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INHIBITION OF GIBBERELLIN 3β-HYDROXYLASE BY NOVEL ACYLCYCLOHEXANEDIONE DERIVATIVES

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Key Word Index—*Cucurbita maxima*; Cucurbitaceae; pumpkin; gibberellin, 2-oxoglutarate-dependent dioxygenase; inhibitors; growth retardants; 2-acyl-5-carboxycyclohexane-1,3-diones.

Abstract—The 2-acyl-5-carboxycyclohexane-1,3-diones are plant growth retardants which are believed to act by competing with the natural co-substrate, 2-oxoglutarate, at the active site of dioxygenases involved in the later stages of the biosynthesis of the gibberellin (GA) plant hormones. A number of 2-acyl-5-carboxycyclohexanediones and related compounds were synthesized and compared with pyridine dicarboxylic acids and hydroxybenzoic acids as inhibitors of a GA 3β-hydroxylase from *Cucurbita maxima* endosperm. The most effective inhibitors of this enzyme possessed a 1,3-dioxo-2-acylcyclohexane-5-carboxylic acid structure. The nature of the 2-acyl group had a relatively minor effect on the potency of enzyme inhibition *in vitro*. A related chloro-substituted compound was shown to be a potential affinity label for this GA hydroxylase.

INTRODUCTION

The later stages of gibberellin (GA) biosynthesis are catalysed by soluble 2-oxoglutarate-dependent dioxygenases which convert GA_{12} to a number of hydroxylated products [1]. In addition to 2-oxoglutarate, these enzymes require O_2, Fe^{2+} and ascorbate for GA hydroxylase activity. Another 2-oxoglutarate-dependent dioxygenase, prolyl 4-hydroxylase, is inhibited by a large number of compounds that act by competing with 2-oxoglutarate and/or ascorbate. Inhibition studies with pyridine carboxylates [2] have shown that these compounds inhibit prolyl 4-hydroxylase competitively with respect to 2-oxoglutarate, which they resemble structurally. On the basis of these results and of theoretical considerations [3], a model of the 2-oxoglutarate binding site was postulated [2]. The terminal carboxylic acid group of 2-oxoglutarate is thought to bind to a positively charged amino acid, e.g. lysine (site I), while the other two carbonyls bind to two co-ordination sites on Fe^{2+} (site II). Good inhibitors appear to mimic this binding. Similar studies with hydroxybenzoic acids [4] showed that these compounds also inhibit prolyl-4-hydroxylase competitively with respect to both ascorbate and 2-oxoglutarate. In contrast, pyridine 2-carboxylates were found to inhibit uncompetitively with respect to ascorbate. It was suggested that the ascorbate binding site consisted of two *cis*-positioned co-ordination sites of the enzyme-bound iron and thus overlapped with the binding site of 2-oxoglutarate [3]. Several of the GA 2-oxoglutarate-dependent dioxygenases are known to be inhibited by 2-acyl-5-carboxycyclohexane-1,3-diones [5, 6], which are believed to act by blocking the 2-oxoglutarate binding site within the enzyme active site [7]. Computer molecular modelling shows that acylcyclohexanediones have close structural similarity to the co-substrate [7] and may compete for its binding site. In this report, we compare the effectiveness of pyridine carboxylates, hydroxybenzoic acids and a set of novel 2-acyl-5-carboxycyclohexane-1,3-diones as inhibitors of GA 3β-hydroxylase from *Cucurbita maxima* endosperm.

RESULTS AND DISCUSSION

Synthesis of 2-acylcyclohexane-1,3-diones and related compounds

3,5-Dioxocyclohexanecarboxylic acid (**1**), a key intermediate in the synthesis of 2-acyl-5-carboxycyclohexane-1,3-diones, could be produced by hydrogenation of 3,5-dihydroxybenzoic acid using the method of van Tamelen and Hildahl [8]. However,

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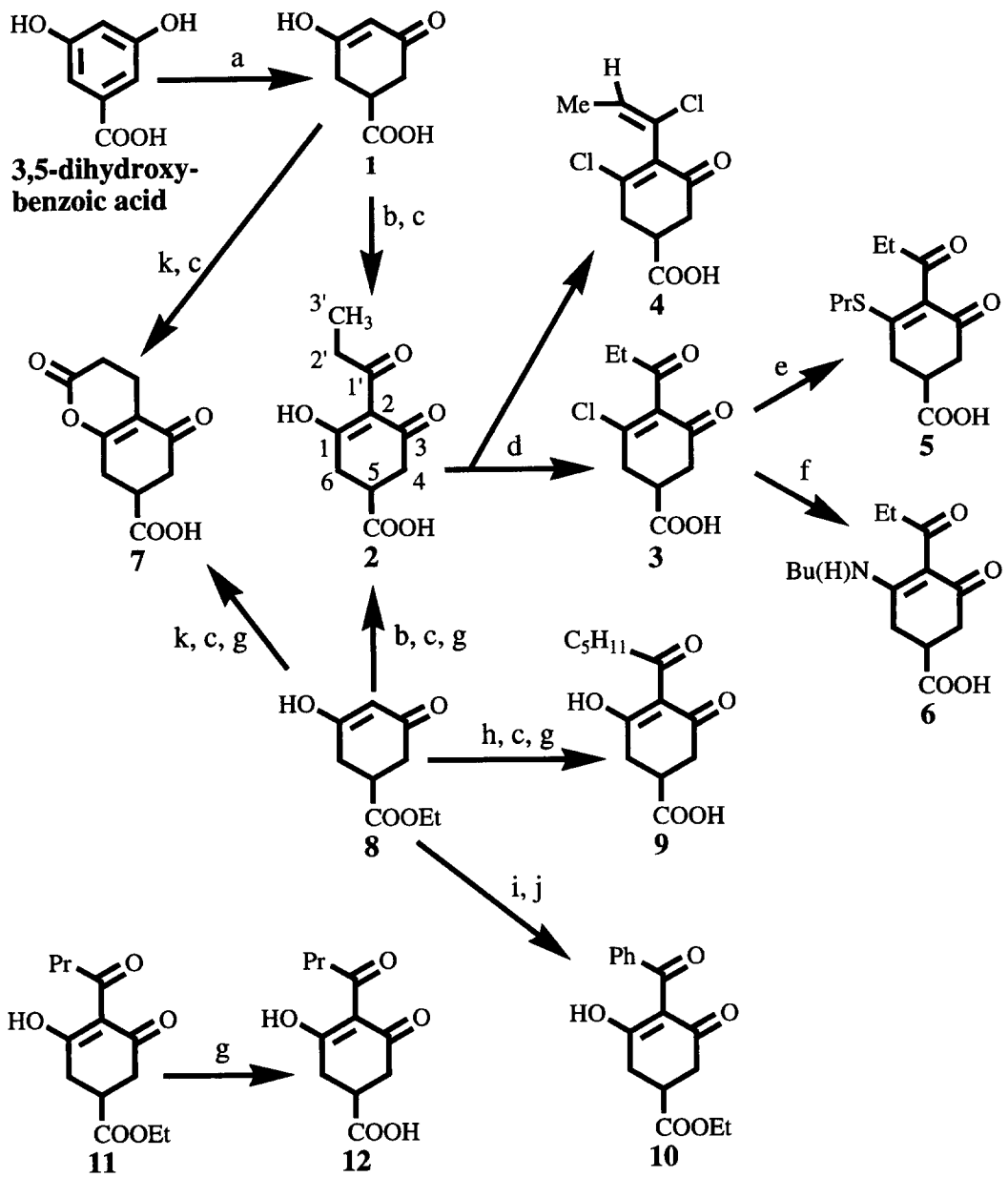
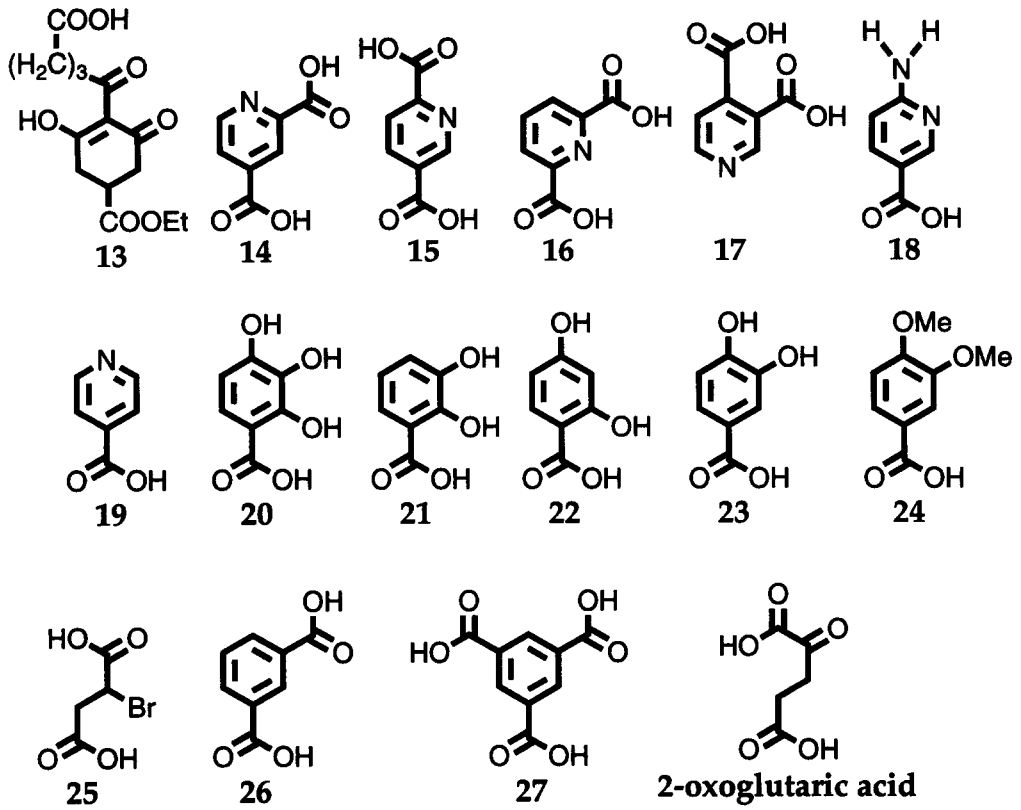


Scheme 1. Structures and synthesis of acylcyclohexanedione-related inhibitors. a: Raney Ni–H_2–NaOH; b: propionyl chloride–Et_3N–CH_2Cl_2; c: DMAP–CH_2Cl_2; d: oxalyl chloride–DMF–CH_2Cl_2; e: nPrSH–Et_3N–THF; f: $nBuNH_2$–Et_3N–THF; g: 1 M NaOH; h: hexanoyl chloride–Et_3N–CH_2Cl_2; i: benzoyl chloride–Et_3N–CH_2Cl_2; j: acetone cyanohydrin–CH_3CN; k: acryloyl chloride–Et_3N–CH_2Cl_2.

most compounds in the present study were synthesized from 3,5-dioxocyclohexanecarboxylic acid ethyl ester (**8**) (Scheme 1). Syntheses of the basic 2-acylcyclohexanediones (**2**, **9**, **10**) from **1** or **8** were a modification of similar routes [9–11] and involved *O*-acylation of **1** or **8** with acyl halides, followed by an O- to C- rearrangement of the acyl group, catalysed by 4-dimethylamino-pyridine(DMAP) (e.g., **1** to **2**) or acetone cyanohydrin (**8** to **10**). Syntheses of the enolthioether (**5**) and enamine (**6**) were a modification of similar work with substituted cyclohexane herbicides [12]. Thus, chlorination of the enol **2** with oxalyl chloride gave the expected 3-chloro derivative **3** along with the dichloro compound **4**, the structures of which were determined by NMR and mass spectrometry. Treatment of **3** with the relevant nucleophiles gave the enolthioether **5** and enamine **6**. The enol lactone **7** was prepared by *O*-acylation of **8** with aryloyl chloride, followed by treatment with DMAP. In this case, O- to C-rearrangement of the acryloyl group did not occur and the favoured reaction was formation of the enol lactone, which appears to arise from Michael addition of the enolate of **8** to the acryloyl residue after dissociation from the C-1 oxygen atom, followed



Scheme 1.—*Continued.*

by cyclization. 2-Butyryl-5-carboxycyclohexane-1,3-dione (**12**) was prepared by hydrolysis of the ethyl ester (**11**) (LAB 198999).

Inhibition of GA 3β-hydroxylase activity

Prohexadione (**2**) was compared with the analogue **3**, in which the 1-hydroxyl had been replaced with chlorine, as inhibitors of a partially purified 3β-hydroxylase from *C. maxima* endosperm [13] (Fig. 1). Activity of this enzyme was measured by determining the amount of conversion of [^{14}C]GA_{25} to [^{14}C]GA_{13} using HPLC-radiocounting. Prohexadione (**2**) was found to be an excellent inhibitor of this enzyme, reducing 3β-hydroxylase activity to 32–47% of the control activity at 10 μM. Compared with prohexadione, there was only slight loss of inhibitory activity with **3** as inhibitor. Thus, only two of the three keto carbonyls are required for activity in these compounds. This fits well with the model [7], which suggests that only the 1′,3-keto groups are required for chelation to Fe^{2+} in site II.

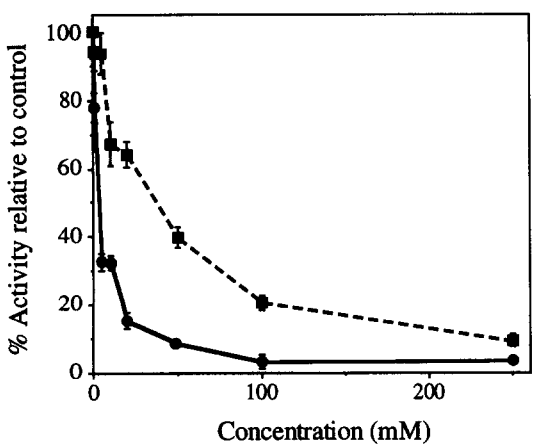


Fig. 1. Comparison of Prohexadione (**2**, solid line) and its chloro-analogue (**3**, dashed line) as inhibitors of partially-purified 3β-hydroxylase from *C. maxima* endosperm.

The 1,1′-dichloro-compound **4** is considerably less active than **2** [Fig. 2(A); Table 1] and apparently is also less active than the 1-chloro analogue **3**, although **3** and **4** were not compared directly. Thus, removal of the 1′-keto group does, as expected, lower activity. However, **4** is not inactive, demonstrating perhaps that an electronegative group on C-1′ is sufficient for some degree of enzyme binding. The inactivity of the cyclic analogue (**7**) [Fig. 2(B)], which has only the C-3 carbonyl and no electronegative function at C-1′, supports this hypothesis. To explore further the scope for modification at C-1, compounds **5** (1-SPr) and **6** (1-N(H)Bu) were examined. These compounds are of similar activity to the 1,1′-dichloro-compound **4** [Fig. 2(A); Table 1] and much less active than **2** or **3**. In the case of these compounds, which retain two ketone carbonyls at C-1′ and C-3, it appears that the increased size and lipophilicity of the 1-substituent is detrimental to inhibitor activity, relative to the 1-

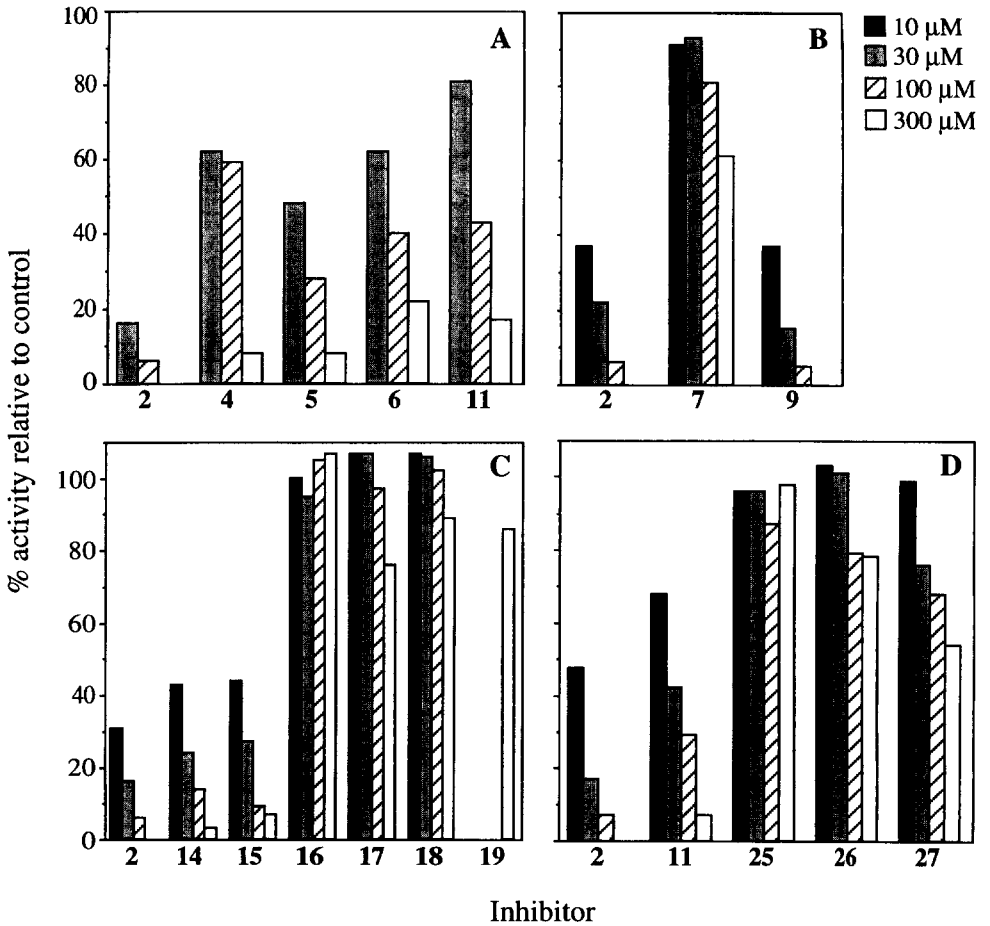


Fig. 2. Comparison of several 1- and acyl-substituted acylcyclohexanediones (**A** and **B**, respectively), substituted pyridines (**C**) and some other commercially-available compounds (**D**) with prohexadione (**2**) and LAB 198999 (**11**) as inhibitors of a partially-purified 3β-hydroxylase from *C. maxima* endosperm.

chloro group. An alternative explanation of the higher activity of **3** relative to **5** or **6** is that the chloro compound, being more electrophilic is capable of irreversibly binding to the active site and inactivating the enzyme (see later).

Comparison between the inhibitory activity of prohexadione (**2**) with the ethyl ester LAB 198999 (**11**) [Fig. 2(A); Table 1] reveals that the 5-carboxylic acid is crucial for binding of these compounds to the 3β-hydroxylase. In contrast to the results of Griggs *et al.* [7], we have found this to be true also for inhibition of a GA 2β-hydroxylase from *Phaseolus vulgaris*, even when comparing the ethyl ester (**11**) with its free acid (**12**) (data not shown).

Kamiya *et al.* [14] suggested that modifications to the 2-acyl side-chain of prohexadione do not seriously affect the potency of the inhibitor. In the present work, we have confirmed this by comparing prohexadione (**2**) with the hexanoyl analogue (**9**), which were equally active [Fig. 2(B)]. In addition, ethyl esters LAB 198999 (**11**), the benzoyl analogue (**10**) and the 4′-carboxybutyryl analogue (**13**) were compared [Fig. 3(A)]. The 2-benzoyl group reduced activity, while the 2-(4-carboxybutyryl) analogue showed no decrease in activity (see Table 1). This may indicate that while the binding site may tolerate larger hydrophobic groups at C-2, a hydrophilic terminus is more favourable.

The inhibitory activities of the acylcyclohexanediones (**2–7** and **9–13**) were compared with those of the carboxypyridines (**14–19**) and hydroxy- (**20–23**), methoxy- (**24**) and carboxy-benzoic acids (**26**, **27**) [Figs 2C, D) and 3(B); Table 1]. The commercially available compounds (**14–27**) are shown in the orientation that may best mimic the structure of the natural co-substrate, 2-oxoglutaric acid.

The results of testing compounds possessing a pyridine ring are shown in Fig. 2(C) 2,4- (**14**) and 2,5- (**15**) pyridine dicarboxylic acids are efficient inhibitors of the 3β-hydroxylase, but are slightly less potent than prohexadione (**2**). 2,6-Pyridine dicarboxylic acid (**16**) is inactive, even at 300 mM, while 3,4-pyridine dicarboxylic acid (**17**), 6-aminonicotinic acid (**18**) and isonicotinic acid (**19**) have little activity. These results indicate that the best pyridine dicarboxylic acid inhibitors have the ring-N atom adjacent to a single carboxyl group, possibly because this configuration allows optimum co-ordination to Fe^{2+}. 2,4-Pyridine dicarboxylic acid (**14**) was as effective an inhibitor as the 2,5-isomer

Table 1. Comparison of the potential of compounds as inhibitors of the activity of a partially-purified 3β-hydroxylase from *C. maxima* endosperm. Activity is representative of an estimate of the I_{50} of each compound: ++++, <30 μM; +++, 30–100 μM; ++, 100–300 μM; +, >300 μM. Compounds within each group are ranked in descending order of relative activity, as estimated by comparing their inhibitory potential at 100 μM from Figs 1–3 and normalizing with respect to compounds **2** or **11** in each case. Compound **19** was ranked according to a comparison at 300 μM

Compound	Activity
2	++++
9	++++
15	++++
14	++++
3	++++
5	++++
13	+++
6	+++
11	+++
4	++
27	+
7	+
26	+
10	+
23	+
20	+
21	+
22	+
17	+
19	+
18	+
16, 24, 25	Inactive

15 in these incubations, contrary to what was found with the GA_1 2β-hyroxylase from *P. vulgaris* [7] and with prolyl 4-hydroxylase [2]. In both these cases, inhibition by 2,4- and 2,5-pyridine dicarboxylic acids was substantially different, with the 2,4-isomer the more potent inhibitor for the 2β-hydroxylase and the 2,5-isomer the more active inhibitor of prolyl 4-hydroxylase.

For maximum activity as inhibitors of GA 3β-hydroxylase activity, it appears that the pyridine dicarboxylic acids should possess a carboxyl group *ortho* to the N atom and a second carboxyl *para* to either the ring-N or the 2-carboxy group. Since **14** and **15** have similar inhibitory activities, the N atom can be *meta* or *para* to the second carboxyl group. The relative positions of these three groups are similar to those of the functional groups in 2-oxoglutarate and prohexadione. It was suggested that the two adjacent groups co-ordinate to Fe^{2+}, whereas the remote carboxylic acid group binds to subsite I within the enzyme active site [2]. This 1,2,4-configuration is not present in the 2,6- and 3,4-pyridine dicarboxylic acids, which have little activity and may not be able to co-ordinate to the Fe^{2+} and bind to subsite I simultaneously. Although the N atom and adjacent carboxyl group are interchangeable, the N atom cannot replace the carboxylic acid group for binding to site I. Thus, the 3,4-isomer **17** has very little activity despite the two adjacent carboxylic acid groups, which would be expected to co-ordinate to Fe^{2+}. Furthermore, since the 2,6- and 3,4-isomers would be predicted to be good iron chelators, but are poor inhibitors of enzyme activity relative to prohexadione or the 2,4- and 2,5-isomers, it follows that good inhibitors do not act simply by chelating iron in solution so that it is unavailable to the enzyme. A good inhibitor must both chelate iron and bind within the 2-oxoglutarate binding site. 6-Aminonicotinic acid (**18**) resembles 2,5-pyridine dicarboxylic acid, but with the 2-carboxyl substituted by a primary amine group. Isonicotinic acid (**19**) resembles 2,4-pyridine dicarboxylic acid, but with the 2-carboxyl absent. Both of these compounds were much weaker inhibitors than 2,4- or 2,5-pyridine dicarboxylic acid, indicating that a primary amine group does not co-ordinate well to Fe^{2+} and that the

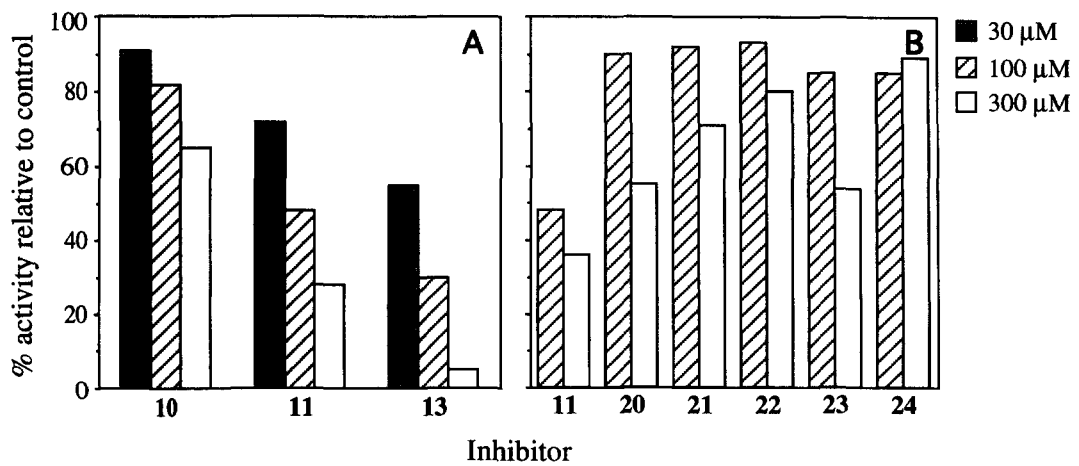


Fig. 3. Comparison of several 1-substituted acylcyclohexanedione ethyl esters (**A**) and hydroxy- and methoxybenzoic acids (**B**) with LAB 198999 (**11**) as inhibitors of a partially-purified 3β-hydroxylase from *C. maxima* endosperm.

adjacent carboxyl group is necessary for maximum inhibition.

Several other related commercially-available compounds were also tested as inhibitors of GA 3β-hydroxylase activity [Fig. 2(D); Table 1)]. Bromosuccinic acid (**25**), an analogue of succinic acid, the product of enzyme turnover of 2-oxoglutarate, was inactive. Isophthalic acid (**26**) structurally resembles 2,4-pyridine dicarboxylic acid (**14**), but without the ring-N, and was chosen so that the importance of the ring N atom could be determined. Both **26** and benzene-1,3,5-tricarboxylic acid (**27**) inhibited the enzyme, but to a lesser extent than did prohexadione (**2**), LAB 198 999 (**11**) and, by extension, 2,4-pyridine dicarboxylic acid (**14**). This result provides further support for the theory that, for this type of inhibitor, highest activity requires both an aromatic N atom and a carboxyl to chelate to the iron; a carboxyl group alone is not sufficient.

LAB 198 999 (**11**) was also compared with a series of hydroxybenzoic acids, which proved to be poor inhibitors [Fig. 3(B); Table 1]. The most active of this type of compound were 2,3,4-trihydroxybenzoic acid (**20**) and 3,4-dihydroxybenzoic acid (**23**). 2,3-Dihydroxybenzoic acid (**21**) and 2,4-dihydroxybenzoic acid (**22**) are weak inhibitors, while 3,4-dimethoxybenzoic acid (**24**) is inactive. The two best inhibitors of this type possess the '1,2,4'-configuration of functional groups, which allows chelation to Fe^{2+} and binding to subsite I. However, these compound are very weak inhibitors, indicating the low capacity of the dihydroxyl system to coordinate Fe^{2+}.

In conclusion, of the three classes of inhibitors studied, the 2-acyl-5-carboxy-cyclohexane-1,3-diones were the most effective. The 2,4- and 2,5-pyridine dicarboxylic acids were also very effective inhibitors. Within the 2-acyl-5-carboxy-cyclohexane-1,3-diones, the 5-carboxy group was important for highest activity. Variation in the size and polarity of the 2-acyl group was tolerated. The 1-chloro derivative **3**, was shown to be an excellent inhibitor. Since the 1-chloro atom in **3** could be replaced by nucleophilic substitution, as in the synthesis of **5** or **6**, it is possible that **3** might act as an affinity label for GA dioxygenases. In theory, a nucleophilic group on amino acid side-chains, such as those of cysteine or lysine, within the enzyme active site could displace the chlorine, leaving the compound bound covalently to the enzyme. To explore this possibility we have prepare [^{3}H]-labelled **3**. Initial results of experiments involving the interaction of labelled **3** with the GA 3β-hydroxylase, in combination with SDS-PAGE and fluorography, indicate that the compound is indeed irreversibly bound (data not shown). Studies to determine the specificity of this binding are in progress.

EXPERIMENTAL

General. Flash CC, unless otherwise stated, was carried out on columns of Merck Keiselgel 60 (40–63 μm). Compounds were loaded, if possible, as solns in CH_2Cl_2 and the columns eluted stepwise with 10% increments of EtOAc in hexane (containing 0.5% HOAc for chromatography of carboxylic acids). ^{1}H and ^{13}C NMR spectra were determined at 400 MHz and 100 MHz, respectively. Spectra were obtained from samples dissolved, where possible, in $CDCl_3$, or, alternatively, in CD_3OD, acetone-d_6 (HDA) or DMSO-d_6 as necessary, with TMS as int. standard.

Reagents. Compounds **14–27** were obtained commercially. 2-Butyryl-5-carboxyethylcyclohexane-1,3-dione (**11**) was a gift from BASF AG. 5-Carboxyethylcyclohexane-1,3-dione (**8**) and 2-(4-carboxybutyryl)-5-carboxyethylcyclo-hexane-1,3-dione (**13**) were gifts from Kumiai chemical company, Japan. Purity was determined by ^{1}H and ^{13}C NMR.

5-Carboxycyclohexane-1,3-dione (**1**) was prepd by the hydrogenation of 3,5-dihydroxybenzoic acid over Raney Ni in aq. NaOH soln, according to ref. [8]; recrystallization from acidified aq. soln gave the required compound. ^{1}H NMR (HDA): δ 2.60 (4H, *d*, J = 6.97 Hz, 2× ring CH_2), 3.14 (1H, *m*, J = 7.0 Hz, H-5), 5.33 (1H, *s*, H-2).

2-*Propionyl-5-carboxy-cyclohexane*-1,3-*dione* (**2**). To a stirred soln of 5-carboxyethylcyclohexane-1,3-dione (**8**) (2 g) and Et_3N (4.5 ml, 3 eq.) in dry CH_2Cl_2 (30 ml), was added dropwise, with ice-cooling, propionyl chloride (1.1 ml, 1.2 eq.). The reaction mixt. was allowed to warm to room temp., stirring for a total of 2 hr. The soln was then washed with 5% aq. citric acid soln, then with H_2O, dried ($MgSO_4$) and the solvent removed *in vacuo*. The residue was taken up in dry CH_2Cl_2 (30 ml) and DMAP (300 mg) added. The reaction was stirred at room temp. for 20 hr and worked up as described above to give 2-propionyl-5-carboxyethyl-cyclohexane-1,3-dione, pure enough to use directly in the next step. ^{1}H NMR ($CDCl_3$): δ 1.13 (3H, *t*, J = 7.3 Hz, H_3-3′), 1.27 (3H, *t*, J = 7.1 Hz, ester CH_3), 2.7–2.9 (4H, *m*, 2× ring CH_2), 3.07 (2H, *q*, J = 7.2 Hz, H_2-2′), 3.07 (1H, *m*, H-5), 4.19 (*q*, 2H, J = 7.1 Hz, ester CH_2). ^{13}C NMR ($CDCl_3$): δ 8.4 (C-3′), 14.3 (ester CH_3), 34.4 and 36.7 (C-4,5,6,2′), 61.6 (ester CH_2), 112.6 (C-2), 172.4 (COOEt), 206.9 (C-1,3,1′). The ester was then stirred in 1 M aq. NaOH (100 ml) at room temp. for 21 hr. The resulting soln was then acidified to pH 1 with conc. HCl and recovered in EtOAc. After evapn, residue was further evapd from $CHCl_3$ and then dried to give 2-propionyl-5-carboxy-cyclohexane-1,3-dione (**2**) as a yellowish solid (1.8 g, 77% yield over three steps). Found $[M]^+$ m/z 212.0681; $C_{10}H_{12}O_5$ requires 212.0685. ^{1}H NMR ($CDCl_3$): δ 1.14 (3H, *t*, J = 7.1 Hz, H_3-3′), 2.68–2.96 (4H, *m*, 2× ring CH_2), 3.07 (2H, *q*, J = 7.3 Hz, H_2-2′), 3.12 (1H, *m*, H-5). ^{13}C NMR ($CDCl_3$): δ 8.2 (C-3′), 34.3, 34.7, 36.2, 40.1 (C-4,5,6,2′), 112.5 (C2), 177.5 (COOH), 192.7, 196.0, 207.0 (C-1,3,1′). EIMS m/z (rel. int.): 212$[M]^+$ (91), 194 (4), 183 (42), 165 (48), 137 (57), 111 (52), 84 (32), 69 (100), 57 (63).

1-*Chloro*-2-*propionyl*-5-*carboxycyclohex*-1-*en*-3-*one* (**3**). To a stirred soln of **2** (271 mg) in dry CH_2Cl_2

(10 ml) was added oxalyl chloride (1.1 ml, 10 eq.), together with a catalytic amount of DMF (2 drops). The reaction was left at room temp. under N_2 with stirring until the disappearance of the starting material (3–5 days), as determined by TLC, using hexane–EtOH–HOAc (80:20:1), and ^{13}C NMR. The reaction was worked-up by first adding it carefully to ice-cooled 5% aq. citric acid soln, with stirring, followed by extracting ×2 with CH_2Cl_2. The organic layer was washed with H_2O, dried ($MgSO_4$) and evapd. The residue was subjected to repetitive flash CC, the column eluted stepwise with 2% increments of EtOH in hexane, containing 0.5% HOAc. The reaction gave two compounds, the less polar being the dichlorinated product, 1-chloro-2-(1-chloroprop-1-enyl)-5-carboxy-cyclohex-1-en-3-one (**4**) (gum, 41 mg, 13% yield). Found $[M]^+$ *m/z* 230.0327; $C_{10}H_{11}{}^{35}ClO_4$ requires 230.0325. ^1H NMR ($CDCl_3$): δ 1.88 (3H, *d*, $J = 6.59$ Hz, H_3-3′), 2.6–2.9 (2H, *m*, ring CH_2), 3.12 (2H, *m*, ring CH_2), 3.22 (1H, *m*, H-5), 5.68 (1H, *q*, $J = 6.78$ Hz, H-2′). ^{13}C NMR ($CDCl_3$): δ 14.5 (C-3′), 36.5, 38.4, 38.8 (C-4,5,6), 123.5 (C-2′), 129.1 (C-1′), 137.9 (C-1), 154.5 (C-2), 176.6 (COOH), 191.20 (C-3). EIMS (rel. int.): 252 ($^{37}Cl^{37}Cl.[M]^+$, 7), 250 ($^{35}Cl^{37}Cl.[M]^+$, 38), 248 ($^{35}Cl^{35}Cl.[M]^+$, 59), 213 (54), 203 (20), 185 (15), 167 (76), 139 (43), 103 (64), 77 (100), 51 (66). Further elution gave the required monochlorinated product, 1-chloro-2-propionyl-5-carboxy-cyclohex-1-en-3-one (**3**) (gum, 124 mg, 42% yield). Found $[M]^+$ *m/z* 248.0009; $C_{10}H_{10}{}^{35}Cl_2O_5$ requires 248.0007. ^1H NMR ($CDCl_3$): δ 1.12 (3H, *t*, $J = 7.33$ Hz, H_3-3′), 2.63 (2H, *q*, $J = 6.96$ Hz, H_2-2′), 2.67–2.82 (2H, *m*, ring CH_2), 3.06–3.09 (2H, *m*, ring CH_2), 3.25 (1H, *m*, H-5). ^{13}C NMR ($CDCl_3$): 7.3 (C-3′), 35.8, 37.1, 38.3, 38.5 (C-4,5,6,2′), 140.4 (C-1), 151.0 (C-2), 176.9 (COOH), 191.9, 202.8 (C-3,1′). EIMS *m/z* (rel. int.): 232 ($^{37}Cl.[M]^+$, 18), 230 ($^{35}Cl.[M]^+$, 53), 201 (94), 185 (22), 173 (30), 155 (100), 129 (55), 99 (80), 57 (92).

1-*Thiopropyl-2-propionyl-5-carboxy-cyclohex*-1-*en*-3-*one* (**5**). To a soln of **3** (20 mg) stirred in THF (3 ml) at room temp., was added *n*-propylthiol (16 μl, 2 eq.) followed by Et_3N (24 μl, 3.7 eq.). The reaction mixt. was then stirred for 3 days. The product obtained was extracted into CH_2Cl_2 and washed with 5% aq. citric acid soln. The organic layer was dried ($MgSO_4$) and the solvent removed *in vacuo*. The residue was purified by flash CC, to give the required 1-thiopropylether **5** (gum, 14 mg, 60% yield). Found $[M]^+$ *m/z* 270.0902; $C_{13}H_{18}O_4S$ requires 270.0920. ^1H NMR ($CDCl_3$): δ 1.04 (3H, *t*, $J = 7.3$ Hz, $C\underline{H}_3(CH_2)_2S$-), 1.10 (3H, *t*, $J = 7.3$ Hz, H_3-3′), 1.68 (2H, *m*, $CH_3C\underline{H}_2CH_2S$-), 2.70 (2H, *q*, H_2-2′), 2.70 (2H, *m*, ring CH_2), 2.87 (2H, *t*, $J = 7.3$ Hz, $CH_3CH_2C\underline{H}_2S$-), 3.00 (2H, *m*, ring CH_2), 3.11 (1H, *m*, H-5). EIMS *m/z* (rel. int.): 270 ($[M]^+$, 5), 241 (56), 227 (100), 212 (13), 195 (13), 181 (21), 165 (25), 153 (25), 127 (23), 97 (22), 69 (32), 57 (60).

1-n-*Butylamino-2-propionyl-5-carboxy-cyclohex*-1-*en*-3-*one* (**6**). To a soln of **3** (28 mg), stirred in THF (3 ml) at room temp. was added *n*-butylamine (24 μl, 2 eq.) followed by Et_3N (34 μl, 3.8 eq.). The reaction mixt. was then stirred for 3 days. The mixt. was then diluted with CH_2Cl_2 and the soln washed with 5% aq. citric acid soln, then H_2O, dried ($MgSO_4$), and evapd. The residue was purified by flash CC, to give the required 1-*n*-butylamino-2-propionyl-5-carboxy-cyclohex-1-en-3-one (**6**) (gum, 14.4 mg, 44% yield). Found $[M]^+$ *m/z*: 267.1471; $C_{14}H_{21}NO_4$ requires 267.1471. ^1H NMR ($CDCl_3$): δ 0.97 (3H, *t*, $J = 7.3$ Hz, $C\underline{H}_3(CH_2)_3N(H)$-), 1.21 (3H, *t*, $J = 7.3$ Hz, H_3-3′), 1.46 (2H, *m*, $J = 7.33$ Hz, $CH_3C\underline{H}_2(CH_2)_2N(H)$-), 1.68 (2H, *m*, $J = 7.3$ Hz, $CH_3CH_2C\underline{H}_2CH_2N(H)$-), 2.75 (2H, *m*, H_2-2′), 2.75 (2H, *m*, ring CH_2), 3.01 (2H, *m*, ring CH_2), 3.01 (1H, *m*, H-5), 3.44 (2H, *m*, $CH3(CH_2)_2C\underline{H}_2N(H)$-). EIMS *m/z* (rel. int.): 267 ($[M]^+$, 67), 250 (11), 238 (51), 224 (19), 210 (100), 194 (11), 166 (17), 111 (15), 95 (21), 55 (25), 44 (49).

2-*Hexanoyl-5-carboxy-cyclohexane*-1,3-*dione* (**9**). To a soln of **8** (50 mg) and Et_3N (113 μl, 3 eq.) stirred in dry CH_2Cl_2 (5 ml), was added slowly, with ice-cooling, hexanoyl chloride (49 μl, 1.3 eq.). The reaction mixt. was allowed to warm to room temp., stirring for a total of 2 hr. The reaction mixt. was then washed with 5% aq. citric acid soln, then H_2O, dried ($MgSO_4$) and evapd *in vacuo*. The residue was taken up in dry CH_2Cl_2 (10 ml) and DMAP (40 mg) added. The reaction mixt. was stirred at room temp. for 20 hr and then worked-up as described above to give 2-hexanoyl-5-carboxyethyl-cyclohexane-1,3-dione, which was used directly in the next step. ^1H NMR ($CDCl_3$): δ 0.88 (3H, *t*, $J = 7.0$ Hz, H_3-6′), 1.25 (5H, *m*, H_2-5′ and ester CH_3), 1.33 (4H, *m*, H_2-4′), 1.60 (2H, *m*, H_2-3′), 2.63–2.76 (2H, *m*, ring CH_2), 2.87–2.92 (2H, *m*, ring CH_2), 3.00 (2H, *t*, $J = 7.0$ Hz, H_2-2′), 3.02 (1H, *m*, H-5), 4.15 (2H, *q*, $J = 7.0$ Hz, ester CH_2). ^{13}C NMR ($CDCl_3$): δ 14.1 (C-6′), 14.3 (ester CH_3), 22.7 (C-5′), 24.5 (C-4′), 31.7 (C-3′), 35.5, 36.6, 40.6, 40.9 (C-4,5,6,2′), 61.7 (ester CH_2), 172.4 (C$\underline{O}$OEt), 192.8, 196.9, 206.3 (C-1,3,1′). The above compound was then stirred in 1 M aq. NaOH soln (10 ml) at room temp. for 20 hr. The resulting aq. soln was then acidified to pH 1 with conc. HCl and extracted ×2 with EtOAc. The organic layer was washed with H_2O, dried ($MgSO_4$) and evapd. The residue was purified by flash CC, to give the required compound, 2-hexanoyl-5-carboxycyclohexane-1,3-dione (**9**) (gum, 36 mg, 60% yield over three steps). Found $[M]^+$ *m/z* 254.1156; $C_{13}H_{18}O_5$ requires 254.1154. ^1H NMR ($CDCl_3$): δ 0.90 (3H, *t*, $J = 7.0$ Hz, H_3-6′), 1.34 (4H, *m*, H_2-5′,4′), 1.62 (2H, *m*, H_2-3′), 2.68–2.84 (2H, *m*, ring CH_2), 2.93–2.95 (2H, *m*, ring CH_2), 3.02 (2H, *t*, $J = 7.5$ Hz, H_2-2′), 3.13 (1H, *m*, H-5). ^{13}C NMR ($CDCl_3$): δ 14.4 (C-6′), 223.0 (C-4′), 24.8 (C-5′), 32.0 (C-3′), 35.5, 36.6, 40.7, 40.9 (C-4,5,6,2′), 113.0 (C-2), 177.7 (COOH), 192.8, 196.9, 206.7 (C-1,3,1′). EIMS *m/z* (rel. int.): 254 ($[M]^+$, 52), 236 (8), 211 (96), 198 (37), 183 (21), 165 (23), 153 (34), 139 (45), 111 (27), 55 (100).

2-*Benzoyl-5-carboxyethyl-cyclohexane*-1,3-*dione* (**10**). To a stirred soln of **8** (50 mg) and benzoyl chloride (31 μl, 1 eq.) in CH_2Cl_2 (5 ml) was added Et_3N (41 μl, 1.1 eq.). The soln was stirred at room temp. for 5

hr and the reaction mixt. was washed with 5% aq. citric acid soln, then H_2O, dried ($MgSO_4$) and evapd to give the required *O*-acylated diketone, which was used directly in the next step. ^1H NMR ($CDCl_3$): δ 1.28 (3H, *t*, ester CH_3), 2.61–3.02 (4H, *m*, 2× ring CH_2), 3.19 (1H, *m*, H-5), 4.21 (2H, *q*, ester CH_2), 6.12 (1H, *s*, H-2), 7.48 (2H, *m*, Ar H-3,5), 7.61 (1H, *m*, Ar 4-H), 8.11 (2H, *m*, Ar H-2,6). The residue was taken up in redistilled MeCN (10 ml), to which was added (83 μl, 2 eq.) Et_3N and a catalytic amount of Me_2CO cyanohydrin (20 μl). The soln was stirred for 18 hr at room temp. The soln was then evapd and the product purified by flash CC, to give 2-benzoyl-5-carboxyethyl-cyclohexane-1,3-dione (**10**) (43 mg, 55% yield over two steps). Found $[M]^+$ *m/z* 288.0985; $C_{16}H_{16}O_5$ requires 288.0984. ^1H NMR ($CDCl_3$): δ 1.28 (3H, *t*, J = 7.1 Hz, ester CH_3), 2.76–2.85 (4H, *m*, 2× ring CH_2), 3.16 (1H, *m*, H-5), 4.21 (2H, *q*, J = 7.3 Hz, ester CH_2), 7.39 (2H, *m*, J = 7.7 Hz, Ar H-3,5), 7.49 (1H, *s*, Ar H-4), 7.52 (2H, *d*, J = 8.4 Hz, Ar H-2,6). EIMS *m/z* (rel. int.): 288 ($[M]^+$, 50), 287 (88), 243 (9), 215 (21), 213 (20), 187 (11), 160 (17), 137 (14), 105 (100), 77 (54), 40 (42).

2-(2′-*Carboxyethyl*)-3-*hydroxy*-5-*carboxy*-*cyclohex*-2-*en*-1-*one*,3′-2 *lactone* (**7**). To an ice-cooled, stirred soln of **8** (200 mg) and Et_3N (214 μl, 1.4 eq.) in dry CH_2Cl_2 (15 ml) was added acryloyl chloride (125 μl, 1.2 eq.). The reaction mixt. was stirred overnight and then washed with 5% aq. citric acid soln, then H_2O, dried ($MgSO_4$) and evapd to give the *O*-acryloyl derivative. ^1H NMR ($CDCl_3$): δ 1.24 (3H, *t*, ester CH_3), 2.58–2.96 (4H, *m*, 2× ring CH_2), 3.16 (1H, *m*, H-5), 4.19 (2H, *q*, ester CH_2), 6.02 (1H, *s*, H-2), 6.06 (1H, *d*, H-3′, *trans* to C=O), 6.22 (1H, *dd*, H-2′), 6.59 (1H, *d*, H-3′ *cis* to C=O). The product was taken up into dry CH_2Cl_2 and DMAP (100 mg) added. The soln was stirred overnight and then worked up as described above. Treatment of this product with 1 M NaOH soln overnight, followed by acidification to pH 1 with conc. HCl and recovery in EtOAc, yielded compound **7** (gum, 90 mg, 39% yield over three steps). ^1H NMR (HDA): δ 2.54 (2H, *m*), 2.62–2.73 (4H, *m*), 2.82 (2H, *m*), 3.25 (1H, *m*, H-5). ^{13}C NMR (HDA): δ 17.2 (C-1′), 30.4, 38.3, 39.1 (2 signals) (C-4,5,6,2′), 114.7 (C-2), 166.1 and 167.1 (C-3′ and COOH), 174.1 (C-1), 194.7 (C-3). EIMS *m/z* (rel. int.): 210 ($[M]^+$, 26), 182 (46), 165 (20), 137 (100), 123 (9), 110 (21), 95 (18), 82 (43), 68 (26), 55 (48).

Preparation of $[^{14}C]GA_{25}$. $[^{14}C]GA_{12}$ was first produced from *R*-[2-^{14}C]mevalonic acid using a cell-free system from *C. maxima* endosperm according to ref. [15]. The product was purified by C_{18} SEP-PAK cartridge and reversed-phase HPLC. The sample was loaded onto a C_{18} pre-column (30 mm × 2.1 mm i.d.) and washed for 5 min with H_2O, pH 3 with HOAc, to remove protein. The sample was then eluted at a flow rate of 1 ml min^{-1} onto a Phenomenex Hypersil 5 mM C_{18} (250 mm × 4.6 mm i.d.) column, using the following gradient: 50 min at 65% MeOH, 1 min step to 100% MeOH, 40 min at 100% MeOH, 1 min step to 0% MeOH, 5 min at 0% MeOH; frs (1 ml) or whole peaks were collected. Products were identified by GC-MS. HPLC R_t (min): GA_{14} 20.0, GA_{15} 23.1, *ent*-6α,7α-dihydroxykaurenoic acid 30.4, GA_{12} 41.1, GA_{12}-aldehyde 53.3. $[^{14}C]GA_{25}$ was produced from $[^{14}C]GA_{12}$ using a partially purified 20-oxidase from *C. maxima* endosperm [13]. The substrate was incubated in buffered enzyme soln, containing 0.5 mM 2-oxoglutarate, 0.5 mM $FeSO_4$ and 5 mM ascorbate for 3 hr. Products were recovered using a C_{18} SEP-PAK cartridge, sepd by HPLC, recovered on a SEP-PAK cartridge again to remove HOAc and the solvent removed *in vacuo*. HPLC gradient: 15 min at 75% MeOH, 1 min steps each to 78, 83, 90 and 100% MeOH, 5 min at 100% MeOH, 1 min step to 0% MeOH, 6 min at 0% MeOH. HPLC R_t (min): GA_{25} 12.2, GA_{12} 18.1.

Enzyme assays. An appropriate concn of partially purified *C. maxima* 3β-hydroylase [13] was used in each assay, such that the conversion of $[^{14}C]GA_{25}$ did not exceed 70% in the control incubation. The enzyme was assayed in a final vol. of 240 μl, containing 0.5 mM 2-oxoglutaric acid, 5 mM L-ascorbic acid, 0.5 mM Fe_2SO_4, inhibitor added in 5 μl MeOH, or 5 μl buffer, where necessary, and appropriate GA substrate (*ca* 10000 dpm), dissolved in 100 mM bis-tris propane buffer, pH 7. Controls contained either 5 μl MeOH and/or 5 μl buffer, without the inhibitor. Assays were performed in duplicate or triplicate, if possible. The mixts were incubated for 30 min at 30° with shaking and reactions were stopped by the addition of 10 μl HOAc. Product and substrate were sepd by HPLC, using the following gradient: 8 min at 65% MeOH, 1 min step to 100% MeOH, 3 min at 100% MeOH, 1 min step to 0% MeOH, 5 min at 0% MeOH. Products were identified by GC-MS. HPLC R_t: GA_{13} 15.4, GA_{25} 19.1.

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