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TETRAHEDRON

New α-Pyrones Produced by Fungal Culture LL-11G219 Function as Androgen Receptor Ligands

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Abstract: This report describes the isolation and characterization of several closely related compounds, produced by fungal culture LL-11G219, with androgen-like activity. Bioassay-guided isolation yielded four novel fermentation products of the α-pyrone class: 11G219α, β, γ, and δ. Structures were assigned to these compounds on the basis of spectroscopic data, particularly those from 1D and 2D NMR experiments. α-Pyrones bearing an olefinic side chain in conjunction with a 6-alkyl substitution were hitherto unknown.

Keywords: Natural products; pyrones; biologically active compounds; fungi

Fermentation broths and other materials have been screened in a newly developed assay[1] designed to discover non-steroidal androgen receptor agonists and antagonists for use as potential animal performance enhancers. One of the first fermentation leads came from culture LL-11G219, an unidentified filamentous fungus.

RESULTS AND DISCUSSION

Isolation. The recovery of the pyrones from tank fermentations was conveniently performed by extraction of the cell cake with organic solvents. Slurrying the harvested fungal pellet with acetone, filtration, and extraction of the concentrated filtrate with ethyl acetate yielded a red-brown viscous oil containing the α-pyrones. Following a pre-purification on silica gel (column or TLC), the α-pyrone complex was separated into individual components by preparative RP-HPLC using a mixture of acetonitrile, methanol and water as eluent. Under these conditions 11G219γ (**3**) eluted first followed by 11G219β (**2**), then 11G219α (**1**). Of the α-pyrones isolated, 11G219α was the major component.

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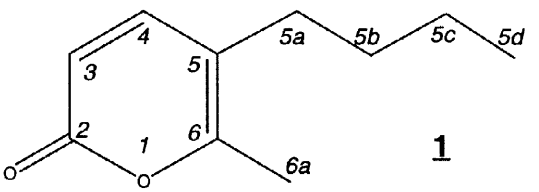
Structure elucidation. The structure determination of 11G219α (**1**) was based on the molecular formula of $C_{10}H_{14}O_2$, which was determined by HREIMS. The number of carbon atoms was also evident from 10 individual ^{13}C-signals and integration of multiple signals in the ^1H-NMR spectrum accounted for 14 hydrogen atoms. The chemical shift values of the 10 carbon resonances (Table 2) suggested the presence of one carboxyl, four olefinic, and five aliphatic carbons. DEPT experiments indicated two olefinic methine, three methylene, and two methyl groups which distinguished **1** clearly from other naturally occurring isomers such as 6-n-pentyl-α-pyrone[2] or 5-n-pentyl-2-furfural (L-1)[3]. The ^1H-NMR spectrum in conjunction with ^1H-^1H COSY data allowed the assignment of a butyl group and a single methyl group as substituents on a heterocyclic moiety. This nucleus was identified as an α-pyrone moiety on the basis of UV-spectrum, ^{13}C data, and ^1H-^{13}C connectivities derived by HETCOR or HMQC measurements. Coupling constants and chemical shifts of the two olefinic protons indicated that the two aliphatic substituents were attached at the same side of the pyrone ring. Therefore, structure **1** was proposed for 11G219α. Any ambiguity in the placement of the aliphatic substituents in **1** was eventually removed by HMBC measurements.



1

Specifically, three bond correlations of the methylene protons at 2.29 ppm (5a) to the low field carbon at 147.0 ppm, and of the olefinic proton at 7.16 ppm to the methylene carbon at 29.1 ppm, placed the butyl group at position 5 of the pyrone ring, 'para' to the carbonyl function. Significantly, the signal for the methyl protons at 2.22 ppm correlated to only two carbon atoms (δ 158.2 and 115.4). Further correlations and linkages were discerned as illustrated in Figure 1 below.

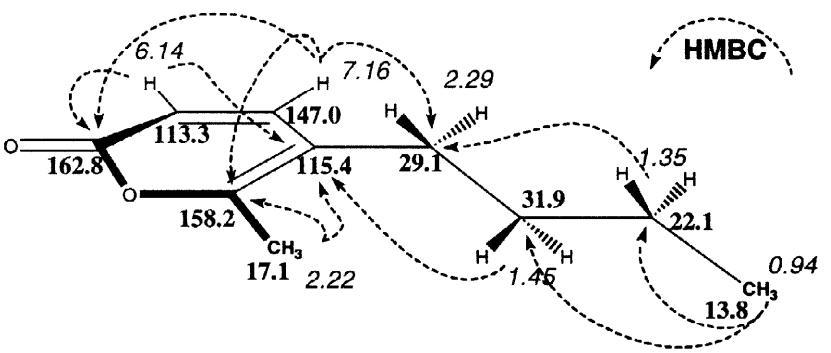
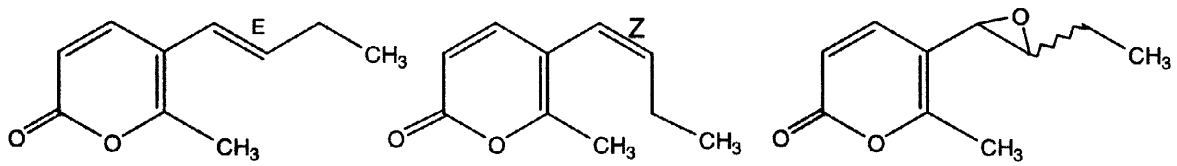


Figure 1. Key HMBC establish the connectivities within the 11G219α molecule.

Structure **1** was recently corroborated by an intramural chemical synthesis using a different method than that employed in the first synthesis[4] of this compound. Resynthesis was necessary

because 1 was originally characterized by b.p. (reduced pressure) and refractive index[4] only, which are not reliable parameters for the identification of 1. Synthetic 1 was identical with the natural product in all parameters. From the evidence presented above, the structure of 11G219α (1) was assigned as shown in Figure 1 and can be referred to by the chemical name 5-butyl-6-methyl-pyran-2-one.



2 3 4

Figure 2. Structures of the minor components 11G219β (2), γ (3) and δ (4).

The structures of 11G219β (2) and 11G219γ (3) were readily elucidated by comparing the observed NMR chemical shifts and coupling patterns to those of 1 (see Tables 1, and 2). The EI (GC/MS) mass spectra of 2 and 3 were virtually identical with a molecular weight of 164, consistent with a molecular formula of $C_{10}H_{12}O_2$. Therefore, both compounds were considered derivatives of 1 with one additional unit of unsaturation. Their ^1H and ^{13}C NMR spectra indicated the presence of a new olefinic function in the side chain of 2 and 3. Although the observed ^1H-^1H coupling patterns suggested that this additional double bond was in the same place, the magnitude of the olefinic ^1H-^1H coupling constants were different indicating the E configuration for 11G219β (2) [15.8 Hz] and Z configuration for 11G219γ (3) [11.1 Hz].

Table 1. Assigned ^1H-NMR shift values of 11G219α–δ dissolved in $CDCl_3$

H	1	2	3	4
3	6.14 *(d, 9.4)*	6.18 *(d, 9.67)*	6.14 *(d, 9.5)*	6.14 *(d, 9.5)*
4	7.16 *(d, 9.4)*	7.49 *(d, 9.67)*	7.17 *(d, 9.5)*	7.24 *(d, 9.5)*
6a	2.22 *(s)*	2.28 *(s)*	2.18 *(s)*	2.28 *(s)*
5a	2.29 *(t, 7.5)*	6.14 *(d, 15.8)*	5.98 *(d, 11.1)*	3.75 *(d, 3.5)*
5b	1.45 *(m)*	5.92 *(dt, 15.8, 6.4)*	5.69 *(dt, 11.1, 7.4)*	3.12 *(dt, 3.5, 6.2)*
5c	1.35 *(m)*	2.21 *(m)*	2.02 *(m)*	1.31 *(m)*
5d	0.94 *(t, 7.2)*	1.06 *(t, 7.4)*	0.99 *(t, 7.5)*	0.98 *(t, 7.4)*

Table 2. Assigned ^{13}C-NMR shift values of 11G219α–δ dissolved in $CDCl_3$

C #	mult (DEPT)	1	2	3	4
2	s	162.8	162.1	162.2	162.0
3	d	113.3	113.6	112.1	112.6
4	d	147.0	142.7	146.4	143.5
5	s	115.4	113.6	113.0	110.4
6	s	158.2	157.8	159.4	160.0
6a	q	17.1	17.2	18.0	17.4
5a	(t)d	29.1	120.7	121.1	53.0
5b	(t)d	31.9	133.4	137.3	59.4
5c	t	22.1	26.2	22.1	21.2
5d	q	13.8	13.8	13.8	10.2

The ^{1}H-NMR spectrum of 11G219δ (4) showed new signals for two protons at 3.75 and 3.12 ppm in place of the olefinic protons seen for 2 or 3 suggesting that in 4 the double bond is oxidized to an epoxide group. The addition of oxygen is apparent from the molecular ion species at M/Z 180 in the mass spectrum of 4 (see Figure 6). The structures of 11G219β (2), γ (3), and δ (4) are therefore as shown in Figure 2.

Characteristics of isolated components. Compounds 1, 2, 3 and 4 can be readily identified by GC/MS on the basis of retention time in conjunction with the electron impact mass spectrum. Molecular ion and characteristic fragmentation patterns easily distinguish 1 from 2 or 3. The most abundant ions in the mass spectra of these pyrones are those of M/Z 123 (1, 4), 121(2), and 121(3), which are apparently derived by decarboxylation of the parent molecule $[M+H - CO_2]^+$ except for 4 where this step occurs after the loss of the epoxide oxygen. An additional loss of 28 mass units then gives rise to the second most intense peaks of M/Z 95 (1), 93 (2), or 93 (3), respectively. An initial loss of 28 mass units from the parent molecule can also explain the occurrence of small fragment ions at M/Z 138 (1) or 136 (2 and 3). The intensities and occurrence of fragment ion for 4 were similar to those of 2 or 3 except that observed molecular ion of was M/Z 180 (compare Figure 6).

Structures 2 and 3 are not readily distinguished by their mass spectra alone, but their UV spectra are unique and readily differentiate them from each other or 1 and 4 (Figure 3). Although the UV curves for 1, 4 and α-pyrone itself have the same shape, the maxima of these curves are different and shift from 215 and 289 nm (α-pyrone) to 220 and 308 nm (1), or to 220 and 304 nm (4), due to the aliphatic substitution. Since the chromophores of 2 and 3 are extended by an additional double bond over that of 1, their UV maxima are shifted to even longer wavelengths. No 6-alkyl substituted

α-pyrones with olefinic substituents at either the 3, 4, or 5 position have been described to allow a comparison of UV data.

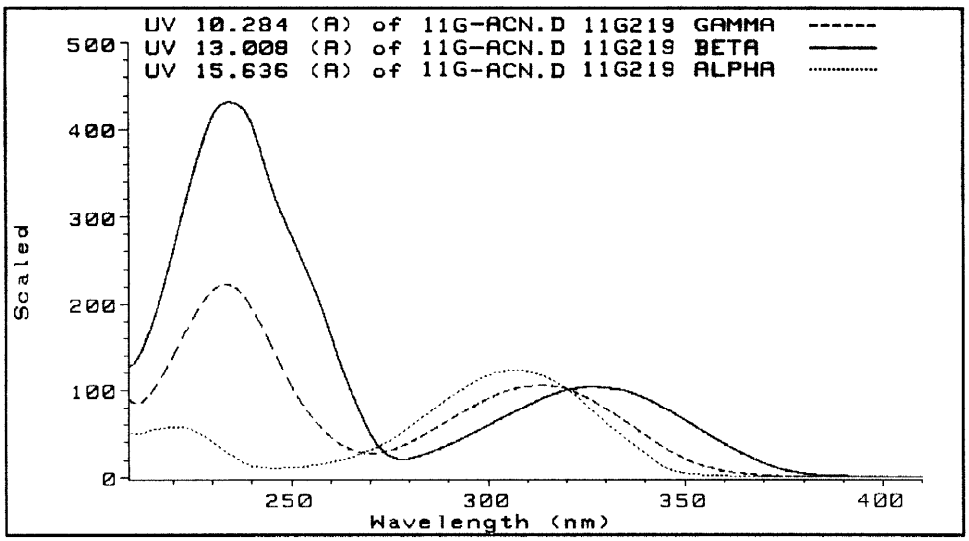


Figure 3. UV spectra of 11G219α(1), β(2) and γ(3).

Biological Activities. The major compound, 11G219α, was determined to bind in the membrane bound androgen receptor assay[1] with affinities 1,000-5,000 fold less than that of 5α-dihydrotestosterone (5αDHT) which showed binding affinity down to 0.2 ng. Compounds **2** and **3** are as active as **1** in this assay, but epoxide **4** appears to be inactive. The latter component seems to exert antifungal activity as judged by the zones of inhibition observed on the yeast based assay plates.

DISCUSSION

A number of relatively simple α-pyrones are produced by various fungi. This class of compounds has shown highly diverse biological activities including antifungal[2a], antibacterial[5], antiprotozoal[6], and fragrance or odor[2b] producing properties. To date, however, no compounds of this class have been reported to exert androgenic activity. The 11G219 compounds are the first pyrones to display this type of activity.

The objective of the screening program was to discover non-steroidal androgen receptor agonists or antagonists, and the isolated 11G219 components have met this criterion. When the structures of 11G219α (**1**) and that of testosterone are overlayed in a way that the ring A of the steroid is matched with the α-pyrone moiety, it becomes apparent that the carbonyl groups are aligned and the butyl side chain of **1** could mimic the remainder of the steroid molecule (see Figure 4). Since the butyl group is flexible, it can fit into any pocket designed to bind the rigid steroid. Thus,

the binding of the pyrone should be facilitated by its "tail portion" with the determining factor being the length and the location of this substituent.

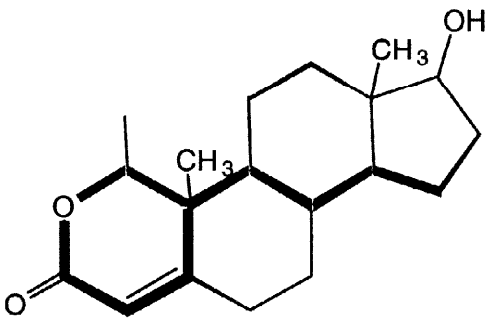


Figure 4. Presumed testosterone mimicry of 11G219α

To lend credence to this hypothesis, we tested commercially available α-pyrones for activity. Although the selected compounds were similar in structure to 11G219α (**1**), only one, 5-ethoxycarbonyl-4,6-dimethylpyran-2-one, had comparable activity, whereas 5-methoxycarbonyl-4,6-dimethylpyran-2-one, differing only by the length of a methyl group, failed to bind to the androgen receptor. It is noteworthy that 5,6,7,8-tetrahydrocoumarin, a compound that can be regarded as having the aliphatic side chain of 11G219α fixed, also failed to interact with the androgen receptor.

The closest natural α-pyrones with a configuration similar to those of the 11G219 compounds are the pyrenocines[7]. These compounds, reported to exhibit antifungal and antibacterial activity in addition to their inhibiting effect of root elongation[8], also possess a butyl substituent at position 5 and a methyl group at 6, which would suggest that they may also bind to androgen receptors.

EXPERIMENTAL

General. A Hewlett-Packard 5890 Series II Gas Chromatograph, fitted with a mass selective detector (series 5971) and a HP-5 column (30 m x 2.5 mm - 2.5μm film thickness) was used for the analysis of fractions or to check the purity of isolated components. Retention time and associated molecular weight was used to identify peaks. With a temperature gradient from 50 °C to 250 °C at a rate of 20 °C/min, typical retention profiles of the 11G219 constituents were observed as shown in Figure 5 below.

Analytical HPLC was performed on a Hewlett-Packard 1090 M LC system with diode array detection employing a Whatman Partisil-5 ODS 3 (C8) reverse phase column (4.5 x 150 mm), eluted isocratically with MeCN/MeOH/water in a ratio of 10:30:60.

Preparative HPLC separations were accomplished on a MODCol™ C18 column (100 Å Kromasil C18, 10μ, 2.54 x 25 cm) using an isocratic system of 50% MeOH/water with the effluent

being monitored at 235 nm by a variable wavelength detector (LDC). Silica gel chromatography was performed on self-packed open columns or Michel-Miller HPLPLC glass columns (21 x 300 mm) by step gradient elution using the indicated solvent mixtures. Fractions from all columns were generally collected by hand and pooled according to peaks observed on GC or HPLC analysis. All solvents were obtained from J. T. Baker, Inc., and were of the highest commercially available purity.

Androgen receptor binding activity was determined using the previously described yeast expression system[1] and recorded with respect to the standard , dihydrotestosterone.

UV spectra were recorded using a Hewlett-Packard Model 8450A spectrometer, or obtained "on the fly" during HPLC analysis on a HP 1090M instrument equipped with a diode array detector. IR spectra were obtained with a Nicolet 20AXB FT-IR spectrometer. Mass spectra were recorded using a VG-ZAB SE high performance mass spectrometer and a VG 11-250 data system. LC-Thermospray mass spectra were obtained on a Finnigan TSP 46 single quadrupole mass spectrometer. NMR spectra were obtained on a Bruker AMX 300 MHz NMR instrument. Chemical shifts of ^{1}H and ^{13}C NMR signals were determined in ppm relative to TMS or referenced to the solvent signals of residual chloroform at δ_H 7.26 ppm and δ_C 77.0 ppm.

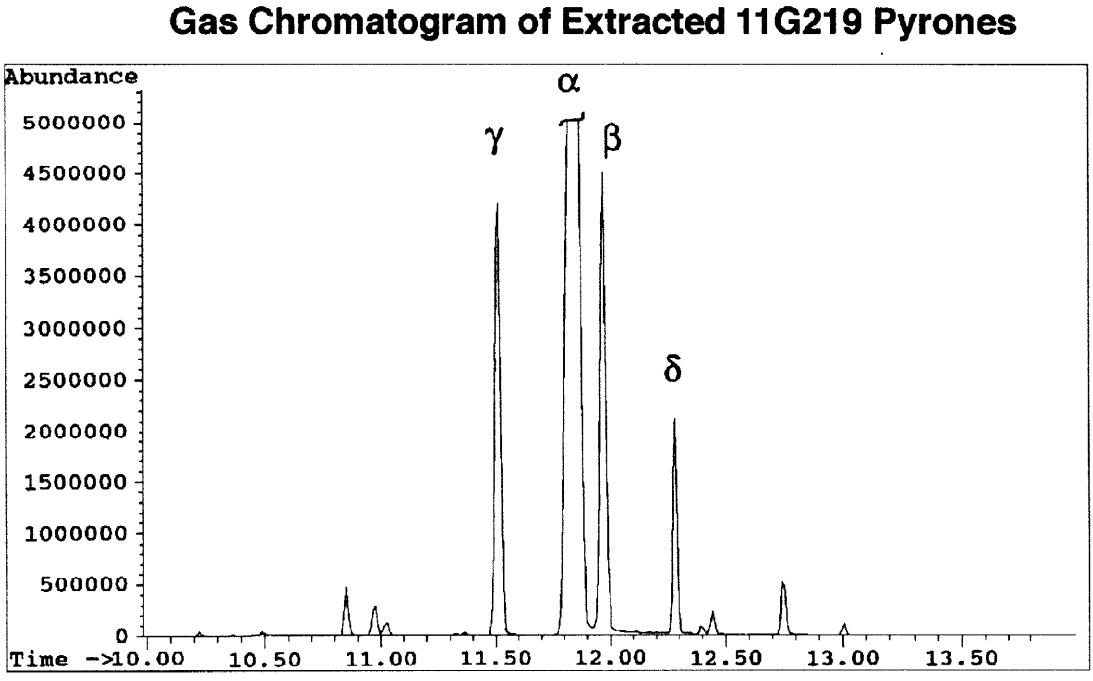


Figure 5. GC/MS profile of α-pyrones produced by culture 11G219

Isolation and Purification of 11G219α(1), β(2) and γ(3) and δ(4). The cell mass from a 300 liter tank fermentation of culture 11G219 was harvested, air-dried, and then slurried with acetone (110 L) followed by mixing with ethyl acetate (100 L). Following filtration of the residual solids, water (150 L) was added and the resulting phases were separated. The organic layer was

subsequently concentrated and then repartitioned between hexane and water. Concentration of the hexane extract containing ca. 12 g solid material yielded one liter of a red-brown solution. This solution was then passed through a silica gel column (15 x 10 cm) followed by a wash with 50% ethyl acetate/hexane (300 mL) to completely elute the compounds. Most of the effluent together with the ethyl acetate containing eluate were concentrated and charged onto a second silica gel column (30 x 4.5 cm) for preparative chromatography. After a wash with one liter of hexane, the column was developed by a step gradient using 500 mL ethyl acetate/hexane mixtures of 5%, 10%, 25%, 50%, and 100% EtOAc in each step. Eight fractions were collected of which the first four were discarded since they contained only inactive material. Fractions 5 and 6 were combined, concentrated, and subsequently re-chromatographed on a self-packed Michel-Miller HPLPLC silica gel column (30 x 2.1 cm) using as eluent CH_2Cl_2 and increasing concentrations of EtOAc from 5 to 50%. Nine fractions of 250 mL each were collected. Four fractions were discarded, and fractions 5, 7 and 8 were concentrated, and prepared for an additional chromatographic step while 6 containing the bulk of **1** was retained.

This chromatography was performed on an open silica gel column (30 x 4.5 cm). A wash with 500 mL hexane preceded the elution of components by a gradient of 5%, 10%, 25%, and 50% EtOAc in hexane using 500 mL solvent with each step. Again, nine fractions of 250 mL each were collected (A to I) of which fractions A and B were discarded. Fraction C contained 445 mg of oily material including small amounts of **3**. Most of the active compounds eluted in fractions D through G of which fraction G provided **1** (600 mg).

Fractions D (445 mg) and H (430 mg) were further purified by reverse phase chromatography employing a MODCol™ C18 column and isocratic elution with MeOH (50 %) to obtain the minor components **2**, **3**, and **4**. Chromatography of fraction D yielded **3** (31 mg) in fraction 11 and **2** (28 mg) in fraction 15, whereas chromatography of fraction H yielded **4** (18 mg) in fraction 6. On GC/MS analysis, **4** produced the mass spectrum shown in Figure 6.

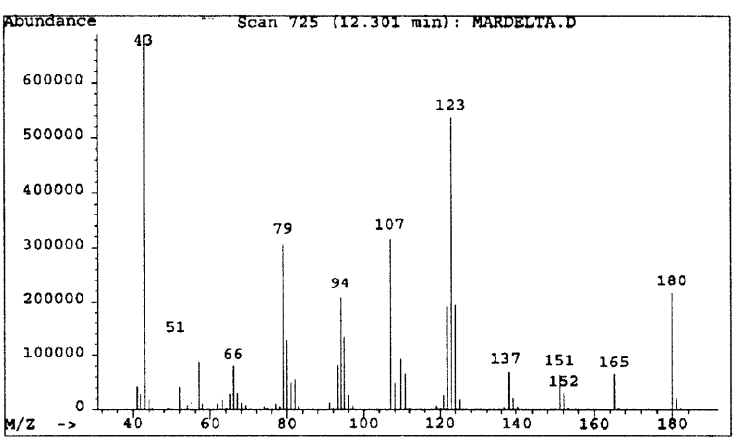


Figure 6. GC-EI mass spectrum of 11G219δ (4)

Hydrolysis of **4** yielded the diol, 5-[1,2-dihydroxybutyl]-6-methylpyran-2-one, as concluded from NMR and LC/UV/MS data. Using an isocratic system with 40% MeCN/0.02 M aqueous TFA on a 25 cm ODS-A column (YMC) separated all components. The diol had a retention time of 3.2 min. versus 6.1 min. for **4**, 12.0 min. for **3**, 12.7 min. for **2**, and 13.2 min. for **1**.

Physico-chemical properties.

5-butyl-6-methylpyran-2-one (1) or **11G219α:** $C_{10}H_{14}O_2$ MW 166
UV (MeOH) λ_{max} nm (ε) 219 (3,790), 308 (5,600); (MeCN) λ_{max} nm (ε) 224 (3,850), 308 (5,500); IR (KBr) 3452, 2957(s), 2931(s), 2866(s), 1738(s), 1642(s), 1555(s), 1465, 1380(s), 1299, 1204, 1187, 1131, 1104(s), 1045, 1012, 867, 826 cm^{-1}; ^1H NMR (see Table 1); ^{13}C NMR (see Table 2); MS: (EI) $[M]^+$ =M/Z 166 (HREI) 166.0993 -Δ[mmu] = 0.1; (pES) [M+1] =M/Z 167, (GC-EI) [M+1] =M/Z 167, GC/MS $[M]^+$=M/Z 166, frag. : 151, 138, 123, 109, 95, 81, 67, 53, 43

5-[1E-butenyl]-6-methylpyran-2-one (2) or **11G219β:** $C_{10}H_{12}O_2$ MW 164
UV (MeOH) λ_{max} nm (ε) 233 (59,100), sh 252, 325 (6,200); (MeCN) λ_{max} nm (ε) 235 (3,790), sh 253, 327 (5,500); ^1H NMR (see Table 1); ^{13}C NMR (see Table 2); (pES) [M+1] =M/Z 165, GC/MS-EI: $[M]^+$=M/Z 164, frag.: 149, 136, 121, 107, 93, 79, 65, 51, 43

5-[1Z-butenyl]-6-methylpyran-2-one (3) or **11G219γ:** $C_{10}H_{12}O_2$ MW 164
UV (MeOH) λ_{max} nm (ε) 233 (60,100), 313 (5,900); (MeCN) λ_{max} nm (ε) 233 (3,790), 314 (5,500); ^1H NMR (see Table 1); ^{13}C NMR (see Table 2); (pES) [M+1] =M/Z 165, GC/MS-EI:$[M]^+$=M/Z 164, frag.: 149, 136, 121, 107, 93, 79, 65, 51, 43

5-[3-ethyloxiranyl]-6-methylpyran-2-one (4) or **11G219δ:** $C_{10}H_{12}O_3$ MW 180
UV (MeOH) λ_{max} nm (ε) 220 (4,400), 304 (5,800); (MeCN) λ_{max} nm (ε) 220 (3,800), 305 (5,500); ^1H NMR (see Table 1); ^{13}C NMR (see Table 2); MS: (pES) [M+1] =M/Z 181, GC/MS-EI:$[M]^+$ =M/Z 180, frag.: 165, 151, 137, 123, 107, 94, 79, 66, 51, 43 (see Figure 6);

5-[1,2-dihydroxybutyl]-6-methylpyran-2-one: $C_{10}H_{14}O_4$ MW 198
UV (MeOH) λ_{max} nm: 220, 302; (MeCN) λ_{max} nm: 219, 302;
^1H NMR ($CDCl_3$): 7.65(*d*, *J* = *9.6Hz*, H-4), 6.21(*d*, *J* = *9.6Hz*, H-3), 4.73(*d*, *J* = *5.8Hz*, H-5a), 3.84(*m*, H-5b), 1.46(*m*, 2H-5c), 1.01(*t*, *J* = *7.3Hz*, 3H-5d); ^{13}C NMR ($CDCl_3$): 161.5(s, C-2), 160.3(s, C-6), 144.2(d, C-4), 113.7(d, C-3), 113.3(s, C-5), 76.1(d, C-5b), 60.1(d, C-5a), 26.6(t, C-5c), 9.9(q, C-5d); MS (pES): [M+1] =M/Z 199

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