Radio-iodinated gibberellin photoaffinity probes

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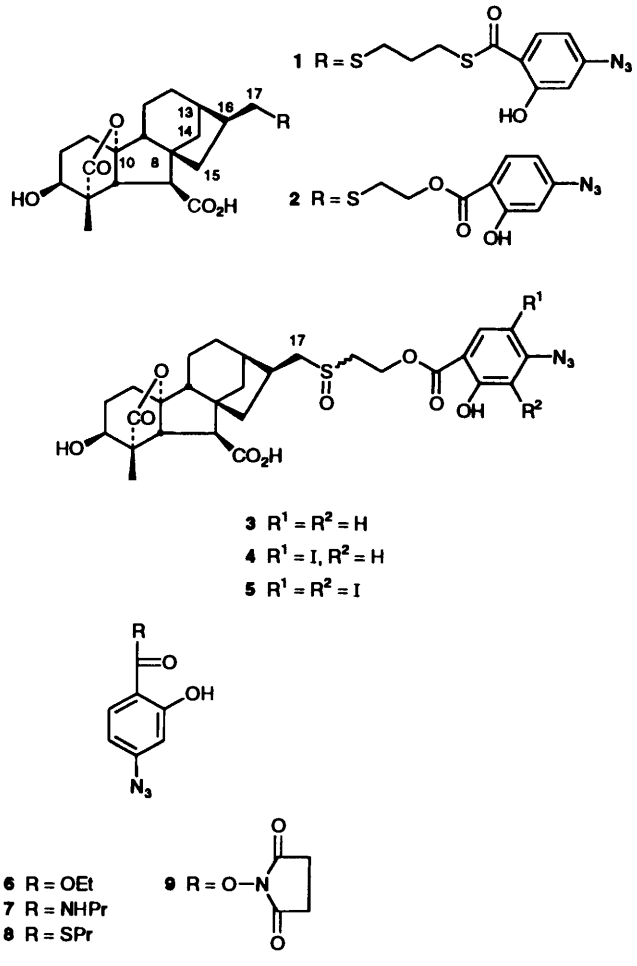
Oxidation of the gibberellin photoaffinity reagent GA_4-17-ylsulfanylethyl 4-azidosalicylate **2** yields separable isomeric sulfoxides. Oxidation at the sulfur atom occurs during the radio-iodination of **2**, leading to low radiochemical yield. The sulfoxides themselves can be efficiently radio-iodinated to specific activities approaching 1000 Ci $mmol^{-1}$ and recovered in high yield after ion-exchange purification. They are biologically active in the induction of α-amylase in aleurone cells of wild oat, *Avena fatua* and, thus, are useful probes for the study of gibberellin perception in this system. The synthesis of 6-[2-(5-iodosalicyloyloxy)ethyl]sulfinylhexanoic acid is also described. This molecule has been designed to act as a mimic of the hydrophobic side-chain of the radioiodinated photoaffinity probe and, thus, may be a useful additive for the reduction of non-specific labelling in photoaffinity experiments.

The gibberellins (GAs) are highly active endogenous regulators of many aspects of plant growth and development. The identification of GA receptors and investigation of GA biosynthetic and metabolic enzymes are important topics in the study of the molecular mechanisms of formation and action of this class of hormones. Affinity labelling, in particular, photoaffinity labelling, is a powerful technique for both the identification of ligand-binding proteins and the characterisation of binding sites within them.[1] We have synthesised GA derivatives as photoaffinity reagents for both receptors and enzymes. The synthesis and bioactivity of several GA_4-C-17-aryl azides (*e.g.* **1** and **2**) have been reported in a previous paper.[2] Having demonstrated the efficacy of radiolabelled **2** for the specific photoaffinity-labelling of a GA_4-binding monoclonal antibody[3] we have been using this compound to probe for GA-binding proteins in aleurone cells of wild oat, *Avena fatua*.[4–6] The *p*-azidosalicyloyl compounds (**1** and **2**) were chosen to enable the use of ^{125}I as a tracer, introduced into the aryl ring by electrophilic substitution. In this paper, we report investigations into the iodination and radioiodination chemistry of both **1** and **2** and describe the preparation of isomeric sulfoxides derived from **2**, which themselves can be radio-iodinated to high specific activity in high radiochemical yield.



Results and discussion

Iodination of salicylates

The early designs of the iodinatable GA photoaffinity probe were based on the efficient radical-chain addition of α,ω-dithiols to the exocyclic double bond of GA_4[2,7] and the subsequent esterification of the terminal thiol to give 4-azidosalicylates, such as **1**. However, we were not able to achieve iodination of the aromatic ring in the thioester **1** with sodium iodide and oxidants, even under forcing conditions. In order to investigate further the failure of this iodination, we prepared the simple salicylates **6–8** by treatment of 2,5-dioxopyrrolidin-1-yl 4-azidosalicylate **9** with the appropriate nucleophile. The oxy ester **6** and amide **7** reacted rapidly with sodium iodide–chloramine-T at 0 °C to give mixtures of 3-iodo- and 5-iodo products with some unchanged starting material. Similarly, reaction with sodium iodide–*tert*-butyl hypochlorite[8] gave the 5-iodo isomer, along with some 3,5-diiodo compound and starting material. However, as observed for **1** we were unable to achieve any reaction of the model thioester **8** with either of these reagents, the starting material being recovered intact even after refluxing. This very large rate difference between the electrophilic substitution of an aromatic oxyester or amide compared with the thioester is surprising. Possibly, it is due to the greater deactivating effect of COSR over COOR arising from the lower conjugative contribution of sulfur compared with oxygen or nitrogen. This iodination, however, is a reaction of phenolate anion (anisoles are

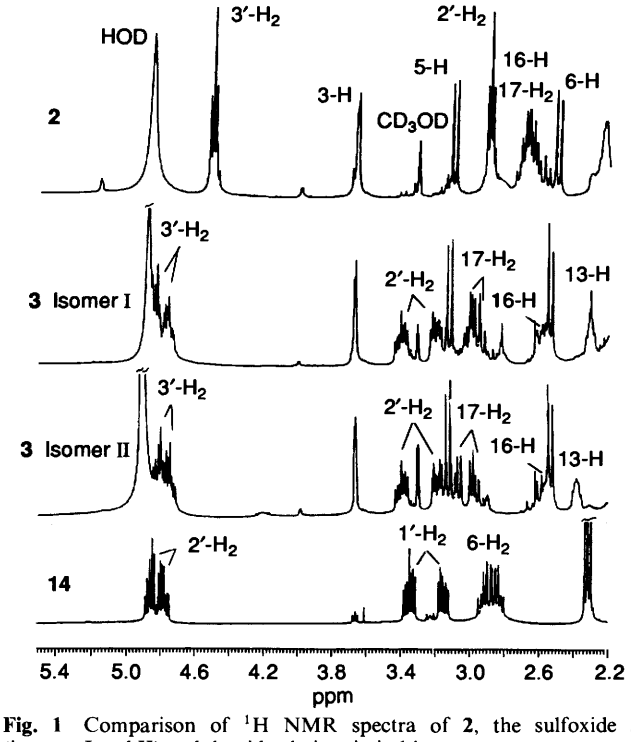


Fig. 1 Comparison of ^{1}H NMR spectra of **2**, the sulfoxide **3** (isomers I and II) and the side-chain mimic **14**

unreactive[8]) and, thus, the reactivity difference could be associated with a difference in intramolecular hydrogen bond strengths in the various esters.

For the preparation of iodinated GA-photoaffinity probes, one solution to this problem was to iodinate **9** and then couple it to the C-17-derivatised GA_4. The radio-iodination of 2,5-dioxopyrrolidin-1-yl 4-azidosalicylate **9** has been reported previously,[9–11] but in our hands this iodination was accompanied by a considerable amount of hydrolysis to iodo-4-azidosalicylic acid. An alternative solution for the preparation of a radioiodinatable GA-photoaffinity probe was to use an oxy ester linkage between the GA and 4-azidosalicyloyl group, thereby allowing the radio-iodination as the final step. This was the route used. The addition of 2-sulfanylethanol to C-17 of GA_4 and the conversion of the adduct into the 4-azidosalicylate **2** 2,5-dioxopyrrolidin-1-yl 4-azidosalicylate was carried out as we described previously.[2]

Radioiodination of compounds 2 and 3

Preliminary attempts to radioiodinate the GA_4-17 *p*-azidosalicylate **2** gave poor radiochemical yields of [^{125}I]-**2**.[2] This warranted more detailed examination, since the model studies above, and larger-scale unlabelled iodinations, indicated that the reaction was fast and high-yielding. A detailed examination of the products of chloramine-T-mediated radio-iodination of **2** by TLC/autoradiography, before and after separation into inorganic and organic fractions by reverse-phase chromatography on a C_{18} Sep-Pak cartridge, revealed that a substantial amount of the recovered radioactivity was not associated with **2** but with two relatively polar, organic compounds. Indeed, Sep-Pak separation of the products of a 5 mCi scale chloramine-T radio-iodination of **2** gave over 1.2 mCi of this 1:1 mixture of new polar compounds. We suspected that these products arose from oxidation at the sulfanyl function of **2**, even though the original larger-scale experiments with unlabelled sodium iodide had indicated that the reaction mediated by 1 mol equiv. of chloramine-T would not cause this. To confirm these observations and achieve our aim to prepare radio-iodinated GA-photoaffinity probes of very high specific activity and in high radiochemical yield, we needed to synthesise and characterise the oxidation products of **2** and investigate their radio-iodination chemistry, as well as their suitability as probes for GA-receptors in *A. fatua* aleurone cells.

Oxidation of **2** with sodium periodate yielded a 1:1 mixture of compounds which co-chromatographed with the above [^{125}I]-labelled by-products of radio-iodination of **2**. The same products were also obtained when **2** was oxidised with 1,3,4,6-tetrachloro-3a,6a-diphenylglycouril (Iodogen™) in a heterogeneous reaction analogous to the common method of radio-iodination.[12] The compounds were separable by flash chromatography and by reverse-phase HPLC. Fast atom bombardment (FAB) mass spectrometry of the compounds revealed that they were isomers. In the negative ion mode, FAB gave strong ions at *m/z* 586 (M_r = 587) for both compounds, while the starting material **2** gave *m/z* 570 (M_r = 571). The addition of one oxygen to **2** was confirmed by FAB mass spectrometry of the corresponding methyl esters, prepared with diazomethane, where the isomeric products both gave the expected molecular ion at *m/z* 600 compared with *m/z* 584 for the methyl ester of **2**. This suggested that the isomeric products have the sulfoxide structure **3**. To confirm this structural assignment, extensive ^{1}H and ^{13}C NMR experiments were carried out. Comparison of the ^{1}H NMR spectra of the less polar isomer (Isomer I) **3** with that of the starting material **2** revealed downfield shifts of the signals assigned to $^{17}CH_2SCH_2CH_2O$ in **2** (see Fig. 1). The methylene signals of the SCH_2CH_2O spacer in **2** occur as two characteristic simple triplets. However, in spectra of **3** (Isomer I), both as the free acid and 7-methyl ester, these signals are much more complex. These data are consistent with the sulfoxide structure **3**, in which these methylene protons are now non-equivalent. Similarly, the ^{1}H NMR spectra of the more polar isomer (Isomer II) (Fig. 1) revealed similar downfield shifts and increases in complexity of the side-chain methylene groups. We concluded that Isomers I and II were epimeric at the sulfur atom. Further NMR and molecular modelling studies were aimed at trying to determine the configuration at the sulfur atom in each isomer. Comparison of the coupling constants in the ^{1}H NMR signals for 17-CH_2 in the isomers showed that the dihedral angles relative to the 16α-H were different in each isomer. In Isomer I, the vicinal coupling constants for the 17-hydrogens are 12.5 and 5.1 Hz, while in Isomer II they are 8.8 and 7.3 Hz. These values indicate that Isomer I takes up a staggered conformation (dihedral angles 180 and 60°), while in isomer II there is a rotation towards a more eclipsed conformation with dihedral angles of 150–160° and 30–40°. Plots of molecule energy *versus* torsion angle for rotation about the 16-17 bond and also contour plots for two bond rotations (16-17 and 17-S), generated using the COSMIC force field (NEMESIS), were similar for both epimers. Examination of ^{1}H and ^{13}C NMR data (Table 1) produced by [^{1}H-^{1}H] and [^{1}H-^{13}C] COSY experiments for the isomers and the unoxidised **2** revealed only a few differences in the chemical shifts of atoms in rings C and D. The position of 13-H in Isomer II (δ 2.38) is significantly downfield of its position in Isomer I (δ 2.31). This, when considered with the decreased dihedral angles for 16-H; 17-H_2 in Isomer II, perhaps indicates that the configuration at sulfur in Isomer II is (*S*), with the 1,3 interaction of S=O and 16-H causing a 20–30° rotation, bringing the oxygen closer to 13-H.

Both isomers of **3** are readily radio-iodinated. For convenience, the heterogeneous method based on Iodogen™ as oxidant can be employed,[13] but more consistent results were obtained with chloramine-T as oxidant. Carrying out the iodination at pH 8.5 allowed the product to be isolated directly from the reaction mixture by adsorption onto a quaternary ammonium ion-exchange cartridge (SAX cartridge). Elution

Table 1 ^{13}C NMR data of **2** and **3** isomers I and II (in CD_3OD)

Carbon	2	Isomer I	Isomer II
1	28.5	28.4	28.4
2	21.3	22.2	21.8
3	70.9	70.9	70.9
4	56.4	56.3	56.4
5	52.5	52.6	52.6
6	54.6	54.5	54.6
7	176.3	176.2	176.3
8	52.8	53.0	53.0
9	58.0	57.7	58.0
10	96.0	95.9	96.0
11	16.4	16.3	16.3
12	29.3	29.3	29.3
13	36.2	37.5	36.5
14	39.6	39.7	39.5
15	44.4	43.1	44.4
16	42.4	36.8	37.3
17	36.6	57.6	56.4
18	15.3	15.3	15.3
19	181.0	180.8	181.0
2'	31.8	52.3	52.4
3'	66.1	59.6	59.7
ArCO	170.6	170.3	170.3
ArC_1	110.6	110.3	110.3
ArC_2	133.1	133.2	133.2
ArC_3	111.8	111.9	111.9
ArC_4	149.1	149.4	149.4
ArC_5	108.2	108.2	108.3
ArC_6	164.3	164.2	164.3

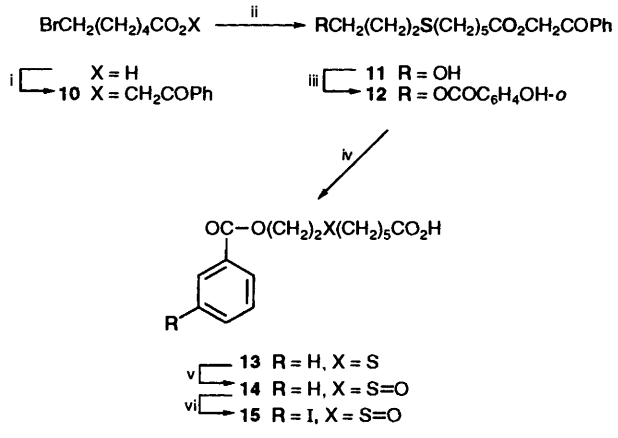
with water at pH 8 followed by organic solvents of increasing polarity and containing acetic acid gave material of high purity suitable for use in most photoaffinity-labelling experiments. TLC/autoradiography of SAX ion-exchange fractions from the radio-iodination of Isomer I is shown in Fig. 2. Most of the radioactivity was recovered in fraction 7, which contained [^{125}I]-**3** of high purity. Fractions 8 and 9, containing less material, were slightly less pure but, nevertheless, could be used in labelling experiments. In this way, a total of 4–4.5 mCi of [^{125}I]-**3** of specific activity ~800–1000 Ci mmol^{-1} could be obtained from a radio-iodination containing 5 mCi of ^{125}I. Iodinated and non-iodinated **3** are not separable on silica gel TLC. However, reverse-phase HPLC analysis of an aliquot of fraction 7, co-injected with a mixture of products from a larger scale non-radioactive iodination, confirmed that the radiolabel was associated with both mono- and di-iodo species **4** and **5** (Fig. 2).

Synthesis of a side-chain mimic

When using aryl azides of this type as photoaffinity reagents, there is a considerable background of non-specific protein labelling, possibly due to hydrophobic interactions with the side-chain. In order to minimise these, we have designed the mimic **15** for addition, in excess, to photoaffinity-labelling experiments in order to block sites that bind the side-chain of the radio-iodinated GA-probe **3**, rather than rings A and B with which bioactivity is associated. The compound was designed to contain the C-17-substituent without the azide to avoid influencing the photoactivation of **3**. The hexanoic acid moiety was included to mimic the GA carbon-chain ^{17}C-^{16}C-^{15}C-^{8}C-^{6}C-$^{7}CO_2H$ and to give the compound the partition properties associated with a hydrophobic carboxylic acid. The synthesis from 6-bromohexanoic acid is shown in Scheme 1 and proceeded *via* protection of the carboxylic acid by phenacylation to **10**. This protecting group was chosen to allow selective deprotection with zinc–acetic acid after introduction of the salicylate at the distal end of the molecule later in the synthesis. Displacement of the bromine atom in **10** by 2-sulfanylethanol yielded the sulfide **11**. Condensation with 2,5-dioxopyrrolidin-1-yl 4-azidosalicylate (→**12**) was not high yielding (30–40%) due to competing hydrolysis of the reagent. Recycling of recovered **11** with fresh reagent, however, allowed acceptable yields of **12**. Reductive removal of the phenacyl group with zinc–acetic acid provided **13** in good yield. Oxidation at the sulfide was carried out as above with periodate to give the sulfoxide **14**, the ^1H NMR spectrum of which contained signals due to the non-equivalent methylene protons in the substructure $CH_2S(O)$-CH_2CH_2OCO at similar chemical shifts and of similar multiplicities to those observed in the isomers of **3** (see Fig. 1). Iodination of **14** with an equimolar mixture of sodium iodide–chloramine-T was incomplete and gave a mixture of 5-iodo derivative **15** and starting material in the ratio 2:1, as determined by analysis of the ^1H NMR spectrum. These compounds were not separable by silica gel chromatography. No efforts were made to purify the iodo compound **15** further because, in fact, the radioiodinated probe **3** used in photoaffinity-labelling experiments is also a mixture containing starting material and iodo compounds.

Biological activity

The biological activity of **2** was determined previously.[2] However, before use of the sulfoxides in photoaffinity-labelling it was important to show that neither oxidation at the sulfur atom nor iodination lowered biological activity. The sulfoxide isomers I and II **3**, and pure samples of unlabelled monoiodo and diiodo compounds **4** and **5**, isolated by preparative reverse-phase HPLC, were examined for activity in the induction of α-amylase in aleurone layers of *A. fatua*, the system in which these C-17-linked aryl-azido photoaffinity compounds are being used to probe for GA-receptors. Before assay, purity of the compounds was assessed by NMR, TLC and HPLC. Analysis of the derivatives by gas chromatography–mass spectrometry, to ensure the absence of contaminating active GAs, was attempted but found to be impracticable due to thermal elimination of the C-17-substituent in the injection port of the gas chromatograph, giving rise to a bleed of GA_4 into the mass spectrometer. This thermal elimination occurred, not unexpectedly, to a high level for the sulfoxides **3**–**5**, and was also observed at low levels for the 17-sulfide **2**. Compounds **2**–**5** were equally active (Table 2). The level of activity is moderate when compared with the highly active GA_4 or GA_1. As controls, the inactive GA_{34} gave very low activity (Table 2) and the side-chain mimic **15** was inactive (data not shown). These results show that the initial substitution of thioalkyl groups at C-17



Scheme 1 *Reagents:* i, $PhCOCH_2Br$, $KHCO_3$, 18c6, MeCN; ii, $HSCH_2CH_2OH$, $KHCO_3$; iii, 2,5-dioxopyrrolidin-1-yl salicylate, Et_3N, MeCN; iv, Zn, AcOH; v, $NaIO_4$, H_2O–MeOH; vi, NaI, chloramine-T

Table 2 Dose–response of aleurone layers to GAs and GA derivatives

GA concentration (mol dm^{-3})	Total α-amylase (units)							
	2	**3** (Isomer I)	**3** (Isomer II)	**4**	**5**	GA_1	GA_4	GA_{34}
10^{-6}	120 ± 5	103 ± 2	94 ± 2	100 ± 3	108 ± 3	nd	nd	20 ± 1
10^{-7}	69 ± 1	73 ± 2	55 ± 2	79 ± 4	83 ± 5	147 ± 4	186 ± 14	9 ± 1
10^{-8}	20 ± 1	50 ± 2	42 ± 1	44 ± 3	41 ± 2	100 ± 15	193 ± 28	2 ± 1
10^{-9}	8 ± 1	23 ± 4	20 ± 2	17 ± 1	17 ± 2	72 ± 1	155 ± 5	0
10^{-10}	nd	nd	nd	nd	nd	45 ± 1	56 ± 1	nd

Aleurone layers of *A. fatua* were incubated for 4 days with the ranges of concentration of GAs and GA derivatives. Total α-amylase was determined in triplicate assays for each of three replicate experiments and are presented as means with standard errors. Aleurone layers incubated without GA produced 1.0 unit of α-amylase and this figure has been subtracted from the above values (nd = not determined).

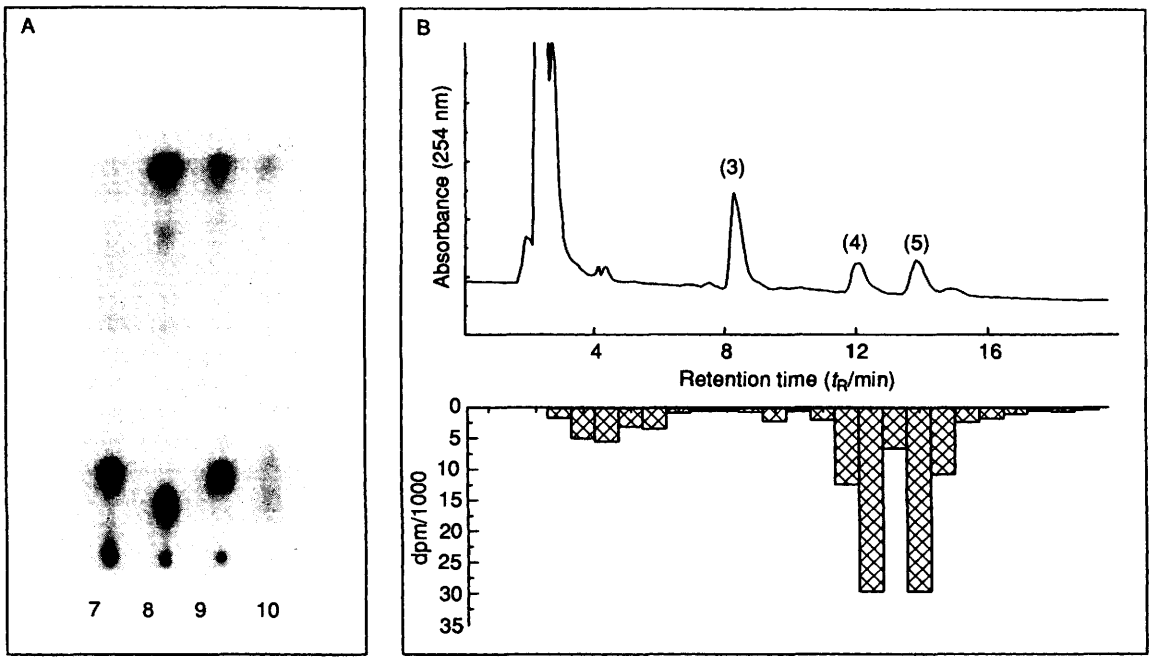


Fig. 2 Chromatography of radioiodination products of Isomer I. A TLC-autoradiography of fractions from SAX ion-exchange purification (see Experimental section). B HPLC-radiocounting of an aliquot from fraction 7, co-injected with a mixture of products from a larger scale unlabelled iodination.

causes a reduction in biological activity, but thereafter extension or changes to the 17-substituent cause no further losses of activity. Current work is aimed at preparing photoaffinity probes with higher biological activity. At the present time, the sulfoxides **3**, because of their efficient radioiodination chemistry, are being used to identify GA-binding proteins. The stereochemistry at the sulfur atom has no effect on biological activity and, thus, either isomer is useful in this respect. However, in the longer term, the probe may be used in mapping the active site of isolated or recombinant proteins. In this case, knowledge of the exact three-dimensional structure will be necessary and future work will be aimed at X-ray crystallographic analysis of the isomers.

Experimental

2,5-Dioxopyrrolidin-1-yl salicylate and 2,5-dioxopyrrolidin-1-yl 4-azidosalicylate **9** and GA_4-17-ylsulfanylethyl 4-azidosalicylate **2** were prepared as described previously.[2] 1,3,4,6-tetrachloro-3α,6α-diphenylglycouril (Iodogen™, Pierce) was purchased from Sigma, UK. Ratios of solvent mixtures for chromatography are on a v/v basis. NMR data are given in ppm relative to TMS internal standard. Coupling constants are in Hz. TLC plates were visualised by spraying with 5% sulfuric acid in ethanol and heating to 110 °C for 5 min.

C_{18} Sep-pak separation of radio-iodination products of GA_4-17-ylsulfanylethyl 4-azidosalicylate 2

CAUTION all work with radioiodine was carried out in a well-ventilated hood and with appropriate lead or lead-impregnated acrylic shielding. Compound **2** was radio-iodinated essentially as described previously.[2] To sodium [^{125}I]iodide (5 mCi in 13 mm^3 dilute NaOH, Amersham) was added **2** (1.5 μg) in phosphate buffer (5 mm^3) (100 nmol dm^{-3}, pH 8.5) and then Chloramine-T (2.5 μg) in the same buffer (2.5 mm^3). After 5 min 1% aq. sodium metabisulfite (5 mm^3) was added. The reaction mixture was then diluted with 70 mm^3 of water containing 50 mmol dm^{-3} formic acid and transferred to a C_{18}-Sep-Pak cartridge (Waters) which had been pre-eluted with methanol (5 cm^3) and then water (5 cm^3) containing 2 mmol dm^{-3} formic acid and sodium iodide (50 nmol dm^{-3}). The cartridge was eluted initially with 2 mmol dm^{-3} formic acid (4 cm^3) to remove unincorporated inorganic iodide and then in 12 × 2 cm^3 fractions comprising increasing amounts of methanol in 2 mmol dm^{-3} formic acid as follows: 20, 30 and 40% (2 cm^3 of each), 60, 70 and 80% (4 cm^3 of each) and 100% (6 cm^3). Frs. 5–10 contained the majority of recovered radioactivity (1.24 mCi, liquid scintillation counting).

TLC/autoradiography was initially carried out using ethyl acetate–hexane–acetic acid (75 : 25 : 1) followed by more detailed

analysis of the polar components of fractions 6–9 with ethyl acetate–acetone–acetic acid (95:5:1).

Oxidation of GA_4-17-ylsulfanylethyl 4-azidosalicylate 2

Sodium metaperiodate (208 mg) was dissolved in water (10 cm^3) and the solution cooled with ice–water. The aryl azide **2** (500 mg), dissolved in methanol (20 cm^3), was added to the solution and the mixture stirred at this temperature overnight. Dil. HCl (pH 3) and ethyl acetate were then added to the reaction mixture and the organic layer was separated and evaporated to give a gum (400 mg) containing two polar compounds [1:1 mixture, TLC analysis R_F 0.30 and 0.25 (ethyl acetate–acetic acid, 100:1)]. These were separated by flash chromatography on a 20 × 3.0 cm silica column eluted in 42 fractions of *ca.* 20 cm^3 each with ethyl acetate–hexane–acetic acid (70:30:1; 80:20:1; 90:10:1; 100:0:1; 75 cm^3 of each) followed by ethyl acetate–acetone–acetic acid (75 cm^3 each of 90:10:1; 80:20:1; 70:30:1; 60:40:1; 50:50:1; 40:60:1; 30:70:1; 20:80:1 and 10:90:1). Frs. 33–35 contained the sulfoxide isomer I **3** (92 mg); Frs. 36–38 contained a mixture (*ca.* 1:1, 110 mg) and Frs. 39–41 the sulfoxide isomer II **3** (83 mg).

Isomer I. R_f 0.30, negative ion FAB MS, *m/z* 586 [M − 1]; δ_H(400 MHz, CD_3OD) 1.10 (3 H, s, 18-H_3), 2.31 (1 H, br, 13-H), 2.55 (1 H, d, *J* 11, 6-H), 2.95 (1 H, t, *J* 12.5, 17-H), 3.02 (1 H, dd, *J* 12.5 and 5.1, 17-H), 3.13 (1 H, d, *J* 11, 5-H), 3.19 (1 H, dt, *J* 13.8 and 4, 2′-H), 3.40 (1 H, ddd, *J* 13.8, 8.8 and 4.8, 2′-H), 3.66 (1 H, br s, 3-H), 4.75 (1 H, ddd, *J* 12.5, 8.8 and 4.0, 3′-H), 4.82 (1 H, dt, *J* 12.5 and 5.1, 3′-H), 6.62 (1 H, s, 3″-H), 6.64 (1 H, d, *J* 8, 5″-H) and 7.88 (1 H, d, *J* 8, 6″-H); δ_C data—see Table 1.

Isomer II. R_F 0.25, negative ion FAB MS, *m/z* 586 [M − 1]; δ_H(400 MHz, CD_3OD) 1.11 (3 H, s, 18-H_3), 2.38 (1 H, br, 13-H), 2.55 (1 H, d, *J* 11, 6-H), 2.98 (1 H, dd, *J* 13.0 and 7.3, 17-H), 3.08 (1 H, dd, *J* 13 and 8.8, 17-H), 3.13 (1 H, d, *J* 11, 5-H), 3.19 (1 H, dt, *J* 13 and 3.9, 2′-H), 3.39 (1 H, ddd, *J* 13, 8.8 and 4.9, 2′-H), 3.78 (1 H, br s, 3-H), 4.74 (1 H, ddd, *J* 12.2, 8.8 and 3.9, 3′-H), 4.80 (1 H, ddd, *J* 12.2, 4.9 and 3.9, 3′-H), 6.63 (1 H, s, 3″-H), 6.64 (1 H, d, *J* 8, 5″-H) and 7.87 (1 H, d, *J* 8, 6″-H); δ_C data—see Table 1.

Oxidation of 2 with iodogen

1,3,4,6-Tetrachloro-3α,6α-diphenylglycouril (iodogen) (20 mg, 0.045 mmol) was dissolved in chloroform (1 cm^3) and the solution evaporated slowly with a stream of N_2 to form a coating on the sides of a small (10 cm^3) round-bottomed flask. The aryl azide **2** (26 mg, 0.045 mmol) in phosphate buffer (pH 8.0, 100 mmol dm^{-3}; 4 cm^3) was added and the solution stirred vigorously for 20 min and then decanted. After acidification to pH 4.0 with conc. HCl the products were recovered in ethyl acetate. TLC analysis (ethyl acetate–acetic acid, 100:1) indicated conversion into a 1:1 mixture of polar compounds. These were separated by flash chromatography as above on a 20 × 1.5 cm column to give the sulfoxide isomer I **3** (3.8 mg) and sulfoxide isomer II **3** (4.6 mg), identified by ^1H NMR comparison with those above.

Iodination of the sulfoxide isomer I

The sulfoxide **3** (52 mg) in acetonitrile–water (4:1, 5.5 cm^3) was treated with sodium iodide (16 mg) and Chloramine-T (22 mg) at 0 °C for 5 min. Sodium bisulfite (5% aq., 5 cm^3) was added to the mixture after which the products were recovered in ethyl acetate. The product in methanol was purified by preparative HPLC on a Partisil M9 10/25 ODS-2 reverse phase column eluted with methanol–water (7:3) containing 0.005% acetic acid to give recovered **3** (8 mg) (t_R 20 min), the mono-iodo derivative **4** (15 mg) [t_R 34 min, ^1H NMR (CD_3OD) δ 6.92 (s) and 8.22 (s), 3″-H and 6″-H] and the di-iodo compound **5** (7 mg) [t_R 51 min, ^1H NMR (CD_3OD) δ 8.31(s), 6″-H].

Radio-iodination of the sulfoxide isomer I, 3

To sodium [^{125}I]iodide (5 mCi in 14 mm^3 dilute NaOH, NEN-Dupont) was added the sulfoxide **3** (3 μg in 10 mm^3 of phosphate buffer, 100 mmol dm^{-3}, pH 8.5), followed by chloramine-T (2.5 μg in 2.5 mm^3 buffer). After 5 min sodium metabisulfite solution (5% in water, 3.5 mm^3) was added. For purification, the reaction mixture was transferred to a Bond Elut 3cc SAX cartridge (Varian) which had been pre-eluted with water at pH 8 (5 cm^3). The reaction mixture was washed onto the column with 100 mm^3 (× 3) of water (pH 8). The cartridge was eluted in 1 cm^3 fractions with the following solvents: water (pH 8), 2 cm^3 (frs. 1,2); hexane (containing 2 mmol dm^{-3} acetic acid), 4 cm^3 (frs. 3-6); ethyl acetate (2 mmol dm^{-3} acetic acid), 2 cm^3 (frs. 7,8) and methanol (2 mmol dm^{-3} acetic acid), 2 cm^3 (frs. 9,10). Fraction 7 contained the [^{125}I]-sulfoxide (3.49 mCi). Fractions 8 (0.77 mCi) and 9 (0.35 mCi) contained slightly less pure material.

TLC-autoradiographic analysis of radio-iodination products. This was carried out as follows. Unlabelled **3** was spotted onto the origin of each track before 2 mm^3 aliquots of each fraction were applied. The plate was developed with ethyl acetate–acetic acid (100:5:1). After drying, the carrier spots were identified under a UV lamp. Direct autoradiography was carried out with X-ray film and exposure times of 5 min to 1 h as necessary.

HPLC analysis of radio-iodination products. This was carried out on a 25 cm × 4.6 mm id Spherisorb S5 ODS2 column (PhaseSep) eluted with methanol–water (7:3) containing 0.005% acetic acid at a flow rate of 1 cm^3 min^{-1}. Compounds were detected by UV absorbance at 254 nm. Retention times: sulfoxide isomer I t_R 8.3 min; sulfoxide isomer II t_R 10.1 min. Iodination of isomer I gave new peaks at 11.5 min (monoiodo, **4**) and 13.2 min (diiodo, **5**) as well as residual un-iodinated compound (8.3 min). An aliquot (180000 dpm) of fraction 7 from the radioiodination was co-injected with product of an unlabelled iodination and the eluted fractions analysed by liquid scintillation counting. Approximately 80% of the applied radioactivity was associated with the peaks at 11.5 and 13.2 min (see Fig. 2).

Phenacyl 6-bromohexanoate 10

To 6-bromohexanoic acid (5 g) in acetonitrile (100 cm^3) was added bromomethyl phenyl ketone (5 g), potassium hydrogen carbonate (2.5 g) and 18-crown-6 ether (50 mg). The suspension was refluxed for 2 h and then evaporated under reduced pressure. The resultant oil was partitioned between ethyl acetate and water. Evaporation of the ethyl acetate layer yielded the title compound **10** (7 g, 86%) as an oil which was used directly in the next step; δ_H(400 MHz, $CDCl_3$) 1.42 (2 H, quint., *J* 7, 4-H_2), 1.76 (2 H, quint., *J* 7, 3-H_2), 1.90 (2 H, quint., *J* 7, 5-H_2), 2.50 (2 H, t, *J* 7, 2-H_2), 3.41 (2 H, t, *J* 7, 6-H_2), 5.37 (2 H, s, OCH_2COAr), 7.50 (2 H, m, *J* 8, 3′-H and 5′-H), 7.61 (1 H, t, *J* 8, 4′-H) and 7.92 (2 H, d, *J* 8, 2′-H and 6′-H).

Phenacyl 6-(2-hydroxyethylsulfanyl)hexanoate 11

The phenacyl ester **10** (7 g) in acetonitrile (100 cm^3) was treated with 2-sulfanylethanol (5 cm^3) and potassium hydrogen carbonate (2.5 g) at reflux for 16 h. After evaporation of the solvent from the mixture, the product was recovered in ethyl acetate. The ethyl acetate extract was washed with water and evaporated to give a thin oil containing residual 2-sulfanylethanol and product. Flash chromatography of this with a stepwise gradient of ethyl acetate in hexane gave the *title compound* **11** (6.05 g, 97%) as a gum. [Found (negative ion

CIMS, isobutane): *m/z* 309.1172. ($C_{16}H_{22}O_4S$ − H) requires 309.1161]; δ_H(400 MHz, $CDCl_3$) 1.48 (2 H, quint., *J* 7, 4-H_2), 1.62 (2 H, quint., *J* 7, 5-H_2), 1.71 (2 H, quint., *J* 7, 3-H_2), 2.49 and 2.53 (4 H, 2 t, *J* 7, 2-H_2 and 6-H_2), 2.70 (2 H, t, *J* 7, 1′-H_2), 3.72 (2 H, t, *J* 7, 2′-H_2), 5.35 (2 H, s, OCH_2COAr), 7.48 (2 H, t, *J* 8, 3″-H and 5″-H), 7.60 (1 H, t, *J* 8, 4″-H) and 7.90 (2 H, d, *J* 8, 2″-H and 6″-H); δ_C(100 MHz, $CDCl_3$) 24.39, 28.11, 29.31, 31.47, 33.65, 34.88 (C-2, C-3, C-4, C-5, C-6 and C-1′), 60.56 (C-2′), 65.94 (OCH_2CO), 127.75 and 128.86 (C-2″, C-6″ and C-3″, C-5″), 133.93 and 134.11 (C-1″ and C-4″), 173.07 (COO) and 192.43 (CO).

Phenacyl 6-(2-salicyloyloxyethylsulfanyl)hexanoate 12

The alcohol **11** (1.15 g) in acetonitrile (50 cm^3) was treated with 2,5-dioxopyrrolidin-1-yl salicylate (1.3 g) and triethylamine (0.75 cm^3) for 3 days at room temperature, at which time TLC analysis indicated that *ca.* 40% of the starting material had been converted into a less polar product. After removal of solvent from the mixture, the product was partitioned between ethyl acetate and 5% aqueous citric acid. The organic layer, after being washed with water, was evaporated to give an oil which was fractionated by flash chromatography with hexane–ethyl acetate mixtures. The *title compound* **12** (340 mg) was eluted first, followed by starting material **11** (920 mg). Data for **12**: [Found (negative ion CIMS, isobutane): *m/z* 429.1383. ($C_{23}H_{26}O_6S$ − H) requires 429.1373]; δ_H(400 MHz, $CDCl_3$) 1.50 (2 H, quint., *J* 7, 4-H_2), 1.67 (2 H, quint., *J* 7, 5-H_2), 1.74 (2 H, quint., *J* 7, 3-H_2), 2.51 (2 H, t, *J* 7, 2-H_2), 2.63 (2 H, t, *J* 7, 6-H_2), 2.89 (2 H. t, *J* 7, 1′-H_2), 4.48 (2 H, t, *J* 7, 2′-H_2), 5.35 (2 H, s, OCH_2COAr); phenacyl aromatics: 7.5 (2 H, t, *J* 8, 3″-H and 5″-H), 7.61 (1 H, t, *J* 8, 4″-H), 7.92 (2 H, d, *J* 8, 2″-H and 6″-H); salicyloyl aromatics: 6.90 (1 H, t, *J* 8, 5″-H), 6.96 (1 H, d, *J* 8, 3″-H), 7.50 (1 H, t, *J* 8, 4″-H) and 7.85 (1 H, d, *J* 8, 6″-H); δ_C(100 MHz, $CDCl_3$) 24.56, 28.26, 29.42, 30.45, 32.30, 33.82 (C-2, C-3, C-4, C-5, C-6 and C-1′), 64.45 (C-2′), 66.03 (OCH_2CO), 169.99 (COO), 173.13 (COO) and 192.39 (CO), phenacyl aromatics: 127.87 (C-2″, C-6″), 128.98 (C-3″, C-5″), 134.02 and 134.27 (C-1″ and C-4″); salicyloyl aromatics: 112.37, 117.69, 119.35, 130.07, 135.96 and 162.11.

6-(2-Salicyloylsulfanyl)hexanoic acid 13

To the phenacyl ester **12** (900 mg) in acetic acid (40 cm^3) was added activated zinc dust (9 g). After being stirred for 1 h at room temperature, the mixture was filtered and the solution evaporated under reduced pressure by means of azeotropic distillation with added toluene. The product was purified by flash chromatography with hexane–ethyl acetate mixtures (containing 0.5% acetic acid) to give the *acid* **13** (558 mg, 90%) [Found (EIMS): *m/z* 312.1018. $C_{15}H_{20}O_5S$ requires 312.1031. Found (negative ion CIMS, isobutane): *m/z* 311.0949. ($C_{15}H_{20}O_5S$ − H) requires 311.0953]; δ_H(400 MHz, $CDCl_3$) 1.45 (2 H, quint., *J* 7, 4-H_2), 1.62 (4 H, m, *J* 7, 3-H_2 and 5-H_2), 2.30 (2 H, t, *J* 7, 2-H_2), 2.66 (2 H, t, *J* 7, 6-H_2), 2.96 (2 H, t, *J* 7, 1′-H_2), 4.54 (2 H, t, *J* 7, 2′-H_2), 6.96 (1 H, t, *J* 8, 5″-H), 6.97 (1 H, d, *J* 8, 3″-H), 7.54 (1 H, t, *J* 8, 4″-H) and 7.88 (1 H, d, *J* 8, 6″-H).

6-(2-Salicyloylethylsulfinyl)hexanoic acid 14

The sulfide **13** (420 mg) in water–methanol (1 : 1; 40 cm^3) at 0 °C was treated with sodium metaperiodate (311 mg). After the solution had been allowed to warm to room temperature it was stirred for 2 days and then diluted with ethyl acetate and water. The *sulfoxide* **14** (417 mg, 93%) was recovered from the organic layer. [Found (EIMS): *m/z* 328.0962. $C_{15}H_{20}O_6S$ requires 328.0981. Found (negative ion CIMS, isobutane): *m/z* 327.0884. ($C_{15}H_{20}O_6S$ − H) requires 327.0902]; δ_H[400 MHz, $(CD_3)_2CO$] 1.53 (2 H, quint., *J* 7, 4-H_2), 1.65 (2 H, quint., *J* 7, 3-H_2), 1.80 (2 H, quint., *J* 7, 5-H_2), 2.33 (2 H, t, *J* 7, 2-H_2), 2.88 (2 H, complex m, 60 Hz width, 15 lines, 6-H_2), 3.15 (1 H, ddd, *J* 14, 5.5 and 4.4, 1′-H), 3.34 (1 H, ddd, *J* 14, 8.4 and 4.8, 1′-H), 4.77 (1 H, ddd, *J* 12.4, 8.4 and 4.4, 2′-H), 4.85 (1 H, ddd, *J* 12.4, 5.5 and 4.8, 2′-H), 6.96 (1 H, t, *J* 8, 5″-H), 6.97 (1 H, d, *J* 8, 3″-H), 7.55 (1 H, t, *J* 8, 4″-H) and 7.90 (1 H, d, *J* 8, 6″-H).

Iodination of the sulfoxide 14

The sulfoxide **14** (40 mg) in acetonitrile–water (4 : 1; 2 cm^3) was stirred at 0 °C and treated with sodium iodide (24 mg) followed by a solution of chloramine-T (28 mg) in water (2 cm^3). After the initially formed brown colour had faded appreciably (5 min), a few crystals of sodium metabisulfite were added to the mixture followed by ethyl acetate and further water. Recovery of material from the organic layer gave crude product which was purified by flash chromatography using ethyl acetate–acetone mixtures (10–40% acetone). Analysis of the ^1H NMR spectrum revealed that the product consisted of a 2 : 1 mixture (15 mg) of the 5-iodosalicylate **15** and starting material. Data for **15**: *m/z* (positive ion CI, isobutane) 455 (M + H); δ_H[400 MHz, $(CD_3)_2CO$] aliphatic signals as in **14**; aromatic signals δ 6.84 (1 H, d, *J* 8.5, 3″-H), 7.83 (1 H, dd, *J* 8.5 and 2.5, 4″-H) and 8.16 (1 H, d, *J* 2.5, 6″-H).

Plant material and bioassay

Seeds from a single harvest of an inbred line of *Avena fatua* were air-dried,[14] and then stored over anhydrous calcium chloride for 3 years at 25 ± 2° C. Half-seeds were surface sterilised[15] and imbibed in sterile water for 60 h. Aleurone layers, isolated as previously described,[16] were incubated (5 per cm^3) for 4 days in 2 mmol dm^{-3} aqueous calcium chloride and a range of concentrations of the aryl azides **2–5**, GA_1, GA_4 and GA_{34}; they were then homogenised. Total α-amylase was determined and data presented as described previously.[16]

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