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Identification of Alkyl Substituted 2*H*-Furo[2,3-*c*]pyran-2-ones as Germination Stimulants Present in Smoke

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The butenolide, 3-methyl-2*H*-furo[2,3-*c*]pyran-2-one (**1**), is a major compound in smoke responsible for promoting the seed germination of a wide range of plant species. We now report the structure of five alkyl substituted variants of **1** that are also present in smoke. The concentrations of these analogues, as well as that of **1**, in a typical smoke−water solution have been determined using high-performance liquid chromatography (HPLC) purification followed by gas chromatography−mass spectrometry (GC-MS) analysis. The analogue, 3,5-dimethyl-2*H*-furo[2,3-*c*]pyran-2-one (**3**), was identified at levels that indicate that it is a contributor to the overall germination-promoting activity of crude smoke extracts.

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

INTRODUCTION

The butenolide, 3-methyl-2*H*-furo[2,3-*c*]pyran-2-one (**1**) has been identified as the compound in cellulose- and plant-derived smoke responsible for promoting seed germination (*1*, *2*). The germination-promoting activity of **1** was demonstrated with the key indicator species *Lactuca sativa* L. cv. Grand Rapids (Asteraceae) as well as a broader range of well-known smoke-responding species from Australia, South Africa, and North America. Subsequent studies have confirmed **1** as a potent germination cue for many species including various weed and crop species (*3*−*9*). While **1** has been established as the major germination stimulant present in smoke (*10*, *11*), it is not known whether smoke contains additional compounds that also promote seed germination.

Smoke is known to contain a complex array of volatile compounds with over 4400 compounds known to be present in cigarette smoke alone (*12*). At least a similar number of compounds would be expected to be derived from the burning of plant material. Given this complexity, it is feasible that more than one compound may contribute to the germination-promoting activity of all smoke-responsive species. Other researchers have previously reported that smoke contains more than one germination-promoting compound. A study by Baldwin et al. (*13*) suggested that at least three compounds in smoke were responsible for promoting the germination of *Nicotiana attenuata* Torr. ex Wats. (Solanaceae). Van Staden et al. (*14*) also showed that at least two different fractions of plant-derived smoke were active toward Grand Rapids lettuce seed (*L. sativa*). During our own work that led to the isolation of **1** using bioassay-guided fractionation (*1*), the presence of other germination-promoting compounds similar to **1** also became evident.

A number of synthetic analogues of **1** have been prepared in our laboratory, and most of these show germination-promoting activity (*15*). We therefore investigated whether any of these analogues were present in plant-derived smoke. We now report that at least five analogues of **1** are present in smoke and that some of these are likely to contribute to the overall germination-promoting activity of smoke extracts. A rationale for the formation of **1** and these naturally occurring analogues from cellulose-derived pyrolysis products is also given.

MATERIALS AND METHODS

General Experimental Procedures. High-performance liquid chromatography (HPLC) was conducted using a Hewlett-Packard 1050 HPLC system equipped with a multiple wavelength detector (MWD). Gas chromatography−mass spectrometry (GC-MS) was performed using a Shimadzu GCMS-QP2010 instrument operating in the electron impact (EI, 70 eV) mode. Analogues **2**−**7** were prepared as previously described (*15*).

Extraction of Smoke−Water (SW). Smoke−water (SW) was prepared from burning straw as previously described (*5*). SW (1 L) was extracted with dichloromethane (3 × 200 mL), and the combined organic extract was washed with 1 M NaOH (3 × 100 mL) to remove the acidic compounds. The resulting organic extract was washed with brine, dried (Na_2SO_4), filtered, and evaporated in vacuo to give the neutral fraction (630 mg).

HPLC Separation of SW Neutral Fraction. Separation was achieved using a 250 × 10 mm i.d., 5 μm, Apollo C_{18} reversed-phase column (Grace- Davison) with a 33 mm × 7 mm guard column of the same material. The column was eluted at 4 mL/min with 10% acetonitrile/water,

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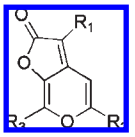
Table 1. Retention Times and Mass Spectrometric Data of 2*H*-Furo[2,3-*c*]pyran-2-one Analogues and Their Relative Concentrations in a Typical Smoke−Water Solution



compound				retention time (mins)				EI mass spectra	
KAR$_n$	R_1	R_2	R_3	HPLC	5 ms[a]	35 ms[a]	wax[a]	mass (relative abundance %)	concentration (μg/L)
1	CH_3	H	H	19.03	19.08	30.43	30.23	150 (93), 121 (100), 122 (20), 66 (15), 65 (23)	39.8 ± 1.4
2	H	H	H	12.52	18.02	29.50	32.19	136 (100), 80 (31), 79 (23), 52 (33), 51 (29)	6.5 ± 0.5
3	CH_3	CH_3	H	24.38	22.05	35.51	33.30	164 (76), 136 (21), 135 (100), 79 (28), 77 (26)	5.2 ± 0.4
4	CH_3	H	CH_3	23.14	21.55	34.66	31.89	164 (64), 136 (18), 135 (100), 79 (10)	3.4 ± 0.2
5	H	CH_3	H	18.45	21.09	34.76	34.81	150 (100), 94 (24), 93 (16), 66 (34), 65 (33)	1.0 ± 0.1
6	H	H	CH_3	17.30	20.56	33.63	33.57	150 (100), 122 (63), 94 (15), 79 (19), 66 (21)	1.3 ± 0.1
7	CH_3	CH_3	CH_3	28.91	24.36	39.07	34.60	178 (52), 150 (18), 149 (100), 79 (11), 77 (16)	N/A

[a] GC stationary phases.

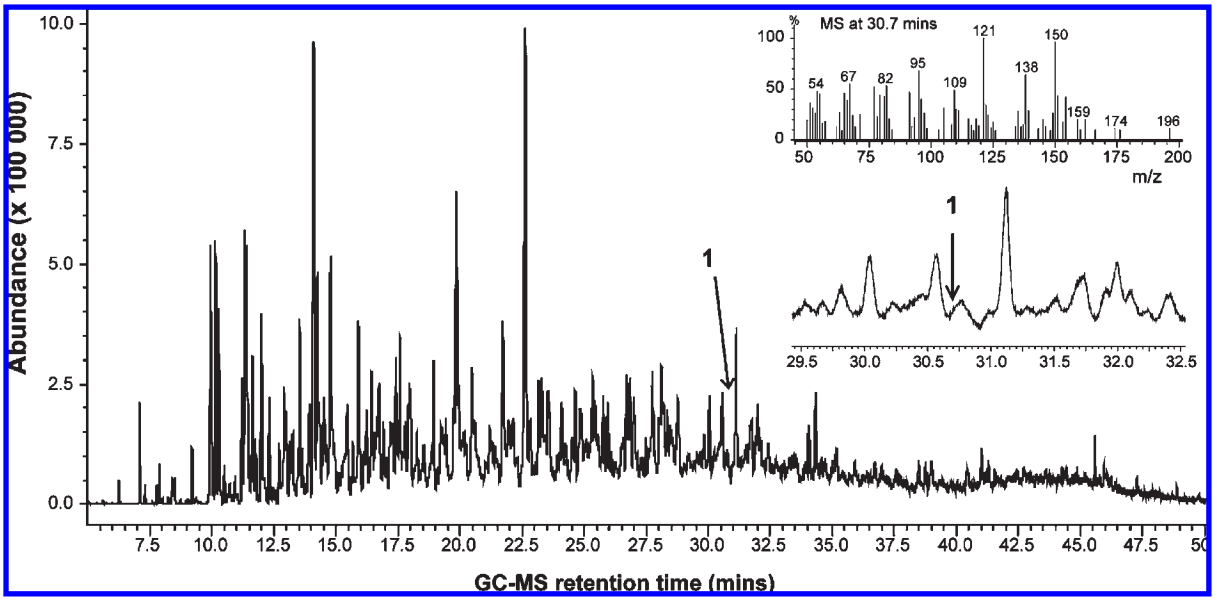


Figure 1. GC-MS total ion chromatogram (TIC) of the smoke−water (SW) neutral fraction showing the location of **1** as only a trace component of the mixture. The bottom inset shows an expansion of the region where **1** elutes and indicates the presence of **1** on the shoulder of a small peak. The top inset shows the mass spectrum indicating the presence of the molecular ion *m*/*z* 150 and major fragment ion *m*/*z* 121 of **1** at 30.7 min retention time using the DB-35 ms column. The retention time was confirmed with an authentic sample of **1** in a subsequent run.

increasing to 50% acetonitrile/water over 30 min, then to 100% acetonitrile at 31 min, and held for 9 min. UV absorbance was measured at wavelengths of 210, 254, and 325 nm. The SW neutral extract (630 mg) was dissolved in 50/50 (v/v) acetonitrile/water (10 mL), and 1 mL of this solution (equivalent to 100 mL SW) was separated by HPLC as described above, and fractions were collected every minute for 40 min. This was repeated several times. From one separation, 400 μL (from 4 mL) was taken from fractions 4 to 40 and purged to dryness under a stream of $N_{2(g)}$. The samples were made up to 10 mL with milli-Q water, which gave a concentration equivalent to that of the initial smoke−water. Each sample was tested in duplicate at the neat concentration and 1/10 dilution level with the bioassay species *Solanum orbiculatum* as previously described (*15*). For analysis by GC-MS, fractions were evaporated to dryness under reduced pressure and resuspended in 100 μL of HPLC grade acetonitrile.

GC-MS Analysis. Separation was achieved using a 30 m × 0.25 mm i.d., 0.1 μm, Rtx-5 ms (Restek), a 30 m × 0.2 mm i.d., 0.33 μm, DB-35 ms (J&W Scientific), or a 30 m × 0.25 mm i.d., 0.1 μm, Rt-Stabilwax (Restek) column with UHP helium as the carrier gas (1 mL/min). The initial oven temperature was set to 40 °C and held for 1 min before increasing at 10 °C/min to 100 °C, then 3 °C/min to 200 °C, then 10 °C/min to 250 °C, and held for 10 min (inlet temperature 250 °C; transfer line 250 °C). The ion source was set at 200 °C, and the spectrometer was set to record between 45 and 400 amu. For quantification, standard solutions of each compound were prepared at 1 mg/mL in HPLC grade acetonitrile, and dilutions were made accordingly to give the following concentrations; 100, 50, 20, 10, 5, and 1 μg/mL. The standards were analyzed using selective ion monitoring (SIM) of the base ion and major qualifier ion for each of the analogues as indicated in **Table 1**. Calibration curves were constructed using the areas obtained for the base ion.

RESULTS AND DISCUSSION

Smoke−water (SW), derived from the combustion of straw material, was partitioned three times with dichloromethane to extract the germination-promoting compounds. The dichloromethane extract was further partitioned against aqueous sodium hydroxide to remove the acidic compounds (carboxylic acids and phenolics) and provide the neutral fraction, which has been shown to contain the germination-promoting compounds (*16*). GC-MS analysis of the neutral fraction revealed a complex

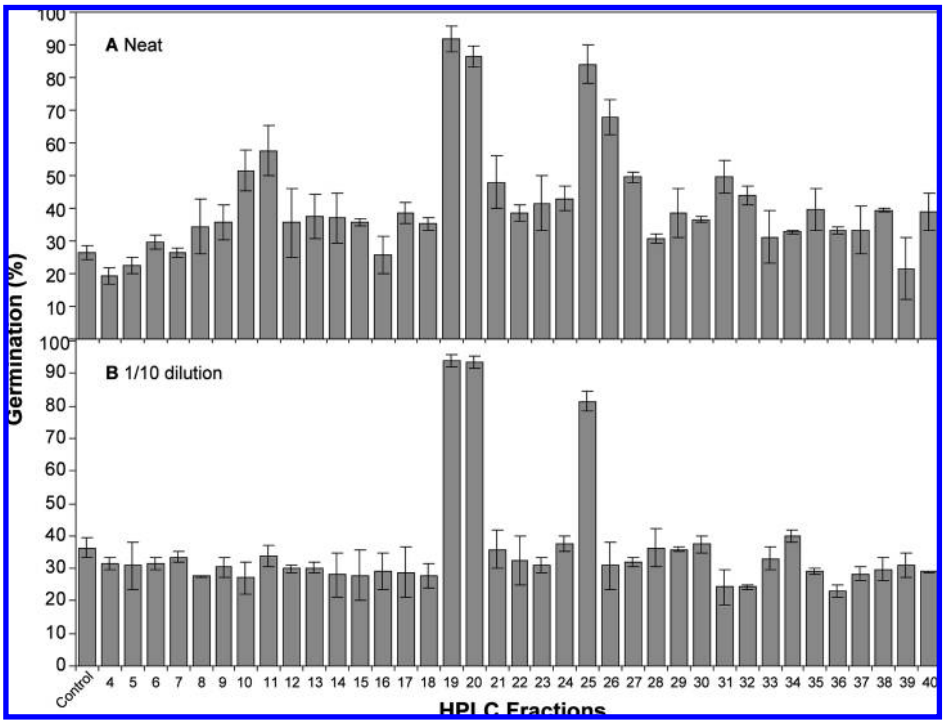


Figure 2. Germination activity of the HPLC fractions derived from the SW neutral fraction and tested with the seeds of *Solanum orbiculatum* at (**A**) neat concentration and (**B**) 1/10 dilution.

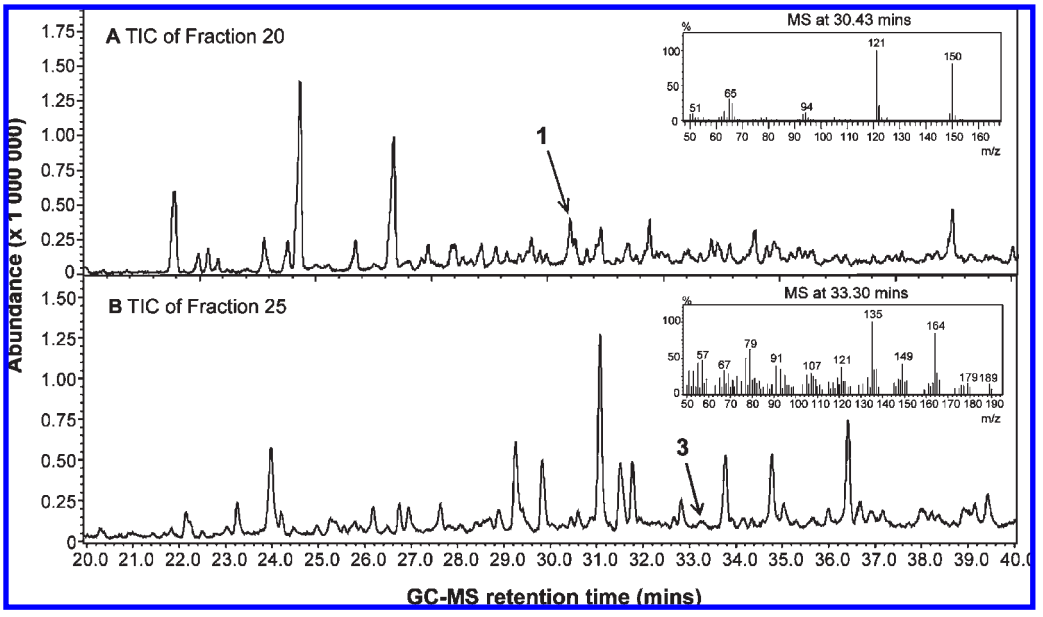


Figure 3. GC-MS total ion chromatograms (TIC) from the analysis of (**A**) fraction 20 and (**B**) fraction 25. Insets show the mass spectrum (**A**) at 30.43 min showing the presence of **1** with molecular ion *m*/*z* 150 and major fragment (base) ion *m*/*z* 121, and (**B**) at 33.30 min showing the presence of **3** with the molecular ion *m*/*z* 164 and major fragment (base) ion *m*/*z* 135.

mixture of compounds. Comparison with an authentic sample of **1** showed that it was present as only a trace component (**Figure 1**). Because of this complexity, all attempts to optimize the separation by varying the temperature ramping rate or using different column phases (5 ms, 35 ms, and wax) failed to completely resolve **1** from other components in the mixture.

During the course of our study on the chemistry and activity of **1**, we had prepared a number of alkyl analogues, some of which showed germination-promoting activity (*15*). Six of these analogues (**2**−**7**) were analyzed by GC-MS, and their retention times and mass spectrometric data (**Table 1**) were compared with those of the neutral fraction. By comparing the retention times obtained using three different column phases (5 ms, 35 ms, and wax), along with the mass spectrometric data (**Table 1**), trace amounts of **2**−**6** were detected, but at levels that were too low for unambiguous identification. To increase the concentration of the analogues and reduce the complexity of the smoke mixture, the neutral fraction was separated into 40 fractions by semipreparative HPLC. A sample from each fraction was tested at concentrations equivalent to that of the original smoke−water and at 1 in 10 dilution with

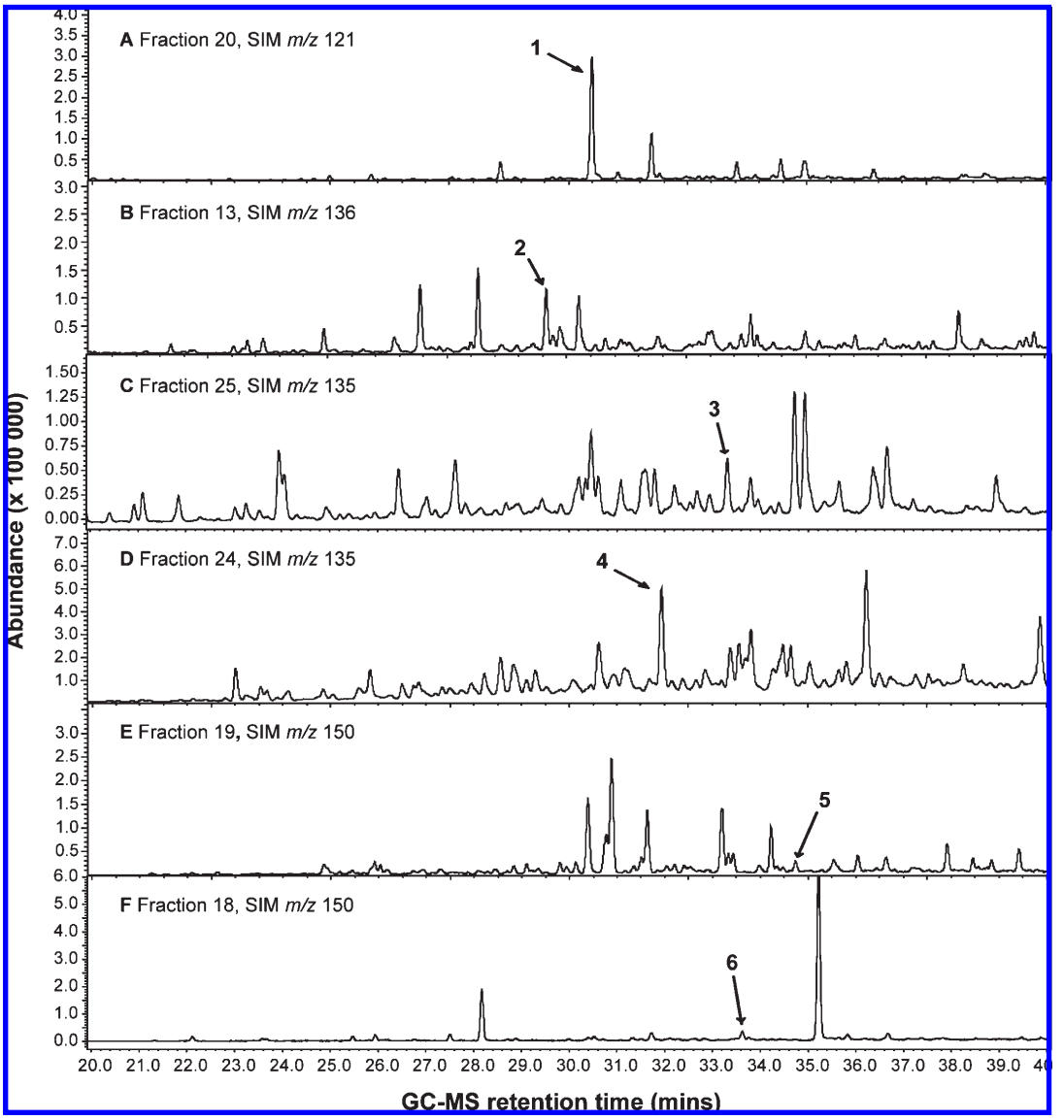


Figure 4. GC-MS chromatograms of selected HPLC fractions showing the presence of each compound using selective ion monitoring (SIM) of the relevant ion as indicated. Chromatograms **A**, **B**, **E**, and **F** were obtained using the DB-35 ms column. Chromatograms **C** and **D** were obtained using the Rt-stabilwax column.

Solanum orbiculatum Poir. (Solanaceae). The germination results showed that the main region of germination activity was found in fractions 19 and 20 for both concentrations tested (**Figure 2**). A second region of activity was also observed for fractions 25 and 26 and, to a lesser extent, for fractions 10 and 11, but only at the neat concentration level.

The main region of activity corresponded to fractions 19 and 20, which was shown to contain **1** by GC-MS (**Figure 3A**). Analysis of **1** by HPLC showed that it eluted at 19.03 min. The desmethyl analogue (**2**) eluted at 12.52 min, corresponding to fraction 13 (12−13 min), which did not show any germination promotion above the control. The 3,5-dimethyl (**3**)- and 3,7-dimethyl (**4**)-analogues eluted at 24.38 and 23.14 min, respectively. The retention time of **3** corresponded with the second region of activity, fraction 25. This analogue has previously been shown to have germination-promoting activity similar to that of **1** (*15*). Analysis of fraction 25 by GC-MS confirmed that **3** was present (**Figure 3B**), and a trace amount was also found in fraction 26, which also showed germination activity. The 5-methyl (**5**)-, 7-methyl (**6**)-, and 3,5,7-trimethyl (**7**)-substituted butenolides eluted at 18.45, 17.30, and 28.91 min, respectively. Fractions corresponding to these retention times, however, did not show any activity above that of the control at the concentrations tested.

The fractions corresponding with the retention times of each of the analogues were analyzed by GC-MS to determine whether these compounds were present. The results confirmed that the analogues **2**−**6** were present in their respective fractions (**Figure 4A**−**F**); however, no trace of **7** was found. To gain some insight into the relative amounts of these compounds with respect to **1** in this smoke−water solution, the fractions corresponding in retention time plus one fraction on either side were combined and quantitatively analyzed in triplicate by GC-MS. Using six standard concentrations (1 μg/L to 100 μg/L), a calibration curve was constructed for each analogue. The amount found for **1** was 39.8 ± 1.4 μg (39.8 ppb) from 1 L of smoke−water (**Table 1**). The desmethyl compound was found to be present at 6.5 ± 0.5 μg/L, while the 3,5 and 3,7-dimethyl analogues returned values of 5.2 ± 0.4 μg/L and 3.4 ± 0.2 μg/L, respectively, from the same 1 L of smoke−water. The concentrations for analogues **5** and **6** were lower at 1.0 ± 0.1 μg/L and 1.3 ± 0.1 μg/L, respectively.

The analogues **2**−**6** have previously been shown to promote the germination of three different plant species, including

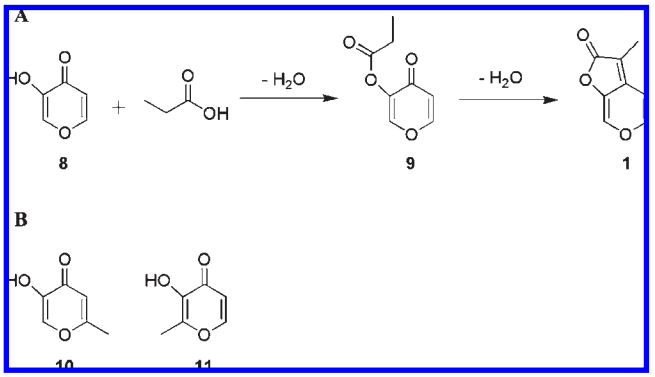


Figure 5. (**A**) Proposed formation of karrikinolide (**1**) from common cellulose combustion products. (**B**) Chemical structures of allomaltol (**10**) and maltol (**11**).

S. orbiculatum (*15*). However, these analogues show no germination-promoting activity at concentrations of 10 μg/L (10 ppb) and below, except for **3**. The 3,5-dimethyl analogue (**3**) shows activity comparable with that of **1**, and the concentration of **3** found (5.2 μg/L, 5.2 ppb) for this smoke−water solution would be expected to promote germination at the levels tested in this study. Hence, **3** is likely to be a contributor to the overall germination-promoting activity of crude smoke extracts. It is also possible that the other analogues may be significant contributors to the germination activity of smoke with other plant species. This is supported by the fact that *Arabidopsis thaliana* is promoted to germinate by smoke solutions and that the desmethyl analogue (**2**) shows activity at concentrations comparable to those observed with **1** and **3** (*17*).

These results show that there are at least 5 additional germination-promoting compounds in plant-derived smoke, all simple analogues of **1**. It should be noted though that **1** is responsible for the majority of activity observed with plant-derived smoke and is present in the highest concentration (40 μg/L compared to less than 7 μg/L for the analogues). The amounts of these analogues are also likely to vary with different smoke−water solutions depending on the source of plant material and duration of smoke capture. As these compounds are derived from smoke, the name karrikin has recently been suggested for this family of butenolides, derived from the Australian aboriginal word for smoke, karrik (*3*, *17*). The main stimulant, **1**, has been termed karrikinolide (KAR_1) to distinguish it from the other karrikins and acknowledge the lactone functional group as a key feature.

Karrikinolide (**1**) and the karrikins are produced from the combustion of pure cellulose (*1*), which as a major component of all plants, represents a universal combustion substrate that is present during natural fires. The formation of karrikinolide (**1**) could be envisaged to arise from two substrates known to be generated upon the pyrolysis of cellulose, pyromeconic acid (**8**), and propionic acid (*18*). Condensation of **8** with propionic acid could lead to the propionyl ester (**9**), which on intramolecular cyclization and dehydration provides **1** (**Figure 5A**). Similarly, the formation of **3** and **4** can be rationalized from the double dehydration of propionic acid with allomaltol (**10**) and maltol (**11**), respectively (**Figure 5B**), which are also known constituents of cellulose-derived smoke (*18*, *19*). Moreover, **2**, **5**, and **6** could be formed in analogous fashion by reacting the appropriate substrates (**8**, **10**, or **11**, respectively) with acetic acid instead of propionic acid.

In summary, we have identified a number of alkyl substituted *2H*-furo[2,3-*c*]pyran-2-ones as germination stimulants present in smoke−water at concentrations below 10 μg/L. In particular, 3,5-dimethyl-*2H*-furo[2,3-*c*]pyran-2-one (*3*) was found at levels that indicate that it is a contributor to the overall germination-promoting activity of crude smoke extracts. In addition, the concentration of the main germination-promoting compound **1** has been determined for the first time and found to be approximately 40 μg/L in a typical smoke−water solution.

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