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Amylase Induction Activity of Fluorescein Labeled Gibberellin in Barley Aleurone Protoplasts

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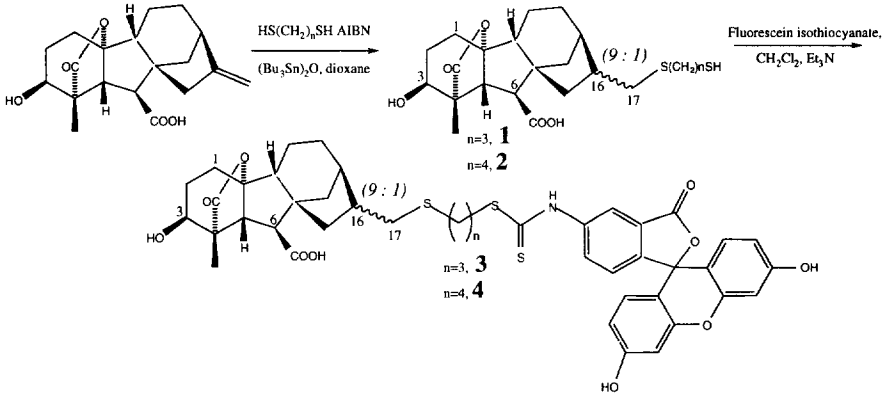
Abstract. The biological activity of two fluorescence-labeled gibberellins where a 1,4-dithiobutylene or 1,3-dithiopropylene chain works as a spacer between the fluorescent and the gibberellin moieties, was evaluated by means of amylase induction in barley aleurone protoplasts. Compound **4**, whose spacer is longer, was found to be more active than compound **3**. Copyright © 1996 Elsevier Science Ltd

The identification of the site where plant hormones are perceived at the cellular level, is a primary task in modern plant physiology, and an essential step towards the isolation and identification of the receptors. Concerning gibberellin (GA), considerable efforts have been devoted to such a purpose. For instance, photoaffinity labeling has led to the identification of several GA-binding proteins[1] which, however, may include also GA metabolic enzymes. Biological activity of some impermeant GAs[2] and especially the response of protoplasts to internally and externally applied hormones,[3] have provided more helpful information, suggesting that the site of perception of GA is on the external surface of the plasma membrane. Nevertheless, a direct evidence for the localization of the receptor is still lacking. Currently, fluorescence-labeled bioactive compounds are widely used for the identification and analysis of receptors' functions;[4] their ability to bind to the receptors in real-time makes them very useful tools for such a purpose. The attainment of a fluorescence-labeled bioactive GA (FLBG) is thus an attractive way for gaining much information about the binding mode as well as the binding site of gibberellin. However, there has been no report on the biological activity of FLBGs. We wish to describe here the activity of two fluorescein labeled gibberellins, which could be powerful tools in understanding the perception of GA by the receptors.

Structure-activity relationships for gibberellins are well known,[5] and show that 3β-hydroxyl and 6-carboxyl groups are essential, while chemical modification of the 16(17)-double bond usually does not affect their biological activity. To prepare our molecular probe, we decided therefore to modify the latter, introducing a mercaptoalkylthio group that could work as a spacer between the fluorescent functional group and the gibberellin moiety, following a method reported by Beale.[6] The properties and synthesis of **1** and **3** have already been reported,[6] while compound **4** can be prepared in a similar way. The free radical addition of 1,4-butanedithiol to GA_4 proceeds smoothly in dioxane, furnishing 17-mercaptobutylthio-3α,10-dihydroxy-20-norgibberella-7,19-dioic acid 19,10-lactone (**2**) in quantitative yields.[7] This is isolated by elution of the crude

reaction mixture through silica gel (hexane : ethyl acetate : acetic acid, 1 : 1 : 0.01).[8] Compound **2** (135 mg) is then coupled with fluorescein as described, and the conjugate (**4**) is purified by dissolving the crude mixture in acetone, and eluting through silica gel with abundant acetone first, and acetone : acetic acid 1 : 0.01 later (164 mg, 65 %).[9]

Separation of the epimers for both **3** and **4** was not attempted, and the two compounds were used as a 9 : 1 diastereomeric mixture for the biological assays.



To evaluate the activity of these compounds the choice of an appropriate biological system is crucial. The aleurone layer of barley (*Hordeum vulgare*) provides a unique opportunity for this purpose because it allows us to assay the hormonal regulation of plant gene expression in an easy and direct way. The aleurone layers synthesize several hydrolytic enzymes, including α-amylase, and secrete them into the endosperm. This process normally takes place during cereal grain germination, and it is induced by active GAs synthesised by the embryo. Therefore the activity of GA analogues could be monitored by measuring their ability to induce α-amylase activity in the embryoless half grains, through the use of a well-established method.[10] However, to study the direct interaction of labeled GA and receptor, it is necessary to exclude from the assay system all the factors, such as transport or metabolism, that might hamper the bioavailability of the probe. The use of half cut barley seeds as an assay system is unsuitable, because the cell walls could act as a barrier against the interaction of biological active chemicals and the membranes. This would make the interpretation of activity results problematical. To overcome such difficulties, we used protoplasts from aleurone cells prepared by the method recently described by Lin et al.[11] The results we obtained are shown in Fig. 1, and can be compared with those obtained with half cut seeds shown in Fig. 2. Protoplasts isolated from the barley aleurone layers respond to 10^{-6} M free GA_3 by synthesizing and secreting amylase at nearly maximal levels. Both **3** and **4** display agonistic activity, however, to obtain a comparable level of induction **3** has to be about 100 times more concentrated than GA_3, while **4** is more potent than **3**. This difference, since the structures of the two compounds are identical except for the length of the spacer, has to be ascribed to the number of methylene groups connecting the two moieties. Considering that the fluorescent functional group is equally bulky as the gibberellin moiety, the longer spacer in compound **4** must allow better interaction between the active GA moiety and the receptor, presumably because of reduced hinderance by the fluorescent moiety.

Fig. 1 Amylase induction activity of GA analogs in protoplasts

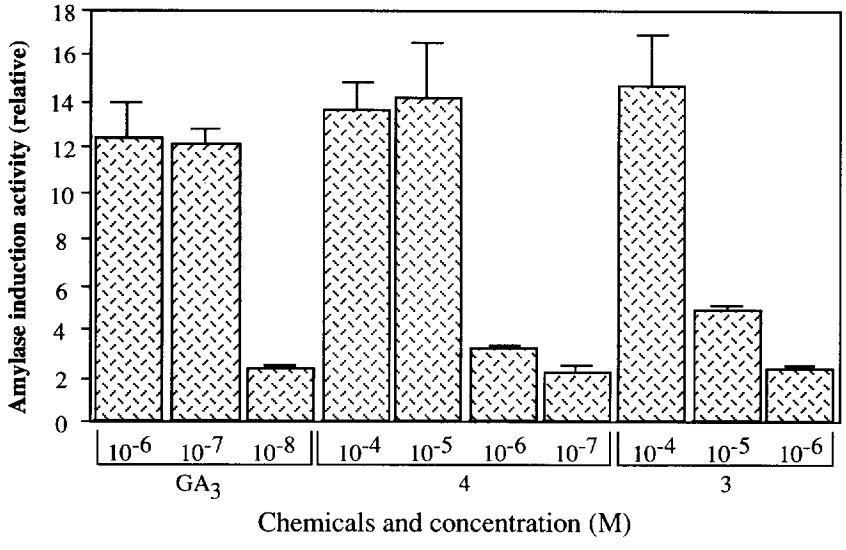
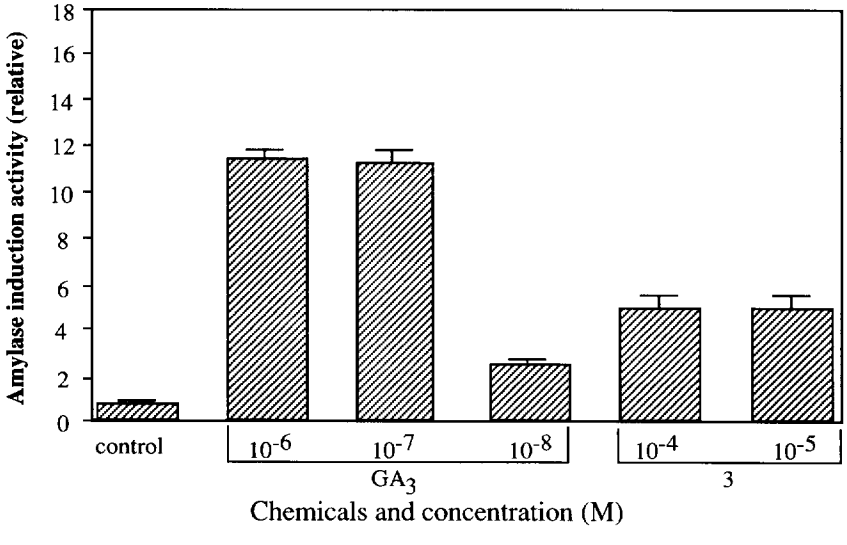


Fig. 2 Amylase induction activity of GA analogs in half cut seeds



The possibility that FLBGs are degraded when incubated with aleurone protoplasts has also been taken into consideration. The resulting free gibberellin could have induce the α-amylase activity. However, while in protoplasts **3** (10^{-4} M) and GA_3 (10^{-6} M) induce approximately the same level of α-amylase activity, at the same concentrations in half grains, activity of **3** is about one half of that of GA_3. This works against the degradation hypotheses because if α-amylase induction was due to free GA_4 released from **3**, we would

expect a similar behavior of FLBG in the two cases. We concluded therefore that while the unhindered GA_3 moves freely through the cell wall, the latter acts as a physical barrier against the probe. As a consequence, activity of **3** appears lower in the half grains experiment.

Synthesis of compounds such as **4** and **3** constitutes the first step toward the analysis of the interaction between FLBG and aleurone protoplasts. These compounds will be of great use in identifying plant hormone receptors, through the use of a flowcytometer or by confocal laser microscopy.

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7) Although we have not yet assigned the stereochemistry at C-16, ^{1}H-NMR spectra of **2** and **4**, as well as those of **1** and **3**, clearly showed a splitting of the signal of H-5, suggesting the presence of two diasteroisomers in approximately a 9 : 1 ratio.

8) ^{1}H-NMR (CD_3COCD_3, 300 MHz): 1.11 (3H, s, H-18), 2.29 (2H, m), 2.56 (7H, m, H-6, CH_2S X 3), 3.18 (1H, d, J = 10.7 Hz, H-5), 3.72 (1H, br. d, J = 3.6 Hz, H-3). ^{13}C-NMR: 15.5, 16.3, 21.1, 24.6, 24.7, 28.5, 32.4, 34.1, 35.8, 36.0, 39.7, 41.8, 44.1, 52.0, 52.4, 54.0, 55.7, 57.5, 70.5, 94.6, 174.3, 178.9, (one signal masked by solvent). FAB-MS m/z (rel. int.): 455 (10, $M+H^+$), 439 (60), 423 (15), 395 (12), 333 (10).

9) ^{1}H-NMR (CD_3COCD_3, 300 MHz): 1.11 (3H, s, H-18), 1.18 (1H, dd, J = 13.0 and 3.0 Hz), 2.26 (2H, m), 2.58 (5H, m, H-6, CH_2S X 2), 3.18 (1H, d, J = 10.7 Hz, H-5), 3.38 (2H, t, J = 6.9, CH_2S), 3.72 (1H, br. d, J = 3.1 Hz, H-3), 6.7 (7H, m), 7.32 (1H, d, J = 8.3 Hz), 8.13 (1H, dd, J = 8.3 and 1.8 Hz), 8.6 (1H, br. s). ^{13}C-NMR: GA signals: 15.4, 16.2, 20.8, 28.3, 29.0, 29.3, 29.7, 30.9, 32.4, 35.6, 35.8, 35.9, 39.3, 41.8, 44.0, 52.0, 52.4, 54.0, 55.6, 57.4, 70.5, 94.7, 174.7, 179.1. Fluorescyl signals: 103.6, 111.6, 113.6, 119.3, 125.5, 128.5, 130.3, 131.4, 142.7, 150.9, 153.5, 160.5, 169.3 (C=O), 199.1 (C=S). FAB-MS m/z (rel. int.): 844 (0.9, $M+H^+$), 453 (0.9), 421 (1.2), 333 (2).

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