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Preparation of 2*H*-Furo[2,3-*c*]pyran-2-one Derivatives and Evaluation of Their Germination-Promoting Activity

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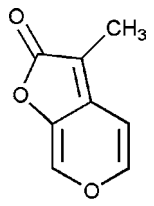
The butenolide, 3-methyl-2*H*-furo[2,3-*c*]pyran-2-one (**1**), has recently been identified as the germination stimulant present in smoke that promotes the germination of seeds from a wide range of plant species. In this paper, we describe the preparation of a number of analogues of **1** and compare their efficacy in promoting seed germination of three highly smoke-responsive plant species, *Lactuca sativa* L. cv. Grand Rapids (Asteraceae), *Emmenanthe penduliflora* Benth. (Hydrophyllaceae), and *Solanum orbiculatum* Poir. (Solanaceae). The results show that the methyl substituent at C-3 in **1** is important for germination-promoting activity while substitution at C-7 reduces activity. In contrast, bioactivity is mostly retained with analogues substituted at C-4 or C-5.

KEYWORDS: Butenolide; seed germination; seed dormancy; smoke; germination stimulant

INTRODUCTION

Plant-derived smoke has been reported to promote the germination of a variety of wild species from regions as diverse as South Africa (*1*), Australia (*2*), North America (*3*), and Europe (*4*). A number of important crop species including lettuce (*5*), maize (*6*), and celery (*7*) have also shown significant increases in germination when treated with smoke, as have selected weed species (*8*, *9*). There have been many attempts to identify the active agent in smoke, and recently, the butenolide, 3-methyl-2*H*-furo[2,3-*c*]pyran-2-one (**1**), was identified as the key stimulant (*10*). Confirmation of the germination activity of **1** has been demonstrated with a variety of smoke-responsive plant species with activities observed at below ppb concentrations ($<10^{-9}$ M), thus providing a new class of potent germination stimulants.

With the identity of the germination stimulant in smoke now known, a better understanding of the way in which **1** acts in promoting seed germination can be investigated. To date, little is known about the mechanism of action of **1** in stimulating seed germination. Furthermore, nothing is known about the activity of analogues of **1**. The preparation of analogues of **1** and assessment of their ability to promote germination will give some insight into the molecular aspects of **1** that are important for its germination-promoting activity. In addition, knowledge of which part of the molecule that can be elaborated without significantly impairing the germination activity would be useful in the design of labeled analogues suitable for determining the site and mode of action.



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In this paper, we report on the synthesis of a number of analogues of **1** and the germination activity of these analogues with seeds of three highly smoke-responsive plant species, *Lactuca sativa* L. cv. Grand Rapids (Asteraceae), *Emmenanthe penduliflora* Benth. (Hydrophyllaceae), and *Solanum orbiculatum* Poir. (Solanaceae). The germination-promoting abilities of the analogues have been compared with the parent butenolide (**1**), and some structure−activity relationships are discussed.

MATERIALS AND METHODS

General. Melting points (mp) were determined using a Kofler hot-stage apparatus and are uncorrected. High-resolution mass spectra (HRMS) were recorded using a VG Autospec mass spectrometer using electron impact (70 eV) ionization. ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded using a Bruker ARX-300, Bruker AV-500, or Bruker AV-600 spectrometer. Chemical shifts are measured on the δ scale (in ppm) in d_6-acetone with residual acetone used as the internal standard (^{1}H, δ 2.04; and ^{13}C, δ 29.8). The signals are described as singlet (s), doublet (d), triplet (t), and quartet (q).

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Solvents used were of technical grade and were distilled before use. Millipore (MP) water was obtained by passage through a Milli-Q ultrapure water system (Millipore, Australia).

Maltol (**7**), 2-chloropropionyl chloride, and 2-chloroacetyl chloride were obtained from Sigma-Aldrich. Pyromeconic acid (**3**), allomaltol (**8**), 2,6-dimethyl-3-hydroxy-4*H*-pyran-4-one (**9**), and 3-hydroxy-6-(methoxymethyl)-4*H*-pyran-4-one (**13**) were prepared from kojic acid (**2**) (Merck) as described by Ellis et al. (*11*). Bromomaltol (**15**) was prepared from **7** as described by Looker et al. (*12*).

Statistical Analyses. Data generated were statistically analyzed by analysis of variance (ANOVA). Percentage germination data were transformed ($arcsine\sqrt{}$) to conform to ANOVA assumptions (untransformed data appears in Figures). Mean comparisons were made using Fisher's protected least significant difference, at the 95% confidence level ($p < 0.05$).

Germination Testing. For testing, 1−2 mg of each analogue was dissolved in MP water to give a stock concentration of 10 mg/L. A series of 10-fold dilutions were performed to give four test solutions of concentrations 1 mg/L and 100, 10, and 1 μg/L.

Grand Rapids Lettuce Seed. Grand Rapids lettuce seeds (Waltham strain) were obtained from R.B. Dessert Seed Co. and were air-dried and stored at −18 °C in sealed laminated foil sachets until required. For testing, 2.5 mL of each test solution was applied to three replicate Petri dishes (90 mm) lined with two layers of Whatman #1 filter paper (7 cm). MP water served as a control for each experiment. In a dark room, 40−50 seeds were added to the Petri dishes, which were sealed and stored in a light-proof container and incubated at 19 ± 2 °C. All manipulations involving the seed were carried out in a dark room, and germinants, based on the appearance of a radicle, were scored after 48 h of incubation.

E. penduliflora and S. orbiculatum. The *S. orbiculatum* seeds were collected in the Shark Bay region (Western Australia) in November 2004 and were stored under ambient laboratory conditions (c. 23 °C and 50% relative humidity) until required. *E. penduliflora* was sourced from garden-collected material in California from Rancho Santa Ana Botanic Garden. Solutions were tested by adding 2.5 mL to two pieces of Whatman #1 filter paper (7 cm) in Petri dishes (9 cm). MP water served as the control. Approximately 20−25 seeds of each species were added to each Petri dish, and each solution was tested in triplicate. The Petri dishes were sealed with a layer of plastic wrap and incubated at 19 ± 2 °C in a light-proof container for 6 days before counting.

General Method for Converting Pyran-4-one Derivatives to Pyran-4-thiones. Phosphorus pentasulphide (1.5 equiv) in tetrahydrofuran was added to a stirred solution of the pyran-4-one compound dissolved in tetrahydrofuran (10 mL) following the general method of Scheeren et al. (*13*). Solid sodium hydrogen carbonate (6 equiv) was added, and the reaction mixture was stirred at room temperature and monitored by thin-layer chromatography for completion (ca. 3 h). The reaction mixture was added cautiously to water (100 mL), and the resulting aqueous mixture was extracted with ethyl acetate (4 × 20 mL). The combined organic extract was washed with 0.2 M $NaHCO_3$ (2 × 20 mL) and saturated brine, then dried (Na_2SO_4), filtered, and evaporated to dryness under reduced pressure. The residue was purified by rapid silica filtration (60% ethyl acetate/light petrol) to yield the pure thione (typical yields, 60−80%).

General Method for Esterification. Triethylamine (1.2 equiv) was added to a stirred solution of the thione (1 equiv) in dichloromethane (10 mL) at 0 °C. A solution of the acyl chloride (1.5 equiv) diluted in dichloromethane (1 mL) was added dropwise to the solution, and the reaction mixture was stirred for a further 10 min at 0 °C. The solution was evaporated to dryness under reduced pressure, and the resulting residue was purified by rapid silica filtration (dichloromethane) to afford the ester (typical yields 80−90%). The ester was prone to hydrolysis when stored and so was used immediately in the cyclization reaction.

General Method for Butenolide Cyclization. A mixture of anhydrous sodium acetate (3 equiv) and triphenyl phosphine (1.1 equiv) in acetic anhydride (20 mL) was heated at reflux for 5 min. A solution of the thione ester (1 equiv) diluted with acetic anhydride (2 mL) was added dropwise to the refluxing mixture. The mixture was refluxed for a further 30 min and allowed to cool. The dark mixture was poured into water (100 mL) and stirred until one phase was formed. The aqueous solution was filtered and extracted with dichloromethane (3 × 20 mL). The organic extract was washed with 1 M $NaHCO_3$ (2 × 20 mL), dried (Na_2SO_4), filtered, and evaporated to dryness. The residue was extracted with 0.2 M potassium carbonate solution (2 × 50 mL) by heating gently, and the resulting yellow solution was filtered and extracted with dichloromethane (3 × 15 mL). The organic extract was washed with brine, dried (Na_2SO_4), filtered, and evaporated to dryness to give a yellow residue. The residue was purified by silica gel chromatography (20−30% ethyl acetate/light petroleum) to afford the appropriate butenolide compound (typical yields, 1−25%).

3,7-Dimethyl-2*H*-furo[2,3-*c*]pyran-2-one (10). Compound **7** was converted to the thione and esterified with 2-chloropropionyl chloride as described above. The thiono-maltol ester (350 mg, 1.5 mmol) was refluxed in acetic anhydride to afford **10** as a white solid (45 mg, 0.27 mmol, 18%), which crystallized from hexane (mp 136−137 °C). ^{1}H NMR (500.1 MHz, d_6-acetone): δ 7.59 (1H, d, J = 5.5 Hz, H-5); 6.73 (1H, d, J = 5.5 Hz, H-4); 2.32 (3H, s, CH_3); 1.84 (3H, s, CH_3). ^{13}C NMR (125.8 MHz, d_6-acetone): δ 171.2 (C═O); 149.6 (C-5); 140.8 (C-3a); 138.9 (C-7a); 137.3 (C-7); 103.7 (C-4); 99.4 (C-3); 13.8 (CH_3); 7.7 (CH_3). HRMS calculated for $C_9H_8O_3$, 164.0473; found, 164.0478.

3,5-Dimethyl-2*H*-furo[2,3-*c*]pyran-2-one (11). Compound **8** was converted to the thione and esterified with 2-chloropropionyl chloride as described above. The thioester (700 mg, 3 mmol) was refluxed in acetic anhydride to afford **11** as a light yellow solid (57.4 mg, 0.35 mmol, 12%), which crystallized from hexane (mp 101−102 °C). ^{1}H NMR (500.1 MHz, d_6-acetone): δ 7.71 (1H, s, H-7); 6.58 (1H, s, H-4); 2.28 (3H, s, CH_3), 1.83 (3H, s, CH_3). ^{13}C NMR (125.8 MHz, d_6-acetone): δ 171.5 (C═O); 159.9 (C-5); 142.4 (C-3a); 142.4 (C-7a); 127.7 (C-7); 101.1 (C-4); 98.4 (C-3); 19.8 (CH_3); 7.5 (CH_3). HRMS calculated for $C_9H_8O_3$, 164.0473; found, 164.0476.

3,5,7-Trimethyl-2*H*-furo[2,3-*c*]pyran-2-one (12). Compound **9** was converted to the thione and esterified with 2-chloropropionyl chloride as described above. The thioester (207 mg, 0.84 mmol) was refluxed in acetic anhydride to afford **12** as a light yellow solid (3.0 mg, 0.02 mmol, 2%), which crystallized from hexane (mp 105−106 °C). ^{1}H NMR (600.1 MHz, d_6-acetone): δ 6.49 (1H, s, H-4); 2.28 (3H, s, CH_3); 2.27 (3H, s, CH_3); 1.79 (3H, s, CH_3). ^{13}C NMR (150.9 MHz, d_6-acetone): δ 171.5 (C═O); 159.5 (C-5); 142.4 (C-7a); 138.2 (C-7); 136.7 (C-3a); 100.7 (C-4); 97.8 (C-3); 19.7 (CH_3); 13.8 (CH_3); 7.6 (CH_3). HRMS calculated for $C_{10}H_{10}O_3$, 178.0630; found, 178.0629.

5-Methoxymethyl-3-methyl-2*H*-furo[2,3-*c*]pyran-2-one (14). Compound **13** was converted to the thione and esterified with 2-chloropropionyl chloride as described above. The thioester (140 mg, 0.5 mmol) was refluxed in acetic anhydride to afford **14** as a light yellow solid (2 mg, 0.01 mmol, 2%). ^{1}H NMR (600.1 MHz, d_6-acetone): δ 7.75 (1H, s, H-7); 6.78 (1H, t, J = 0.8 Hz, H-4); 4.27 (2H, d, J = 0.8 Hz, CH_2); 3.40 (3H, s, OCH_3); 1.87 (3H, s, CH_3). ^{13}C NMR (150.9 MHz, d_6-acetone): δ 171.3 (C═O); 158.8 (C-5); 142.6 (C-7a); 141.4 (C-3a); 127.6 (C-7); 101.3 (C-4); 100.3 (C-3); 71.0 (CH_2); 58.8 (OCH_3); 7.6 (CH_3). HRMS calcd for $C_{10}H_{10}O_4$, 194.0579; found, 194.0586.

4-Bromo-3,7-dimethyl-2*H*-furo[2,3-*c*]pyran-2-one (16). Compound **15** was converted to the thione and esterified with 2-chloropropionyl chloride as described above. The thioester (90 mg, 0.3 mmol) was refluxed in acetic anhydride to afford **16** as a light yellow solid (1.2 mg, 0.01 mmol, 2%). ^{1}H NMR (500.1 MHz, d_6-acetone): δ 7.85 (1H, s, H-5); 2.32 (3H, s, CH_3); 2.07 (3H, s, CH_3). ^{13}C NMR (125.8 MHz, d_6-acetone): δ 170.7 (C═O); 148.5 (C-5); 138.2 (C-3a); 137.7 (C-7); 137.2 (C-7a); 102.4 (C-3); 100.3 (C-4); 13.7 (CH_3); 8.3 (CH_3). HRMS calcd for $C_9H_7BrO_3$, 241.9579; found, 241.9570.

2*H*-Furo[2,3-*c*]pyran-2-one (17). Pyromeconic acid thione (**4**) (200 mg, 1.6 mmol) was esterified with 2-chloroacetyl chloride as described above. Attempts to purify the ester by rapid silica filtration were unsuccessful; therefore, the ester reaction mixture was evaporated to dryness under reduced pressure, and the residue was used directly in the cyclization step. Acetic anhydride was added, and the mixture was filtered through a plug of cotton wool. The filtered solution was added dropwise to a mixture of refluxing acetic anhydride, triphenylphosphine, and sodium acetate as described above. Workup and purification

afforded the desmethyl butenolide (**17**) as a light yellow solid (14.2 mg, 0.1 mmol, 7%), which crystallized from hexane (mp 105−106 °C). ^{1}H NMR (300.1 MHz, d_6-acetone): δ 7.92 (1H, d, J = 1.5 Hz, H-7); 7.70 (1H, d, J = 5.4 Hz, H-5); 6.90 (1H, dd, J = 5.4, 0.5 Hz, H-4); 5.40 (1H, dd, J = 1.5, 0.5 Hz, H-3). ^{13}C NMR (75.5 MHz, d_6-acetone): δ 170.5 (C═O); 151.1 (C-5); 146.1 (C-3a); 144.1 (C-7a); 129.4 (C-7); 105.5 (C-4); 90.7 (C-3). HRMS calculated for $C_7H_4O_3$, 136.0160; found, 136.0164.

7-Methyl-2*H*-furo[2,3-*c*]pyran-2-one (18). Compound **7** was converted to the thione (100 mg, 0.7 mmol) and esterified with 2-chloroacetyl chloride as described above. The reaction mixture was evaporated to dryness under reduced pressure, and the residue was used directly in the cyclization step. Acetic anhydride was added, and the mixture was filtered through a plug of cotton wool. The filtered solution was added dropwise to a mixture of refluxing acetic anhydride, triphenylphosphine, and sodium acetate as described above. Workup and purification afforded **18** as a light yellow solid (1.3 mg, 0.01 mmol, 1%). ^{1}H NMR (600.1 MHz, d_6-acetone): δ 7.67 (1H, d, J = 5.4 Hz, H-5); 6.82 (1H, d, J = 5.4 Hz, H-4); 5.34 (1H, s, H-3); 2.36 (3H, s, CH_3). ^{13}C NMR (150.9 MHz, d_6-acetone): δ 170.6 (C═O); 150.7 (C-5); 146.0 (C-3a); 140.2 (C-7a); 138.9 (C-7); 105.1 (C-4); 90.1 (C-3); 14.0 (CH_3). HRMS calculated for $C_8H_6O_3$, 150.0317; found, 150.0314.

5-Methyl-2*H*-furo[2,3-*c*]pyran-2-one (19). Compound **8** was converted to the thione (200 mg, 1.4 mmol) and esterified with 2-chloroacetyl chloride as described above. The ester reaction mixture was evaporated to dryness under reduced pressure, and the residue was used directly in the cyclization step. Acetic anhydride was added, and the mixture was filtered through a plug of cotton wool. The filtered solution was added dropwise to a mixture of refluxing acetic anhydride, triphenylphosphine, and sodium acetate as described above. Workup and purification afforded **19** as a light yellow solid (1.7 mg, 0.01 mmol, 1%). ^{1}H NMR (600.1 MHz, d_6-acetone): δ 7.84 (1H, d, J = 1.4 Hz, H-7); 6.67 (1H, q, J = 0.7 Hz, H-4); 5.26 (1H, d, J = 1.4 Hz, H-3); 2.32 (3H, d, J = 0.7 Hz, CH_3). ^{13}C NMR (150.9 MHz, d_6-acetone): δ 170.9 (C═O); 161.3 (C-5); 147.8 (C-3a); 143.4 (C-7a); 128.9 (C-7); 102.6 (C-4); 89.2 (C-3); 14.0 (CH_3). HRMS calculated for $C_8H_6O_3$, 150.0317; found, 150.0312.

3-Methylfuro[2,3-*c*]pyridin-2(3*H*)one (21). Compound **1** (15 mg, 0.1 mmol) was heated with a concentrated NH_4OH solution (28% NH_3, 3 mL) on a steam bath for 5 h following the method of Hwang et al. (*14*). The resulting solution was evaporated to dryness under reduced pressure and purified by silica gel chromatography (20% MeOH/ethyl acetate/1% triethylamine) to give **21** as a light brown solid (1.5 mg, 0.01 mmol, 10%). ^{1}H NMR (600.1 MHz, d_6-acetone): δ 8.14 (1H, s, H-7); 7.97 (1H, d, J = 4.9 Hz, H-5); 7.05 (1H, d, J = 4.9, H-4); 3.90 (1H, q, J = 7.2 Hz, H-3); 1.48 (3H, d, J = 7.2 Hz, CH_3). ^{13}C NMR (150.9 MHz, d_6-acetone): δ 178.7 (C═O); 153.3 (C-7a); 142.0 (C-5); 140.9 (C-7); 135.2 (C-3a); 124.3 (C-4); 44.6 (C-3); 17.4 (CH_3). HRMS calculated for $C_8H_7NO_2$, 149.0477; found, 149.0479.

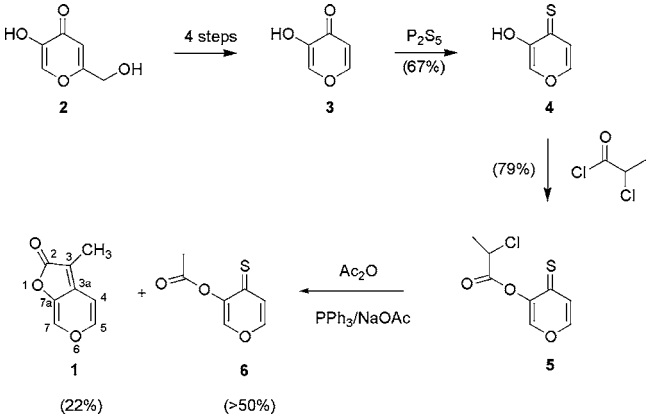
3,6-Dimethylfuro[2,3-*c*]pyridin-2(6*H*)one (22). Compound **1** (10.6 mg, 0.07 mmol) was heated in a sealed tube with aqueous methylamine solution (24%, 3 mL) overnight at 70 °C following the method of Liu et al. (*15*). The resulting solution was evaporated to dryness under reduced pressure and purified by silica gel chromatography (10% MeOH/ethyl acetate/1% triethylamine) to give **22** as a light brown solid (1.6 mg, 0.01 mmol, 14%). ^{1}H NMR (600.1 MHz, d_6-acetone): δ 7.38 (1H, dd, J = 6.9, 1.4 Hz, H-5); 7.32 (1H, d, J = 1.4 Hz, H-5); 6.54 (1H, d, J = 6.9, H-4); 3.81 (3H, s, N−CH_3); 1.79 (3H, s, CH_3). ^{13}C NMR (150.9 MHz, d_6-acetone): δ 172.0 (C═O); 144.2 (C-3a); 143.7 (C-7a); 135.8 (C-5); 114.5 (C-7); 103.3 (C-4); 86.0 (C-3); 44.1 (N−CH_3); 7.4 (CH_3). HRMS calcd for $C_9H_9NO_2$, 163.0633; found, 163.0628.

RESULTS AND DISCUSSION

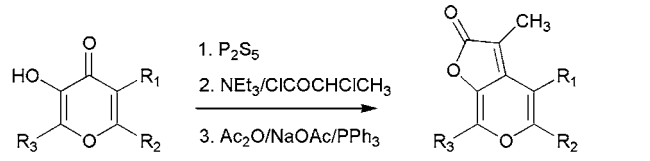
Synthesis of Analogues. The synthesis of the butenolide, **1**, has only recently been reported (*10*) and, to date, represents the only method for preparing this new class of compound. The overall synthesis was achieved in seven steps from **2**, a fungal metabolite (*16*) that is commercially available. In the synthesis, **2** was converted over four steps to **3** (*11*), which was subsequently treated with phosphorus pentasulphide to give **4** (**Scheme 1**). Esterification of **4** with 2-chloropropionyl chloride in the presence of triethylamine afforded the ester **5**, which was refluxed in acetic anhydride with triphenyl phosphine and sodium acetate to give **1**. The yield of **1** was low (22%) mainly due to the formation of the transesterification product **6**, which can be recovered and hydrolyzed back to **4** if required.

For preparing analogues of **1**, the use of alternative starting materials would provide a simple route. In particular, substituting **3** for simple variants such as **7**, **8**, and **9** should provide

Scheme 1. Synthesis of **1** from **2**



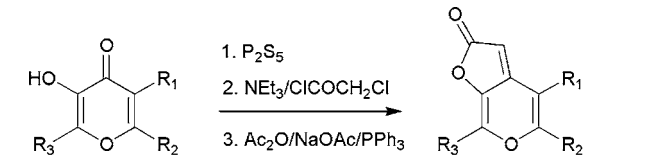
Scheme 2. Synthesis of Analogues of **1** from Variants of **3**



7: R_1, R_2=H, R_3=CH_3
8: R_1, R_3=H, R2=CH_3
9: R_1, R_2, R_3=CH_3
13: R_1, R_3=H, R_2=CH_2OCH_3
15: R_1=Br, R_2=H, R_3=CH_3

10: R_1, R_2=H, R_3=CH_3
11: R_1, R_3=H, R_2=CH_3
12: R_1, R_2, R_3=CH_3
14: R_1, R_3=H, R_2=CH_2OCH_3
16: R_1=Br, R_2=H, R_3=CH_3

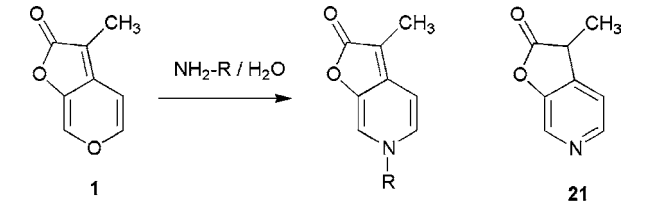
Scheme 3. Synthesis of 3-Desmethyl Analogues from Variants of **3**



4: R_1, R_2, R_3=H
7: R_1, R_2=H, R_3=CH_3
8: R_1, R_3=H, R_2=CH_3

17: R_1, R_2, R_3=H
18: R_1, R_2=H, R_3=CH_3
19: R_1, R_3=H, R_2=CH_3

Scheme 4. Synthesis of Some Nitrogen Containing Analogues of **1**



1

20: R=H
22: R=CH_3

21

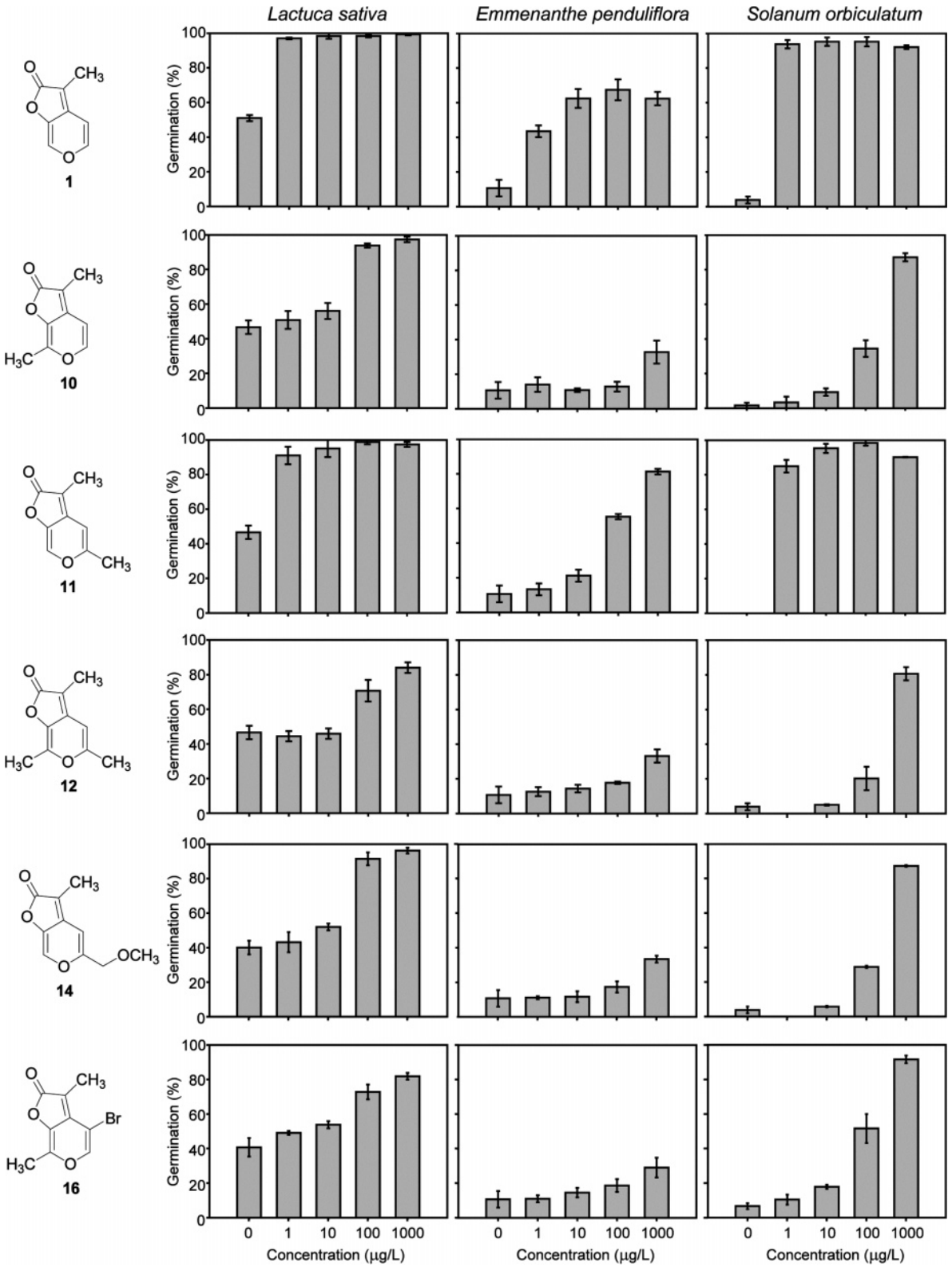


Figure 1. Germination activity of the butenolide (**1**) and some 3-methyl analogues. Error bars represent the standard error of the mean (SEM), and MP water served as the control (0 μg/L).

analogues with methyl substituents at the corresponding positions. Conveniently, **7** is commercially available, while **8** and **9** are easily prepared from **2** (*11*).

Following the butenolide annulation methods in **Scheme 1**, **7** provided the 3,7-dimethyl analogue (**10**) in moderate yield (**Scheme 2**). Similar treatment of **8** and **9** returned the corresponding 3,5-dimethyl (**11**) and the 3,5,7-trimethyl (**12**) analogues, respectively. Attempts to treat **2** in a similar fashion proved unsuccessful, as did treatment of the acetyl-protected **2**. Alternatively, conversion of **2** into the methyl ether (**13**) and subsequent treatment provided the 5-methoxymethyl analogue (**14**).

While use of this methodology on different starting materials allowed for easy elaboration of the C-5 and C-7 positions of the 2*H*-furo[2,3-*c*]pyran-2-one skeleton, it was difficult to substitute at the C-4 position. A simple pyrone that would lead

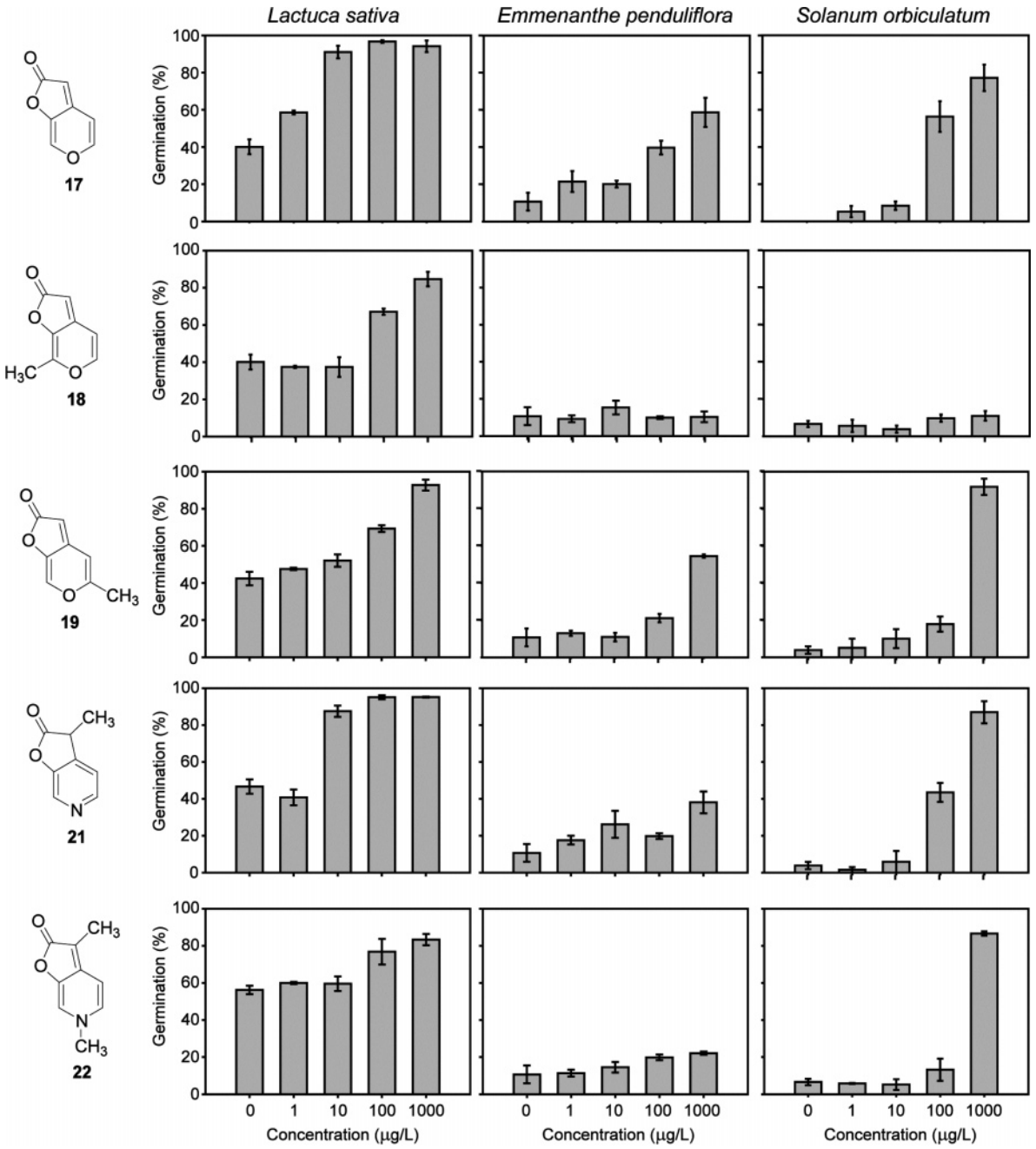


Figure 2. Germination activity of 3-desmethyl analogues and nitrogen analogues of **1**. Error bars represent SEM, and MP water served as the control (0 μg/L).

to an analogue substituted at C-4 was **15** (*12*). Hence, use of **15** returned the **16** derivative containing a substituent at C-4.

The butenolide analogues prepared so far all contain a methyl group at C-3. To determine the importance of this methyl group for biological activity, 2-chloroacetyl chloride was used as the esterifying agent instead of 2-chloropropionyl chloride. Refluxing the chloroacetyl-thione ester in acetic anhydride afforded the desmethyl butenolide (**17**) in very low yields, lower than obtained using the chloropropionyl-thione ester; however, enough material was obtained to enable characterization and germination testing. Similar treatment of **7** and **8** gave the 7-methyl (**18**) and the 5-methyl (**19**) analogues, respectively, in similar low yields.

Compound **3** and its analogues can be easily converted to the corresponding pyridone derivatives upon treatment with amines (*11*). It was thought that similar treatment of **1** may provide the pyridone analogue **20**. However, heating the butenolide in concentrated ammonium hydroxide yielded the pyridine **21**. In an analogous reaction using aqueous methylamine solution, the expected N-methyl-pyridone **22** was obtained.

Germination-Promoting Activity of Analogues. The analogues were tested for germination-promoting activity with the three highly smoke-responsive species *L. sativa* Grand Rapids lettuce, *E. penduliflora*, and *S. orbiculatum*. Grand Rapids lettuce was used as a key bioassay species for the isolation and identification of **1** (*10*) and represents an excellent test species for assessing the germination-promoting activity of butenolide analogues. Likewise, *E. penduliflora* has been used extensively for assessing the activity of smoke extracts (*17*) and for probing the mode of action of smoke-induced seed germination (*18*). The third species, *S. orbiculatum*, is a West Australian native species that has recently been found to be highly responsive to smoke and, now, to **1**. Typical control germination for this

species is less than 10%, while butenolide-treated seeds are promoted to germinate in excess of 90% even at 1 ppb, thus providing a reliable and sensitive test species for evaluating the bioactivity of analogues.

The germination responses of the three test species used for evaluating the activity of the analogues of **1** were broadly similar, although the sensitivity of each species to the analogues varied somewhat. The *L. sativa* bioassay was more sensitive to the germination-promoting effects of the analogues, while *E. penduliflora* was the least sensitive of the three species, requiring higher concentrations of analogues for promotion of germination to be observed. The *S. orbiculatum* gave the largest range between control (<10%) and butenolide-like enhancement (>80%), indicating that this species could provide a useful assay for evaluating the activity of analogues of **1** in future work.

For each of the test species, the natural stimulant (**1**) showed significantly higher germination activity than the control (**Figures 1** and **2**) for each of the concentrations tested (1 mg/L to 1 μg/L). A number of the analogues were also found to promote germination. In particular, the 3,5-dimethyl analogue (**11**) showed similar levels of activity to those observed for **1** (**Figure 1**). In contrast, introduction of a methyl at C-7, as in **10**, resulted in a 100-fold reduction in activity. A similar effect was observed for the 3-desmethyl variant (**17**) and analogues with a methyl at C-7 (**18**) and C-5 (**19**). Introduction of a bromine at C-4 had little effect as shown by comparison of the activities of the 3,7-dimethyl (**10**) and 3,7-dimethyl-4-bromo analogues (**16**). Replacement of the pyran oxygen by nitrogen, as in **21** and **22**, does not eliminate the activity. It is also worth noting that none of the starting materials or intermediates used in the preparation of the butenolide analogues showed germination stimulation on these test species.

It appears that some variation of substitution at C-5 can be tolerated, since introduction of a methyl at this position does not reduce activity. Although the analogue containing a methoxymethyl (**14**) at C-5 had reduced activity, it is still active at 100 μg/L. Importantly, substitution at C-5 is rather simple and may lend itself to the introduction of various labeling groups, such as fluorescent dyes or affinity labels (*19*). In addition, the fact that the 3,5-dimethyl analogue (**11**) shows similar levels of activity to the parent butenolide (**1**) may be of some interest since the synthesis of **11** is simpler to that developed so far for **1**.

In summary, the results (**Figures 1** and **2**) show that the methyl group at C-3 is important for germination-promoting activity of **1** and substitution at C-7 reduces germination activity. Substitution at C-5 and C-4 can be tolerated more and does not affect bioactivity to the same extent as substitution at C-7. Furthermore, replacement of the pyran oxygen with a nitrogen atom gives pyridine/pyridone analogues that also have germination activity. In terms of labeling the butenolide, substitution through C-5 appears to be more promising and should provide analogues that retain some germination-promoting ability.

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