phosphate-buffered saline (PBS). Versene (5 mL) was added to the flasks for 3 min. The detached cells were pipetted into plastic universals and spun down for 5 min at 1100 rev min^{-1}. The media were poured off and 10 mL of fresh culture media were added to the cells. Cells were counted by the trypan blue exclusion method using a plastic Kova counting chamber. The MAC13 and MAC16 cells were suspended in appropriate volumes and were seeded at 0.5×10^4 and 2×10^4/200 μL, respectively, in flat-bottomed 96-well plates. Depending on solubility, test compounds were dissolved in water, alcohol or dimethyl sulfoxide (DMSO) to give stock solutions of 100 mM (10^{-1} M). Dilution series from 10^{-4} to 10^{-9} M were made so that each compound was tested at six concentrations and in triplicate.

5-Fluorouridine (5-FU), a known anti-cancer agent, was used as a control and tested in the 20–0.02 μM range. Plates were then incubated at 37°C in 5% CO_2 for three days. Compounds were tested on at least two separate occasions. On day three, 20 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (7.5 mg MTT/mL of PBS) was added to each well and plates were allowed to incubate for a further 2 h. Culture supernatant (120 μL) was carefully removed from each well with a pipetter and 100 μL of acidified i-propanol containing 10% Triton-X100 was then added to each well. Plates were agitated for 10 min at 800 rev min^{-1} on a plate shaker. Following this solubilisation step all plates were then read, within 15 min, on an Anthos AW200 plate reader at 540 nm with a reference wavelength of 590 nm.

The results are expressed as mean ± s.d. and the data were subjected to repeated measures of the one-way analysis of variance. If the probability level (*P*-value) is less than 0.05, a statistical significance was attained.

Results and Discussion

Chemistry

Mucochloric acid was reacted with phenyl, naphthyl and i-butyl isocyanate to form the pseudocarbamates **2a–c** under reflux conditions in benzene. Crystallisation of the targets **2a–2c** was achieved by diluting the mixture with hexane (Figure 2).

An excess of thionyl chloride was reacted with mucochloric acid **1a** to afford the acid chloride **3** in good yields. The excess of thionyl chloride was distilled off and the desired chloride **3** was purified by vacuum distillation. This useful tri-chlorinated building block **3** was reacted further with aziridine at ambient temperature in THF to give the bis-aziridinyl compound **8**.

The pseudoanhydrides **4a–4e** were readily obtained from the mucochloric acid **1a** and the mucobromic acid **1b** (e.g. **4d**) by refluxing the parent acetic anhydride/propionic anhydride or with benzoyl chloride at 110°C. The benzoate **4a** was recrystallised from methanol and the acetate **4b** was distilled in vacuum for further purification.

Mucohalogen acids were reacted to form the pseudoesters (Lattmann et al 1999a, b) **5a–p** by using the selected alcohol according to method A or B in the presence of a catalytic amount of sulfuric acid. Volatile alcohols, which were used in excess and refluxed, gave the esters **5a–l**. Alcohols **m**, **n**, **o** and **p** (1.3 equiv.) were heated in toluene (Semonský et al 1961). The pseudoesters **5o–p** were purified by column chromatography (Method B). The allyl-furanone (Mowry 1950, 1953) **5f** was converted into the epoxide **7** with 2.5 equiv. m-CPBA in DCM at room temperature. The isoxazolidinyl-furanone **6** was generated in a (3 + 2)-cyclo-condensation by reacting the nitriloxide with the allyl ester **5f** in THF at ambient temperature. The phenylnitriloxide, as dipole, was generated in-situ from benzaldehyde oxime with N-bromosuccinimide (NBS) according to standard reaction conditions (Harwood et al 1999). No attack of the dipole on the butenolide double bond was observed.

The aziridine **8** was obtained from the pseudo acid chloride **3** by adding **3** to an excess of aziridine at ambient temperature. Using this method, no other by-products were obtained.

The reaction of **1a** with simple formamides and acetamide furnished a series of amides **9a–e** by refluxing the mixture for 2 h or heating the reaction at 50°C over a period of 48 h in the presence of a catalytic amount of sulfuric acid. Formanilide formed in nearly quantitative yield the phenylimine of mucochloric acid, 2,3-dichloro-4-phenylimino-but-2-enoic acid (entry **9d**).

The methyl ester **5a** was reacted with an excess of ammonia in THF at ambient temperature overnight to the tetramic acid chloride **11**. The direct conversion of

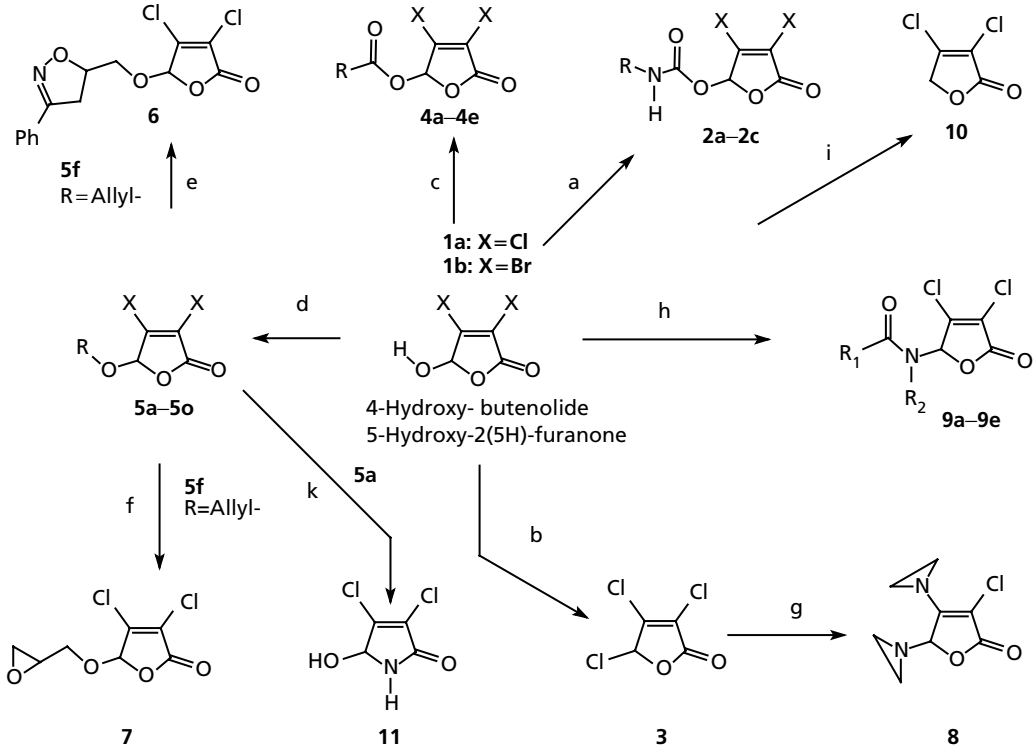

Figure 2 Synthesis of 3,4-dihalogenated 2(5H)-furanones. Reaction conditions: a, R-NCO, benzene, reflux; b, thionyl chloride, $ZnCl_2$, reflux; c, acid chloride or anhydride, reflux; d, excess alcohol, cat. H_2SO_4 or toluene, alcohol, reflux; e, nitriloxide 3 + 2-cycloaddition: benzaldehyde oxime, NCS, ethylacetate, room temp.; f, *m*-CPBA, DCM, room temp.; g, aziridine, TEA, THF, room temp.; h, *N*-formamides or acetamide, cat. H_2SO_4, toluene, reflux or 50°C; i, $Al(OPr\text{-}i)_3$, i-propanol; k, ammonia, room temp.

mucochloric acid into **4** with ammonia was unsuccessful. No replacement of chlorine in the 4-position was observed.

The Meerwein Pondorf reduction of **1a** with aluminium isopropylate furnished the furanone **10**, which is the chlorinated acid chloride of tetronic acid (Jerris et al 1979).

Pharmacology–cytotoxicity

Colorimetric MTT (tetrazolium assay): In principle, this assay is based on the cellular reduction of MTT (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) by the mitochondrial dehydroxygenase of viable cells to a blue formazan product. The production of formazan can be measured photometrically following solubilisation. The method described by Mossmann (1983) was applied with some modifications. The in-vitro screening results, based on two selected murine colon adenocarcinoma (MAC13, MAC16) cell lines, are outlined in Table 1.

The mucochloric acid **1a** and mucobromic acid **1b**, as readily available starting material, showed a cytotoxicity $>100\,\mu$M in the MMT assay. The maximum cytotoxicity was found in the carbamate series for the naphthyl

Table 1 Yields and inhibitory concentrations of tested 2(5H)-furanones

Entry	X	R		Yield (%)	MAC 13*	MAC16*
1a	Cl	—		—	>100	>100
1b	Br	—		—	>100	>100
Pseudo-carbamates						
2a	Cl	Ph-		74	40 ± 2	90 ± 4
2b	Cl	Naphthyl-		65	6 ± 1	13 ± 1
2c	Cl	t-Bu		54	40 ± 3	58 ± 3
3	—	—		56	13 ± 1	20 ± 2
Pseudo-acid anhydrides						
4a	Cl	Ph-		90	30 ± 2	30 ± 2
4b	Cl	Me-		91	1.0 ± 0.5	8 ± 2
4c	H	Me-		74	30 ± 3	70 ± 3
4d	Br	Me-		81	2.0 ± 0.5	18 ± 2
4e	Cl	Et-		78	10 ± 1	10 ± 1
Pseudo-esters						
5a	Br	Me-		67	50 ± 3	70 ± 6
5b	Br	Et-		69	6 ± 1	8 ± 1
5c	Cl	Me-		80	6 ± 1	7 ± 1
5d	Cl	Et-		84	8 ± 1	16 ± 2
5e	Cl	Vinyl-		59	20 ± 3	80 ± 5
5f	Cl	Allyl-		65	8 ± 1	50 ± 4
5g	Cl	Propargyl-		36	2.0 ± 0.5	3 ± 1
5h	Cl	i-Pr		79	5 ± 1	20 ± 2
5i	Cl	n-Bu		85	3 ± 1	3 ± 1
5j	Cl	n-Hexyl		82	3 ± 1	4 ± 1
5k	Br	n-Hexyl-		63	4 ± 1	4 ± 1
5l	Cl	n-Nonyl-		52	20 ± 2	30 ± 3
5m	Cl	Dodecyl-		49	20 ± 2	40 ± 3
5n	Cl	Cyclopentyl-		69	25 ± 2	40 ± 3
5o	Br	Benzyl-		51	7 ± 1	16 ± 2
5p	Br	Menthyl-		48	3 ± 1	40 ± 6
Bis-cyclic furanones						
6	—	—		62	20 ± 2	30 ± 2
7	—	—		27	0.05 ± 0.01	0.6 ± 0.1
8	—	—		36	0.03 ± 0.01	0.2 ± 0.01
Pseudo-amides						
		R_1	R_2			
9a	—	Me	H	10	6 ± 1	12 ± 1
9b	—	Me	Me	6	16 ± 2	50 ± 4
9c	—	t-Bu	H	12	25 ± 2	90 ± 7
9d	—	—	—	98	32 ± 2	93 ± 6
9e	—	Bz	H	15	22 ± 2	67 ± 3
10	—	—		33	3 ± 1	5 ± 1
11	—	—		37	7 ± 1	13 ± 2

*IC50 in μM, n = 5; 5-FU (5-fluorouridine) as standard.

derivative **2b** containing a large and flat aromatic system, suitable for DNA intercalation.

The trichloro furanone derivative **3** displayed a moderate bioactivity and provided three electrophilic centres in the 3-, 4- and 5-position. The acetate **4b** displayed an IC50 (50% inhibitory concentration) of 1 μM for the MAC13 cell line and the replacement of chlorine atoms by bromine in the 3- and 4-position did not enhance the cytotoxicity, as shown for acetate **4d**.

The size of the 5-substituent and the lipophilicity of the pseudo esters (Farina et al 1983; Font et al 1990a) **5a–o** was systematically varied and evaluated in-vitro. A maximum cytotoxicity was determined for the n-butyl ester **5i** and n-hexyl derivatives, containing either chlorine (ester **5j)** or bromine (ester **5k**), attached to the 2(5H)-furanone structure. The ester **5g**, containing a 5-propargyl group, displayed the same range of cytotoxicity, but this additional functionality may result in side reactions. Above C_6, more lipophilic side chains exhibited a decreased activity in cell culture assays.

Based on these biologically active esters, a further structure–activity relationship (SAR) optimization was carried out using the allyl ester **5f**. The allyl ester **5f** was converted in a (3 + 2)-cycloaddition into the isoxazolyl-furanone **6**, which is biologically inactive. The isoazolyl system was supposed to be inert and it acted as an aromatic side chain, simply attached to the butenolide moiety in the 5-position.

The epoxidation of the allyloxy-2(5H)-furanone **5f** furnished epoxide **7**, a highly potent agent, which has shown an IC50 of about 50 nM on the MAC13 cell line. It was expected that a bi-functional molecule with two chemically reactive centres would give an enhanced bioactivity. It was assumed that the butenolide moiety may have interacted with the cell's DNA, possibly through cross-linking of DNA or the formation of protein DNA cross-linked complexes.

The chlorinated furanone (Smith et al 1981) **3**, which itself was only moderately potent, had been transferred into the azidirine **8** resulting in a 1000-times increased potency in cell line experiments (Lattmann et al 2001a).

The formamide **9a** showed the best inhibition of cell growth and further analogues had shown a decreased bioactivity. The 3,4-dichlorinated 2(5H)-furanone **10** displayed an IC50 of 3 μM and 5 μM for the MAC13 and MAC16 cell line, respectively. Furanone **10** was crystalline, unlike the esters, anhydrides and amides, and was lipophilic and moderately soluble in water. The amide **11**, which is the aza-analogue of mucochloric acid, depicted a 10-time higher cytotoxicity than the parent compound, the mucochloric acid **1a**. As reported (Lattmann et al 2003), **1a** produced a good inhibition of cell growth in-vivo and further studies of this novel aza-analogue are in due course.

The in-vitro results concerning the cytotoxicity and the chemical yields are outlined in Table 1.

Alkylating antineoplastic agents display a correlation of chemical reactivity and bioactivity. The chemical reactivity in the 4-position was explained by attacking the 2(5H)-furanone system in an *ipso*-substitution (Lattmann et al 1999b).

Conclusions

In a previous publication (Lattmann et al 2003) the anticancer properties of 3,4-dihalogenated furanones were reported. The cytotoxicity was optimized here further for various series of 3,4-halogenated (mainly chlorinated) furanones. Brominated analogues (Font et al 1990b) were obtained in lower yields and did not show an enhanced bioactivity.

The epoxide **7** and aziridine **8** were derived from two simple 2(5H)-furanones displaying a very high potency in the nanomolar range.

All compounds were obtained in good overall yields and further in-vivo evaluation is currently ongoing.

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