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Structure−Activity Studies of Antitumor Agent Irofulven (Hydroxymethylacylfulvene) and Analogues

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Many analogues of the antitumor agent irofulven have been readily prepared by replacing the allylic hydroxyl with a variety of nucleophiles. Analogues of acylfulvene (the precursor to irofulven) were also prepared by Michael reaction with acrolein. The toxicity of the analogues was determined, as well as preclinical antitumor activity. Several analogues exhibited good activity in mouse xenografts. Structural requirements for activity are discussed.

Introduction

The toxic sesquiterpenes illudin S and illudin M (**1** and **2**) were discovered many years ago at the New York Botanical Garden during a search for antibiotic substances in the basidiomycete, *Omphalotus illudens* (*Clitocybe illudens*, *Omphalotus olearius*).[1,2] The compounds are believed to be responsible for poisoning that occurs when *Omphalotus* is mistaken for an edible mushroom.[3]

Illudins and certain derivatives have been evaluated for antitumor activity in the National Cancer Institute Developmental Therapeutics Program. Illudin M significantly increased the life span of rats with Dunning leukemia, but had a low therapeutic index in solid tumor systems.[4] In contrast, we have found derivatives of illudins, including hydroxymethylacylfulvene (**5**), now called irofulven, which produced complete tumor regression in a variety of xenograft models.[5,6] Compound **5**, irofulven, in a phase II human clinical trial, demonstrated efficacy against pancreatic carcinoma, a malignancy that is resistant to all other forms of chemotherapy. On the basis of these outstanding results, a large-scale phase III trial was initiated in February 2001.[8] In June 2001 the Food and Drug Administration granted "fast track" designation for the use of Irofulven in patients with gemcitabine-refractory pancreatic cancer.

Irofulven rapidly enters tumor cells, where it binds to cellular macromolecules and inhibits DNA synthesis.[9−11] Treatment of the human CEM leukemic cell line with irofulven results in cell-cycle arrest in the S phase and inhibition of DNA, RNA, and protein synthesis with 50% inhibitory concentration values of 2, 20, and 70 μmol/L, respectively.[9] Radiolabeled irofulven is localized predominantly in the nuclear compartment followed by the cytosol and membrane compartments: more than 60%, 27%, and 11% of drug binds to proteins, DNA, and RNA, respectively.[10] Irofulven induces cytotoxicity principally by generating DNA strand breakage, as exposure of CEM cells to the drug does not result in the formation of DNA intrastrand cross-links or DNA−protein cross-links.[10]

The most unique aspect of irofulven's antitumor activity seems to be its ability to act as a selective inducer of apoptosis in human tumor cell lines, and in contrast to conventional antitumor agents, irofulven retains this activity against tumor cell lines regardless of their p53 or p21 expression.[12,13] The illudins and acylfulvenes also differ from other agents with alkylating activity in their preferential cytotoxicity toward cell lines deficient in the excision repair cross-complementing (ERCC) DNA repair enzymes ERCC2 and ERCC3, which indicates that the repair of irofulven-induced DNA damage requires a full

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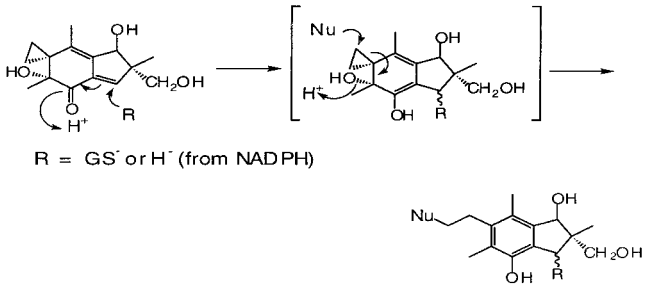


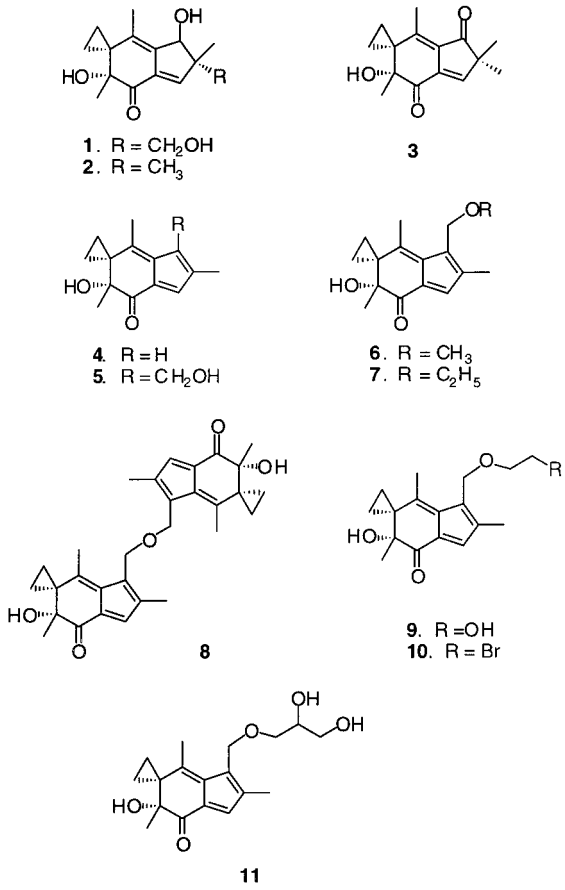
Figure 1.

complement of DNA repair mechanisms that may be absent in sensitive cell lines.[14,15]

Investigation of reactions of illudins indicate that the toxicity of these compounds is due to their behavior as alkylating agents.[16] At room temperature, the compounds react spontaneously with thiol nucleophiles, such as cysteine or glutathione (GSH), at an optimum pH of about 6. Toxicity to myeloid leukemia cells (HL60) can be modulated by altering glutathione levels in the cells.[17] The reaction of illudin S with GSH is illustrated in Figure 1. Michael-type addition to the α,β-unsaturated ketone gives a cyclohexadiene intermediate that is rapidly converted to a stable aromatic product. Concurrent ring opening of the cyclopropane results in alkylation of nucleophiles such as water, DNA and protein.[18] Enzymatic reduction of illudin S leads to a similar reactive intermediate and to a stable aromatic product. NADPH is the coenzyme involved in this bioreductive alkylation (Figure 1).[19]

Synthesis of Analogues Involving Replacement of the Primary Hydroxyl of Irofulven. Many analogues of illudin S and M have been investigated with the aim of finding compounds more selective in their toxicity toward tumor cells than normal cells. From the outset, it was evident that key features, viz. α,β-unsaturated ketone and cyclopropylmethyl carbinol, are ncessary for toxicity and antitumor activity.[18] However, certain modifications could be made that resulted in a greatly improved therapeutic index. Thus, dehydroilludin M (**3**)[20] and acylfulvene (**4**)[21,22] (the latter prepared by treatment of illudin S with dilute sulfuric acid) inhibited growth of tumors in mouse xenografts at doses well tolerated by the animals. The enhanced efficacy and lower toxicity correlates with decreased reactivity of dehydroilludin M and acylfulvene with thiols and also in the enzymatic reaction with NADPH.[23]

Acylfulvene reacts with formaldehyde in dilute sulfuric acid giving irofulven (**5**) (NSC 683863). The efficacy of this compound as an antitumor agent far surpasses that of acylfulvene.[6] The hydroxymethyl group in **5** results in increased toxicity, the compound being five times more toxic than **4**. Improved efficacy (and higher toxicity) can be attributed to the reactivity of the allylic primary hydroxyl. Increased hydrophilic character may also be a factor. The very facile displacement of the hydroxyl allowed us to prepare a large number of analogues. Reactions with various nucleophiles were conveniently carried out by dissolving the reactants in acetone with dilute H_2SO_4 (1 M) at room temperature.



Products from reactions with thiols have been reported previously.[24,25] When the nucleophile was methanol or ethanol, corresponding ethers **6** and **7** were obtained. The latter was also formed with diethyl ether, instead of ethanol, providing the ethoxy group. A byproduct of this reaction was the ether **8** formed from two molecules of irofulven. Ethylene glycol, ethylene bromohydrin, and glycerol afforded the expected products **9**, **10**, and **11**, respectively.

Reaction of irofulven with fructose was investigated. When the compound and a large excess of fructose were dissolved in acetone−dilute H_2SO_4 and kept overnight, the condensed product (from one mole of each reactant) was obtained. NMR spectral analysis indicated the product was a mixture. It has been resolved by HPLC (isocratic elution with CH_3CN and H_2O) into four com-

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Table 1. Assignments for ^{13}C NMR Spectra of Irofulven (5) and Compounds 12 and 14 (DEPT Assignments Are Also Given)

carbon	irofulven (**5**)	12	14	DEPT
1	199.82	199.58	199.57	C
7	161.58	162.160	162.48	C
9	143.20	144.27	144.19	C
5	140.28	140.12	140.13	C
8	136.03	135.38	135.38	CH
6	134.43	131.20	131.89	C
4	128.86	129.09	128.92	C
2	77.83	77.24	77.82	C
15	56.30	64.49	64.82	CH_2
3	38.78	38.90	38.90	C
10	27.73	27.79	27.88	CH_3
14	16.85	17.14	17.43	CH_3
11	14.65	14.85	14.84	CH_2
13	13.03	13.39	13.43	CH_3
12	9.80	10.01	9.99	CH_2

ponents: two major and two minor compounds. The ^{13}C NMR spectrum and DEPT analysis of the first major component (**12**) indicated that the fructose moiety had replaced the primary allylic hydroxyl (change in chemical shift of δ 56.3 to δ 64.49 for CH_2 and δ 134.43 to 131.20 ppm for a vinyl carbon, Table 1). Signals for the fructose moiety suggested a β-furanose structure bonded to irofulven via C-1′ (change in chemical shift of δ 63.6 to 73.06 ppm for CH_2), though bonding via C-6′ cannot be excluded. Chemical shift values of the β-furanose moiety compared to those of β-fructofuranose are given in Table 2.[26]

Rearrangement of **12** to tautomer **13** occurred when the solution in CD_3OD was allowed to stand for several days. This led to change in the chemical shifts of the fructose moiety, most notably the change from δ 63.6 to 71.1 for one of the CH_2 carbons. Spectral data for the other major component (**14**) indicated a pyranose structure with bonding of the pyranose via C-5 (change in chemical shift of δ 70.0 to δ 78.78 ppm, Table 3). The β-furanose and β-pyranose forms (**12** and **14**) are expected to be the major tautomers. The two minor components may possibly be the corresponding α-tautomers.[27]

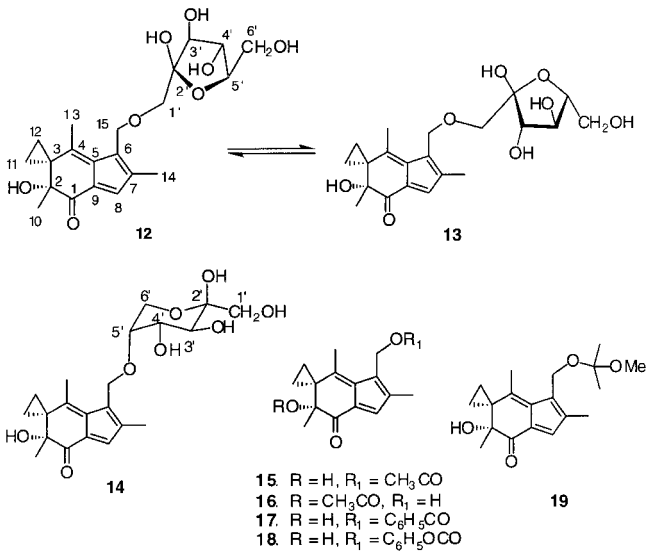


Table 2. Assignments for ^{13}C NMR Spectra of the β-Furanose Moiety of Compound 12

carbon	**12** (furanose moiety)	β-fructofuranose	DEPT
2′	103.42	102.6	C
5′	81.58	81.6	CH
3′	77.84	76.4	CH
4′	77.58	75.4	CH
1′	73.06[a]	63.6	CH_2
6′	63.50[a]	63.2	CH_2

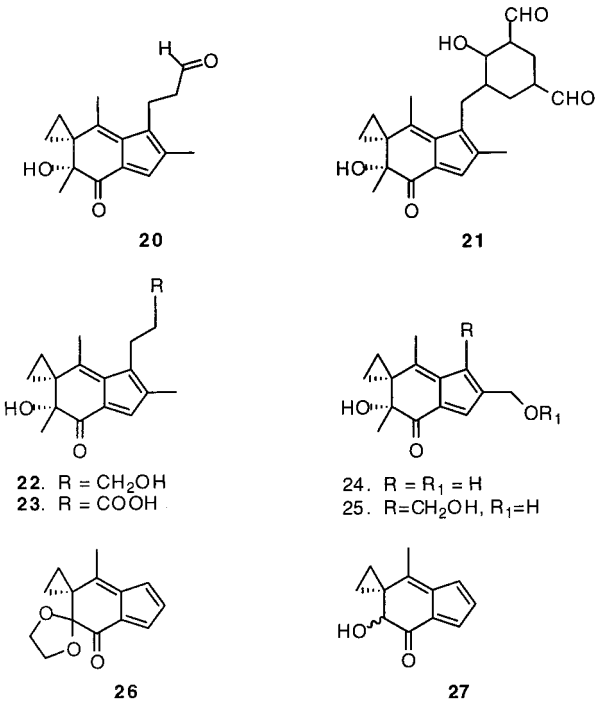
[a] Assignments may be reversed.

Table 3. Assignments for ^{13}C NMR Spectra of β-Pyranose Moiety of Compound 14

carbon	**14** (pyranose moiety)	β-fructopyranose	DEPT
2′	99.15	99.1	C
5′	78.78	70.0	CH
4′	72.05	70.5	CH
3′	69.98	68.4	CH
6′	66.00	64.1	CH_2
1′	61.77	64.7	CH_2

Other analogues possessing a primary allylic oxygen substituent included the acetate **15** prepared from irofulven and sodium acetate in acetic anhydride. Compound **15** was also obtained with acetic anhydride and $BF_3{\cdot}Et_2O$. A byproduct was the tertiary acetate **16**. With benzoyl chloride, irofulven formed the benzoate **17**, and with phenylchloroformate, the corresponding carbonate **18** was obtained. The acetal **19** was prepared by reaction of irofulven and 2-methoxypropene with $POCl_3$ as catalyst.

Analogues from Acylfulvene (4). Irofulven was obtained by nucleophilic addition of acylfuvene **4** to formaldehyde. Attempts to synthesize analogues by similar reaction with acetaldehyde, glyoxal, or acrylonitrile were unsuccessful. However, reaction of **4** with acrolein proceeded smoothly to give the Michael adduct **20**. A byproduct **21** was obtained from this reaction. Reduction of **20** with sodium cyanoborohydride yielded the alcohol **22** while oxidation of **20** with Jones reagent gave the corresponding acid **23**.



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Studies of metabolism of acylfulvene and of irofulven by rat liver cytosol and NADPH have yielded aromatic compounds as well as hydroxylated metabolites including **24** and **25**.[28] The structures of these compounds was confirmed by partial synthesis from acylfulvene. This provided enough material for structure−activity studies.

Toxicity of Analogues Prepared by Total Synthesis. We recently reported a total synthesis of (±)-hydroxymethylacylfulvene.[21] The method makes it possible to prepare a wider variety of analogues. Thus, two analogues **26** and **27** have been made. The in vitro toxicity of these analogues was found to be lower than that of acylfulvene on short (2 h) or long (48 h) exposure of MV 522 cells to these compounds. However, analogue **26** was found to have good in vivo activity, similar to that of mitomycin C, in mice implanted with MV522 cells.[30]

It is interesting to note the synthesis by Kinder et al. of even simpler analogues, bicyclic structures containing spirocyclopropane and unsaturated ketone. Several of these analogues were reported to have IC_{50} values less than 10^{-6} M against a number of human tumor cell lines.[31]

Biological Activity. There are a number of ways for determining biological activity of hydroxymethylacylfulvene analogues. Cytotoxicity tests were used in our initial studies with illudins and were applied to acylfulvenes as well. Cells were exposed to the compounds for various times, and the IC_{50} values were determined. Thus, illudins were added to cultures of MV522 (human lung carcinoma) and HL60 (myeloid leukemia) cells. After 48 h of continuous exposure, cell growth/viability was measured by trypan blue exclusion. As an alternative to 48 h exposure, cells were exposed to the compounds for 2 h. This shorter exposure revealed selective toxicity in these cells due to active uptake of the toxin. For example, the IC_{50} values of illudin S in HL60 cells were 10 ± nM and 3 ± 1 nM for 2 h and 48 hours, respectively. For a human fibroblast (GM637) cell line the corresponding IC_{50} values were >1000 nM and 4 ± 1 nM. This indicated rapid uptake into HL60 cells compared to GM637 cells.[4]

The in vitro toxicity of most of the analogues was determined with MV522 cells for 48 h exposure, and the results are given in Table 4. None of the analogues was as toxic as irofulven itself. This suggests that the free primary allylic hydroxyl might contribute to the toxicity of irofulven because of increased hydrophilicity. Also, its inherent reactivity might lead to substitution by a macromolecular cellular nucleophile at a suitably low pH. Analogue **15** with an acetoxy rather than hydroxyl group was expected to be more reactive than irofulven, acetoxy being a better leaving group. It was actually found to be slightly less toxic. Thus, there is no simple correlation between reactivity of the allylic oxygen function and toxicity. Furthermore the toxicity value found for **20**, which does not possess an allylic oxygen function, is on the same order of magnitude as that of irofulven.

Table 4. IC_{50} Values for Analogues When Tested in MV 522 Cells[a]

compd	IC_{50} (nM)
1 (illudin S)	4 ± 1
2 (illudin M)	4 ± 1
3 (dehydroilludin M)	310 ± 3
4 (acylfulvene)	350 ± 20
5 (irofulven)	73 ± 8
7	440 ± 80
8	320 ± 60
9	680 ± 180
10	930 ± 250
12	18100 ± 5700
15	1400 ± 200
16	480 ± 110
19	170 ± 80
20	165 ± 55
21	270 ± 130
22	850 ± 180
24	660 ± 200
25	580 ± 250
26	4600 ± 200

[a] For cytotoxicity tests the compounds were dissolved in DMSO (1 mg/mL stock solution) and the solutions diluted in 20% DMSO/phosphate buffered saline just prior to addition to cultures of MV 522 cells. Control cells received equal amounts of the DMSO/phosphate buffered saline. After incubation for 48 h, the cells were washed, trypan blue was added, and the cells were counted. These values correlate closely with those determined by colony forming assay.

Analogues **24** and **25** obtained from metabolism of acylfulvene and irofulven were tested and found to be slightly less toxic than the parent compounds. Thus, introduction of a second allylic hydroxyl in the acylfulvene structure appears to partly detoxify the compound.

While in vitro cytotoxicity is a necessary property for anticancer drugs, selective toxicity of the compounds in malignant cells compared to normal cells is of much greater significance. Therefore, a further stage in the development of new drugs requires in vivo studies, usually done with mouse xenografts. Such studies have been carried out with illudin S and M, dehydroilludin M, acylfulvene, and irofulven. As reported in previous publications, illudin S and M, although extremely toxic to malignant cells, had poor efficacy, being extremely toxic to the host animal. The efficacy of acylfulvene and, particularly of irofulven, was much greater.

Since the analogues described in this paper all possess the key structural features found in acylfulvene we expected them to display comparable efficacy. Several of the analogues have therefore been tested in vivo using the MV522 xenograft model. The acetate of irofulven (analogue **15**) was found to be as efficacious as the parent compound. Likewise, analogue ethers **7**, **9**, **11**, and **19** were comparable to irofulven. Thus, the free allylic hydroxyl is not essential for good antitumor activity.

An even more definite indication that the allylic hydroxyl is not necessary was found in analogues **20** and **22**, neither of which possesses an allylic oxygen function. Analogue **20** had an IC_{50} 170 ± 60 nM (cf. irofulven, 75 ± nM) and both **20** and **22** showed good activity in the xenograft model.

As mentioned previously, the product of irofulven and fructose was obtained as a mixture of isomers (**12** and **14**) that was found to be appreciably less toxic than the parent compound. This mixture was found to be moder-

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ately active in MV522 xenograft tests. The mixture was subsequently resolved into two main components that have similar IC_{50} values. It is likely that they will have similar in vivo activity as well, though this has not been tested.

Biological Tests Carried out by NCI: Cancer Drug and Development Program. Because of the promising results obtained with many of the irofulven analogues, they were submitted to the NCI for further evaluation. They have been found to demonstrate potent broad-spectrum activity across many of the human cell lines respresented in the NCI 60-cell panel, with the majority of GI_{50} values below 10^{-6} M. Mouse hollow fiber test results have demonstrated good activity (total score ranging from 18 to 46) across a variety of tumor types and positive net cell kill (9 of 9 analogues tested). In vivo activity has been demonstrated in the drug-resistant MV522 mdr 1/gp 170^+ human lung carcinoma xenograft model in which acylfulvene analogues have led to increased life span and tumor growth inhibition or tumor shrinkage. Three acylfulvene analogues **22** NSC-690179, **20** NSC-690180, and **15** NSC-688985 have displayed significant activity each producing tumor cures in the early stage NCI-H123 nonsmall cell lung tumor, the early stage OVCAR 3 ovarian tumor, and the early stage RXF 393 renal tumor xenograft models.

Conclusions

Our studies of illudins, acylfulvene, irofulven, and analogues, led to the following conclusions:

Toxicity and antitumor activity are a result of their behavior as electrophiles. Bioreductive activation followed by cyclopropane ring-opening and alkylation of protein and DNA, are responsible for biological activity.

Attenuation of reactivity in acylfulvenes and dehydroilludin M (compared to illudin S) presumably leads to lower toxicity and greater selectivity toward malignant cells versus normal cells.

The hydroxymethyl group in irofulven confers remarkable efficacy on this compound. However, a free allylic hydroxyl is probably not the sole reason for this efficacy since analogues with ether or thioether groups retain antitumor activity. Moreover, analogues with longer chain substituents, e.g., hydroxypropyl derivative (**22**), have produced tumor cures as mentioned above. Therefore, an acylfulvene with a side chain at C-6 containing an oxygen or other heteroatom substituent results in enhanced efficacy.

Further structure activity studies are planned with preparation of analogues having side chains of varying length and with heteroatoms including nitrogen. It is possible that there is interaction of the side chain with a receptor and there is a side chain that possesses optinum binding, which will give the best therapeutic activity.

Experimental Section

General directions can be found in previous papers; for example, see ref 25.

Ether 6 from Irofulven 5 and Methanol. To a stirred solution of **5** (320 mg, 1.3 mmol) in 3 mL of Me_2CO were added MeOH (3 mL) and dilute H_2SO_4 (1 M, 3 mL). The reaction mixture was stirred at room temperature for 24 h. It was then extracted with Et_2O, and the combined extracts were washed with saturated $NaHCO_3$, followed by saturated brine, and dried over $MgSO_4$. After concentration, the residue was chromatographed on SiO_2 with EtOAc−hexanes affording compound **6** (290 mg, 86%) as a dark orange gum: ^{1}H NMR ($CDCl_3$) δ 0.62 (ddd, J = 9.7, 6.7, 4.1 Hz, 1H), 0.98 (ddd, J = 9.9, 6.7, 4.9 Hz, 1H), 1.24 (ddd, J = 9.7, 6.1, 4.9 Hz, 1H), 1.37 (ddd, J = 9.9, 6.1, 4.1 Hz, 1H), 1.27 (s, 3H), 2.00 (s, 3H), 2.04 (s, 3H), 3.26 (s, 3H), 3.91 (br s, 1H), 4.29 (dd, 2H), 7.0 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 197.6, 159.7, 142.6, 138.8, 134.3, 129.7, 129.6, 75.9, 65.3, 57.3, 37.3, 27.3, 15.7, 13.9, 12.9, 9.1; EI-HRMS for $C_{16}H_{20}O_3$ calcd 260.1407, found 260.1408 (M^+); UV λ_{max} (EtOH) 331 nm (ϵ 5439).

Ether 7 from 5 and Diethyl Ether. To a stirred solution of **5** (22 mg, 0.89 mmol) in 3 mL of acetone and 1 M H_2SO_4 solution (1:1) was added 7.5 mL of Et_2O. The mixture was stirred at room temperature for 24 h and was then partitioned between EtOAc and water. The organic extracts were washed with saturated $NaHCO_3$ and saline, respectively, until neutral. After being dried over $MgSO_4$, the solution was concentrated and chromatographed to give 17 mg of **7** (80%) as a yellow gum: IR (KBr) 3457, 2968, 1659, 1592, 1502, 1284, 1097 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 0.72 (m, 1H), 1.08 (m, 1H), 1.23 (t, J = 6.9 Hz, 3H), 1.33 (m, 1H), 1.38 (s, 3H), 1.48 (m, 1H), 2.11 (s, 3H), 2.14 (s, 3H), 3.53 (q, J = 6.9, Hz, 2H), 3.91 (s, 1H), 4.42 (q, J_{AB}=10.7 Hz, 2H), 7.10 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 197.4, 159.5, 142.2, 138.8, 134.3, 130.0, 126.4, 75.8, 65.0, 63.5, 37.2, 27.2, 15.6, 14.8, 13.8, 12.7, 9.0; EI-HRMS for $C_{17}H_{22}O_3$ calcd 274.1569, found 274.1568; UV λ_{max} 330 nm (ϵ 7225). The same ether (**7**) was obtained with EtOH instead of Et_2O.

Synthesis of Dimer Ether 8 of Irofulven. To a solution of **5** (36 mg, 0.146 mmol) in 3 mL of acetone and 1 M H_2SO_4 solution (1:1) was added 0.5 mL of Et_2O. The mixture was stirred at room temperature for 30 h and was partitioned between EtOAc and water. The organic extracts were washed with saturated $NaHCO_3$ and saline, respectively, until neutral. After being dried over $MgSO_4$, the solution was concentrated and chromatographed to give 5 mg of **8** (14%), 11 mg of **7**, and 13 mg of **5**. Compound **8** was a yellow gum: IR (KBr) 3433, 2920, 1659, 1592, 1502, 1350, 1163 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 0.67 (m, 1H), 1.08 (m, 1H), 1.31 (m, 1H), 1.37 (s, 3H), 1.48 (m, 1H), 2.07 (s, 3H), 2.11 (s, 3H), 4.48 (s, 2H), 7.10 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 197.9, 159.9, 143.3, 139.1, 134.6, 129.6, 126.8, 76.1, 63.2, 37.6, 27.5, 15.9, 14.2, 13.1, 9.4; EI-HRMS for $C_{30}H_{35}O_5$ (M + H) calcd 475.2535, found 475.2467; UV λ_{max} 330 nm (ϵ 12 905).

Reaction of 5 with Ethylene Glycol. To a stirred solution of **5** (9 mg, 0.037 mmol) in 9 mL of acetone and 1 M H_2SO_4 solution (1:1) was added 4.5 mL of ethylene glycol. The mixture was stirred at room temperature for 2 h and was partitioned between EtOAc and water. The organic extracts were washed with saturated $NaHCO_3$ and saline, respectively, until neutral. After being dried over $MgSO_4$, the solution was concentrated and chromatographed to give 11 mg of **9** (100%) as a yellow gum: IR (KBr) 3439, 2914, 1665, 1598, 1508, 1344, 1103 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 0.71 (m, 1H), 1.06 (m, 1H), 1.32 (m, 1H), 1.36 (s, 3H), 1.47 (m, 1H), 2.11 (s, 3H), 2.14 (s, 3H), 2.55 (s, 1H), 3.57 (t, J = 4.5 Hz, 2H), 3.73 (t, J = 4.5 Hz, 2H), 3.98 (s, 1H), 4.50 (q, J_{AB} = 12 Hz, 2H), 7.09 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 197.9, 160.0, 142.9, 138.9, 134.5, 129.6, 126.8, 76.1, 70.9, 64.2, 61.6, 37.5, 27.4, 16.0, 14.1, 13.1, 9.3; EI-HRMS for $C_{17}H_{22}O_4$ calcd 290.1518, found 290.1515; UV λ_{max} 331 nm (ϵ 9404).

Reaction of 5 with 2-Bromoethanol. To a stirred solution of **5** (188 mg, 0.764 mmol) in 10 mL of acetone and 1 M H_2SO_4 solution (1:1) was added 5 mL of 2-bromoethanol. The mixture was stirred at room temperature for 4.5 h and was partitioned between EtOAc and water. The organic extracts were washed with saturated $NaHCO_3$ and saline, respectively, until neutral. After being dried over $MgSO_4$, the solution was concentrated and chromatographed to give 179 mg of **10** (66%) and a trace amount of a diether ($C_{15}H_{17}O_2$−$OCH_2CH_2OCH_2CH_2$Br). Compound **10** formed yellow crystals: mp 92−94 °C; IR (KBr) 3445, 2914, 1650, 1592, 1502, 1097 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 0.71 (m, 1H), 1.07 (m, 1H), 1.35 (m, 1H), 1.38 (s, 3H), 1.48 (m, 1H), 2.15 (s, 3H), 3.47 (t, J = 6.0 Hz, 2H), 3.77 (t, J = 6.0 Hz, 2H), 3.91 (s, 1H), 4.54 (q, J_{AB} = 12 Hz, 2H), 7.09 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 198.1, 160.6, 143.2, 138.9, 134.4, 129.3, 127.0, 76.3, 69.4, 64.1, 37.7, 30.6, 27.6, 16.4, 14.3, 13.2, 9.5; EI−HRMS

for $C_{17}H_{20}BrO_3$ calcd 352.0674, found 352.0671; UV λ_{max} 332 nm (ϵ 7777).

The diether was a yellow gum: ^{1}H NMR ($CDCl_3$) δ 0.72 (m, 1H), 1.05 (m, 1H), 1.32 (m, 1H), 1.37 (s, 3H), 1.50 (m, 1H), 2.13 (s, 3H), 2.15 (s, 3H), 3.46 (t, J = 6.3 Hz, 2H), 3.65 (m, 4H), 3.79 (t, J = 6.3 Hz, 2H), 3.90 (s, 1H), 4.51 (q, J_{AB} = 12 Hz, 2H), 7.09 (s, 1H); EI-HRMS for $C_{19}H_{26}BrO_4$ calcd 397.1015, found 397.0996.

Reaction of Irofulven with Glycerol. To a stirred solution of **5** (110 mg, 0.447 mmol) in 15 mL of acetone and 1 M H_2SO_4 solution (1:1) was added 5 mL of glycerol. The mixture was stirred at room temperature for 22 h and was partitioned between EtOAc and water. The organic extracts were washed with saturated $NaHCO_3$ and saline, respectively, until neutral. After being dried over $MgSO_4$, the solution was concentrated and chromatographed (adding 5% methanol to the solvent system) to give 79 mg of **11** (55% mixture of isomers) as a yellow gum (with 40 mg of **5** recovered): IR (KBr) 3415, 2926, 1659, 1586, 1103 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 0.72 (m, 1H), 1.08 (m, 1H), 1.26 (m, 1H), 1.37 (s, 3H), 1.50 (m, 1H), 2.10 (s, 3H), 2.15 (s, 3H), 2.57 (s, 1H), 3.58 (m, 4H), 3.86 (m, 1H), 3.91 (s, 1H), 4.51 (q, J_{AB} = 12.9 Hz, 2H), 7.10 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 198.0, 160.1, 143.2, 138.8, 134.6, 129.4, 126.9, 76.2, 70.9, 70.6, 64.4, 63.8, 37.6, 27.4, 16.1, 14.2, 13.1,9.4; EI-HRMS for $C_{18}H_{24}O_5$ calcd 320.1623, found 320.1616; UV λ_{max} 331 nm (ϵ 7920).

Reaction of 5 with Fructose. To a stirred solution of **5** (1.5 g, 6.098 mmol) in 66 mL of acetone and 40 mL of 1 M H_2SO_4 solution was added 20 g of fructose. The mixture was stirred at room temperature overnight and worked up the usual way employing methylene chloride and methanol as solvents. Chromatography afforded 350 mg of **12** and **14** (14%, mixture) as a yellow gum (701 mg HMAF recovered): EI-HRMS for $C_{21}H_{29}O_8$ $(M + H)^+$ calcd 409.1863, found 409.1869; UV λ_{max} 332 nm (ϵ 4745).

The mixture was resolved by HPLC (isocratic elution with CH_3CN and H_2O) into two major compounds **12** and **14** and two minor compounds (structures undetermined). See Tables 1−3 for spectral data.

Reaction of 5 with Acetic Anhydride. To a stirred solution of **5** (300 mg, 1.22 mmol) in acetic anhydride (4 mL) was added sodium acetate (287 mg, 3.5 mmol). The mixture was stirred overnight at room temperature and then filtered, and the filtrate was concentrated under reduced pressure. Chromatography of the residue afforded the acetate **15** (261 mg, 74%) as a yellow gum: ^{1}H NMR ($CDCl_3$) δ 0.740 (m, 1H), 1.103 (m, 1H), 1.324 (m, 1H), 1.385 (s, 3H), 1.507 (m, 1H), 2.037 (s, 3H), 2.086 (s, 3H), 2.171 (s, 3H), 3.898 (s, 1H), 5.100 (s, 2H), 7.109 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 197.56, 170.46, 159.39, 144.25, 138.54, 134.34, 127.23, 126.93, 76.68, 58.21, 37.78, 27.55, 20.99, 16.16, 14.42, 13.27, 9.62. EI-HRMS for $C_{17}H_{20}O_4$ calcd 288.1362, found 288.1364; UV λ_{max} 330 nm (ϵ 5904).

When **5** was treated with $BF_3{\cdot}Et_2O$ in acetic anhydride at −78 °C, **15** was the major product and **16** was obtained in low yield as a yellow gum: ^{1}H NMR ($CDCl_3$) δ 0.97 (m, 1H), 1.16 (m, 2H), 1.46 (m, 1H), 1.51 (s, 3H), 2.10 (s, 3H), 2.14 (s, 3H), 2.19 (s, 3H), 4.60 (s, 1H), 4.65 (s, 2H), 7.18 (s, 1H); EI-HRMS for $C_{17}H_{20}O_4$ calcd 288.1362, found 288.1364

Reaction of 5 with Benzoyl Chloride. To a stirred solution of irofulven (116 mg, 0.447 mmol) in 10 mL of methylene chloride was added 0.10 mL of pyridine and 0.25 mL of benzoyl chloride at room temperature under argon. The mixture was stirred for 2 h and was partitioned between EtOAc and water. The organic extracts were washed with saturated saline. After being dried over $MgSO_4$, the solution was concentrated and chromatographed to give 20 mg of **17** (92%) as a yellow gum (with 13 mg of **5** recovered): ^{1}H NMR ($CDCl_3$) δ 0.65 (m, 1H), 1.02 (m, 1H), 1.18 (m, 1H), 1.32 (s, 3H), 1.44 (m, 1H), 2.03 (s, 3H), 2.16 (s, 3H), 3.86 (s, 1H), 5.28 (q, J_{AB} = 13.2 Hz, 2H), 7.06 (s, 1H).

Reaction of 5 with Phenyl Chloroformate. To a stirred solution of **5** (163 mg, 0.663 mmol) in 10 mL of methylene chhloride was added 0.18 mL of pyridine and 0.34 mL of phenyl chloroformate at 0 °C under argon. The mixture was stirred for 3 h and was partitioned between EtOAc and water. The organic extracts were washed with saturated saline. After being dried over $MgS0_4$, the solution was concentrated and chromatographed to give 20 mg of **18** as a yellow gum: ^{1}H NMR ($CDCl_3$) δ 0.85 (m, 1H), 1.18 (m, 1H), 1.43 (m, 1H), 1.52 (s, 3H), 1.61 (m, 1H), 2.12 (s, 3H), 2.28 (s, 3H), 4.04 (s, 1H), 5.06 (q, J_{AB} = 11.1 Hz, 2H), 6.93−7.47 (m, 6H).

Reaction of 5 with 2-Methoxypropene. To a stirred solution of **5** (260 mg, 1.057 mmol) in 6 mL of 2-methoxypropene was added two drops $POCl_3$. The mixture was stirred at room temperature for 6 days and was then partitioned between EtOAc and water. The organic extracts were washed with saturated $NaHCO_3$ and saline, respectively, until neutral. After being dried over $MgSO_4$, the solution was concentrated and chromatographed to give 133 mg of **19** (40%) as yellow crystals (with 87 mg of **5** recovered): mp 93−95 0 °C; IR (KBr) 3457, 2980, 1665, 1598, 1502, 1091 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 0.72 (m, 1H), 1.06 (m, 1H), 1.25 (m, 1H), 1.38 (s, 3H), 1.41 (s, 3H), 1.42 (s, 3H), 1.49 (m, 1H), 2.15 (s, 3H), 3.25 (s, 6H), 3.95 (s, 1H), 4.43 (s, 2H), 7.11 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 197.7, 159.5, 142.2, 134.9, 134.8, 130.5, 126.7, 100.3, 76.1, 54.4, 48.6, 37.4, 27.5, 24.4, 24.3, 15.9, 14.0, 13.0, 9.3; EI-HRMS for $C_{19}H_{26}O_4$ calcd 318.1831, found 318.1823; UV λ_{max} 330 nm (ϵ 8728).

Reaction of Acylfulvene with Acrolein. To a stirred solution of acylfulvene (**4**, 1 g, 4.63 mmol) in 5 mL of acetone and 2.5 mL of 1 M H_2SO_4 solution was added 2.5 mL of acrolein. The mixture was stirred at room temperature for 7 h and was partitioned between EtOAc and water. The organic extracts were washed with saturated $NaHCO_3$ and saline, respectively, until neutral. After being dried over $MgSO_4$, the solution was concentrated and chromatographed to give 378 mg of **20** (30%) and 241 mg of **21** (14%).

Compound **20** was a yellow gum: ^{1}H NMR ($CDCl_3$) δ 0.68 (m, 1H), 1.07 (m, 1H), 1.32 (m, 1H), 1.36 (s, 3H), 1.46 (m, 1H), 2.01 (s, 3H), 2.06 (s, 3H), 2.65 (t, J = 7.8 Hz, 2H), 3.00 (m, 2H), 3.93 (s, 1H), 7.12 (s, 1H), 9.83 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 200.4, 196.3, 157.3, 139.4, 138.3, 135.4, 133.7, 125.3, 75.4, 43.5, 36.9, 27.0, 19.5, 15.4, 13.4, 12.4, 8.6; EI-HRMS for $C_{17}H_{20}O_3$ (M^+) calcd 272.1413, found 272.1416; UV λ_{max} 331 nm (ϵ 8500).

Compound **21** was also a yellow gum (isomeric mixture): EI-HRMS for $C_{23}H_{28}O_5$ (M^+) calcd 384.1937, found 384.1947; UV λ_{max} 329 nm (ϵ 6000).

Reduction of Aldehyde 20. To a stirred solution of **20** (30 mg, 0.110 mmol) in 5 mL of THF was added 5 drops of HOAc and excess sodium cyanoborohydride. The mixture was stirred at room temperature for 1 h and was partitioned between EtOAc and water. The organic extracts were washed with saturated NH_4Cl and saline, respectively, until neutral. After being dried over $MgSO_4$, the solution was concentrated and chromatographed to give 21 mg of **22** (69%) as a yellow gum: ^{1}H NMR ($CDCl_3$) δ 0.67 (m, 1H), 1.06 (m, 1H), 1.26 (m, 1H), 1.36 (s, 3H), 1.46 (?,1H), 1.73 (m, 2H), 2.06 (s, 3H), 2.07 (s, 3H), 2.74 (m, 2H), 3.70 (t, J = 6.3 Hz, 2H), 3.96 (s, 1H), 7.14 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 197.0, 157.7, 139.6, 139.0, 136.6, 136.5, 128.2, 75.9, 62.0, 37.3, 33.0, 27.5, 24.0, 15.9, 13.8, 12.8, 9.0; EI-HRMS for $C_{17}H_{22}O_3$ calcd 274.1569, found 274.1557; UV λ_{max} 330 nm (ϵ 6700).

Oxidation of Aldehyde 20. To a stirred solution of aldehyde **20** (44 mg, 0.162 mmol) in acetone (6 mL) in an ice bath was added 6 drops of Jones reagent. The mixture was stirred for 20 min at 0 °C and was quenched by addition of methanol. The mixture was then partitioned between EtOAc and saline. The organic extracts were washed twice with saline and dried over $MgSO_4$. After concentration, the crude product was chromagraphed to give the acid **23** (29 mg, 62% yield) as a yellow gum: ^{1}H NMR ($CDCl_3$) δ 0.69 (m, 1H), 0.88 (m, 1H), 1.05 (m, 1H), 1.36 (s, 3H), 1.47 (m, 1H), 2.06 (s, 3H), 2.07 (s, 3H), 2.52 (m, 2H), 3.03 (m, 2H), 7.13 (s, 1H).

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