

Synthesis and biological evaluation of potential substrates for the isolation of the strigol receptor

PERKIN

Jan Willem J. F. Thuring, Rolf Keltjens, Gerard H. L. Nefkens and Binne Zwanenburg*

NSR-Centre for Molecular Structure, Design and Synthesis, Department of Organic Chemistry, University of Nijmegen, Toernooiveld, 6525 ED Nijmegen, The Netherlands

A series of analogues derived from (+)-strigol, which is a germination stimulant for seeds of the parasitic weeds *Striga* and *Orobanche*, has been prepared. For the isolation and characterization of the strigol receptor, labelled analogues are required in which a photoreactive function may be incorporated. The synthetic strategy allows for the synthesis of a range of A-ring substituted analogues of GR24 (which is a strigol analogue), including fluorescent dansyl GR24 10. Bioassays reveal that the stimulatory activity of these analogues in the seed germination of *Striga hermonthica* is retained.

Introduction

Parasitic weeds belonging to the genera *Striga* and *Orobanche* severely reduce yields of economically important crops in tropical and semitropical areas of the eastern hemisphere.^{1,2} The parasitic process begins with the seed germination of the weeds, induced by a stimulant which is present in the root exudate of the host plant. Following the isolation and identification of the naturally occurring germination stimulant (+)-strigol (Fig. 1),^{3,4} several structure-bioactivity studies have been

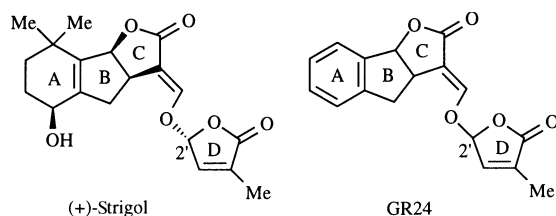
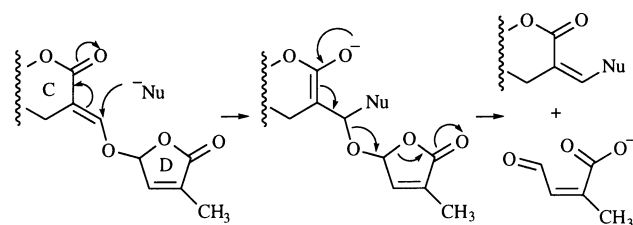


Fig. 1

conducted, which revealed that the bioactiphore resides in the CD-fragment and the vinyl ether moiety.⁵⁻¹⁰

In particular, GR24 (Fig. 1) turned out to be a highly potent synthetic strigol analogue.^{5,6,11} Based on the structural requirements for retaining stimulatory activity a tentative molecular mechanism for germination has been proposed⁸ which is depicted in Scheme 1.



Scheme 1

According to this mechanism a nucleophilic site in the receptor cavity reacts with the Michael acceptor unit, followed by elimination of the D-ring. The C- and D-rings as well as the connecting vinyl ether unit play an important role in inducing the germination. It is thus suggested that the chemical reaction at the receptor site is of crucial importance at the very beginning of the signal-transduction chain. However, nothing is

known about the protein structure nor of its localization within the seeds. Detailed knowledge of the receptor protein would enable the design of a perfectly fitting substrate. Current strategies in the purification of plant proteins involve several types of affinity chromatography and photoaffinity labelling.¹² In photoaffinity labelling the substrate of the protein is converted into a photoaffinity ligand by covalently attaching a photoreactive moiety, such as an azido group, to the natural ligand.¹³ After exposure to a cell extract, a very short-living, highly reactive intermediate is generated upon irradiation, which will be covalently bonded onto the protein in the vicinity of the putative ligand-binding site. A radioactive or fluorescent tag can be introduced in the photoreactive ligand in order to allow detection during isolation and enable the characterization of the protein.¹⁴ It has been demonstrated that this technique is a useful tool in plant hormone research, exemplified by the photoaffinity labelling of auxin binding proteins¹⁵ and gibberellin binding proteins.¹⁶ In the case of the strigol receptor, incorporation of a photoreactive moiety may not strictly be necessary. If the molecular mechanism (Scheme 1) is correct, treatment of the seeds with a radioactive or fluorescent strigol analogue may directly lead to covalent attachment to the receptor protein without the need to generate a highly reactive species by irradiation. When the bioactiphore of the ligand is known, incorporation of the tag and eventually a photolabile moiety should be such that the bioactivity is retained. Structure-activity relationship studies, which have previously been performed⁸⁻¹⁰ will, therefore, provide a firm basis for the design of suitable compounds for the isolation of the strigol receptor.

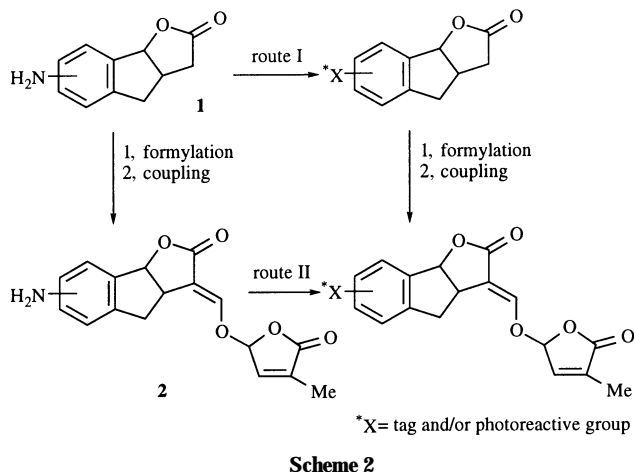
Here synthetic approaches are described for the preparation of biologically active, labelled strigol analogues, suitable for, at least in principle, the identification of the strigol receptor. The synthetic concept is a general approach to a wide range of substrates containing a tag and eventually an additional photolabile moiety. In addition, the activity of the stimulation of seed germination of *Striga hermonthica* (Del.) Benth. and *Orobanche crenata* Forsk. of some of the thus obtained strigol analogues is evaluated.

Results and discussion

Strategy

In designing potential labelled germination stimulants, GR24 (Fig. 1) was used as the lead molecule. The bioactivity of GR24 is very high and its preparation is well documented.¹¹ Since the CD-part as well as the connecting enol ether unit are

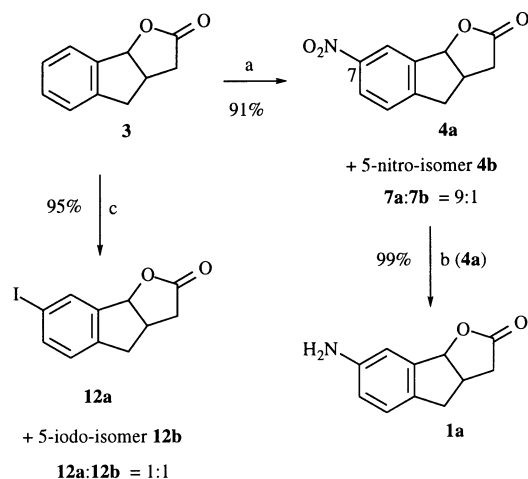
essential for full biological activity,^{8,17} it was suggested that the tag and/or photoreactive group can best be incorporated into the A-ring of the stimulant molecule. Amino tricyclic lactone **1** and amino GR24 **2** were selected as appropriate compounds to serve this purpose as a wide range of transformations are feasible with these synthons, such as acylation, sulfonylation or alkylation, whereby coupling with an external tag can be achieved. The strategy for the synthesis of labelled GR24 analogues is outlined in Scheme 2. The label may be introduced



either before (route I) or after (route II) coupling with the D-ring.

Synthesis

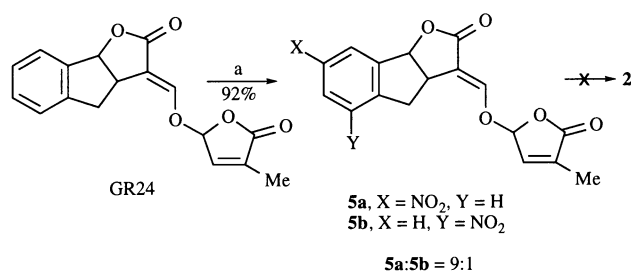
In our strategy the main issue is the introduction of an amino function into the A-ring of tricyclic lactone **3**¹¹ in a regiocontrolled manner (Scheme 3).



Scheme 3 Reagents: a, NaNO₃, TFA; b, Sn-HCl (aq.), heat; c, I₂, PhI[OC(O)CF₃]₂

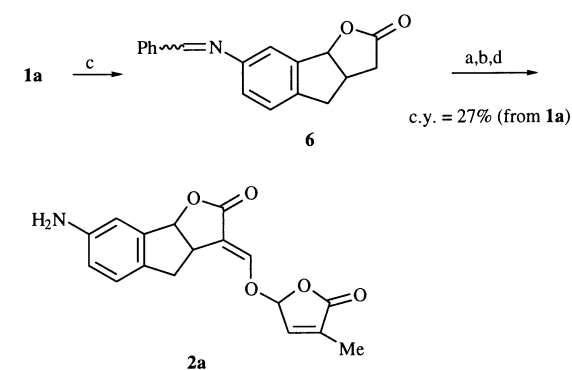
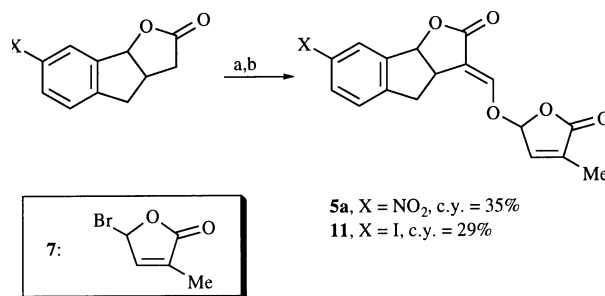
This was achieved *via* nitration of **3** under relatively mild conditions (Scheme 3),¹⁸ to give two regioisomeric lactones **4a** and **4b** in a ratio of 9:1 in excellent yield, which could readily be separated. The structure of **4a** was deduced unambiguously from a 2D-NOESY experiment. The preferred formation of **4a** can be explained by the fact that C-7 is the least electron-deficient carbon atom in the aromatic ring. The nitration under similar conditions took place also starting from GR24 to give 7-nitro GR24 **5a** and its 5-nitro isomer **5b** in a ratio of 9:1 (Scheme 4).

However, several attempts to obtain amino GR24 **2** by reduction of the nitro function in **5** were unsuccessful. In contrast, reduction of the nitro group in **4a** was accomplished employing aqueous Cu(OAc)₂ and NaBH₄ in methanol¹⁹ in yields in the range 34–95%. A more reliable procedure (Scheme 3) involves



Scheme 4 Reagents: a, TFA, NaNO₃

the use of Sn-HCl under reflux,²⁰ which gave the 7-amino tricyclic lactone **1a** in a reproducible yield of 99% (77% after recrystallization). The preparation of amino GR24 **2a** from amino tricyclic lactone **1a** involves a four-step procedure as is depicted in Scheme 5.

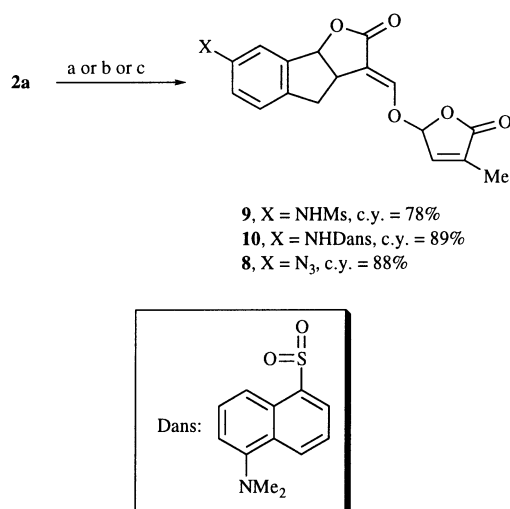


Scheme 5 Reagents: a, KOBu^t, HCO₂Me; b, bromo butenolide **7**; c, PhCHO, mol. sieves; d, SiO₂

The amino group in **1a** was first protected as a Schiff base **6** by reaction with benzaldehyde, then followed by formylation, coupling with bromo butenolide **7**, similar to that described for GR24,¹¹ and finally deprotection. Crude 7-benzalimino GR24 was isolated in an overall yield of 78%, based on **1a**. Deprotection was not as straightforward as expected. Several conventional methods, such as 5% oxalic acid, failed to give the desired result and only starting imine was recovered. However, during purification of 7-benzalimino GR24 by flash chromatography, the deprotection took place on the silica gel column and amino GR24 **2a** was isolated in an overall yield of 27%. The diastereoisomers could not be separated, neither by flash chromatography nor by recrystallization, in contrast to GR24.¹¹

The key intermediate amino GR24 **2a** was then utilized for further derivatization (Scheme 6), especially for the purpose of receptor identification.

Diazotation and substitution²¹ in the presence of NaN₃ gave **8**, which is thermally rather unstable, in high yield. Applying the appropriate sulfonyl chloride, mesyl GR24 **9** and dansyl GR24 **10** were synthesized in high yields.²² The alternative procedure for the preparation of the labelled GR24 analogues **8**, **9** and **10**, involving modification of the amino function of **1a** into the azido, mesylamino and dansylamino group, respectively, fol-



Scheme 6 Reagents and conditions: a, X = NHMs: pyridine, MsCl; b, X = NHDans: pyridine, DansCl; c, X = N₃: 1, NaNO₂, 80% HOAc; 2, NaN₃

lowed by coupling with the D-ring (route I in Scheme 2), is less attractive, as the yields are low to moderate²³ and the purification of the compounds prepared *via* route II is much simpler than by route I (Scheme 2).

The synthesis of 7-iodo GR24 **11** could not be accomplished starting from **2a**, since the substitution of the diazo moiety by iodide, in a similar procedure to that used for the preparation of azido GR24 **8** (*vide supra*), was unsuccessful. Therefore, the tricyclic lactone **3** was iodinated (*cf.* route I in Scheme 2) employing iodine in the presence of [bis(trifluoroacetoxy)iodobenzene] (Scheme 3) by adopting the procedure of Merkushev *et al.*²⁴ A 1:1 mixture of the regioisomeric 7-iodo lactone **12a** and the 5-iodo lactone **12b** was obtained in an excellent yield, which could readily be separated. Structural assignments were performed by comparison with the ¹H NMR spectra of the 7-nitro and 5-nitro counterparts **4a** and **4b** respectively. Coupling reactions of **4a** and **12a** to give the desired GR24 analogues *via* route I (Scheme 2) involve a two-step procedure (Scheme 5), similar to that described for the synthesis of GR24.¹¹ For the preparation of 7-nitro GR24 **5a** this sequence was carried out in a one-pot procedure to give **5a** as an approximately 1:1 mixture of diastereoisomers in a moderate overall yield of 35%. However, for the preparation of 7-iodo GR24 **11** it was found more appropriate to isolate the intermediate hydroxymethylene lactone, which could readily be purified by washing with diethyl ether to remove unchanged starting material, although some loss of material had to be accepted. Alkylation with the bromo butenolide **7** provides 7-iodo GR24 **11** as an approximately 1:1 mixture of diastereoisomers, which could readily be separated by flash chromatography.

Biological activity

The stimulatory activity of nitro GR24 **5a**, amino GR24 **2a** and dansyl GR24 **10** was determined using seeds of *Striga hermonthica* and *Orobancha crenata*. The germination percentages are collected in Tables 1 and 2, together with those obtained for GR24 under the same conditions in the same bioassay. This reference to GR24 enables a comparison between results obtained in different test series, which is important, because the response of seeds of parasitic weeds, in particular of *Striga hermonthica*, varies considerably from test to test. For bioassays of nitro GR24 **5a** and dansyl GR24 **10**, the fast-moving diastereoisomers were used, whereas amino GR24 **2a** was tested as a mixture of diastereoisomers. It was shown for GR24 that the activity of the most active (fast-moving) diastereoisomer is not seriously influenced by the presence of the less active diastereoisomer.¹¹

The data in Tables 1 and 2 reveal that compounds **2a**, **5a** and

Table 1 Germination percentages for seeds of *Striga hermonthica* after exposure to solutions of **2a**, **5a** and **10** at different concentrations^a

Entry	Compound	% Germination ± SE at		
		1 mg l ⁻¹	0.01 mg l ⁻¹	0.001 mg l ⁻¹
1	10	70.0 ± 1.9 (40.3 ± 2.4) ^b	57.8 ± 3.0 (50.1 ± 2.8) ^b	19.4 ± 1.3 (21.4 ± 1.7) ^b
2	5a	54.1 ± 4.3 (51.1 ± 2.4) ^b	59.3 ± 4.6 (60.1 ± 3.0) ^b	44.1 ± 2.2 (32.4 ± 2.0) ^b
3	2a	46.9 ± 3.8 (50.5 ± 1.2) ^b	17.5 ± 2.2 (54.8 ± 1.4) ^b	7.1 ± 1.9 (29.0 ± 5.5) ^b
4	Control ^c	9.8 ± 0.5	11.2 ± 1.3	9.8 ± 0.8

^a Germination percentages given are the mean of two replicate tests. In each test the percentage was determined 12 times by counting the number of germination seeds. ^b The values in parentheses are the mean germination percentages for seeds tested under the same conditions and at the same time, with GR24 as stimulant. ^c Aqueous control containing 0.1, 0.001 and 0.0001% (v/v) acetone.

Table 2 Germination percentage for seeds of *Orobancha crenata* after exposure of **2a**, **5a** and **10** at different concentrations^a

Entry	Compound	% Germination ± SE at		
		1 mg l ⁻¹	0.1 mg l ⁻¹	0.01 mg l ⁻¹
1	10	1.5 ± 0.6 (60.7 ± 2.8) ^b	0.0 ± 0.0 (27.3 ± 3.6) ^b	0.2 ± 0.2 (1.2 ± 0.9) ^b
2	5a	56.7 ± 3.5 (60.0 ± 3.1) ^b	9.3 ± 1.6 (27.3 ± 3.6) ^b	1.2 ± 0.3 (1.6 ± 0.4) ^b
3	2a	33.6 ± 3.5 (58.1 ± 3.8) ^b	2.7 ± 1.0 (24.7 ± 2.4) ^b	0.0 ± 0.0 (0.1 ± 0.1) ^b
4	Control ^c	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

^a Germination percentages given are the mean of two replicate tests. In each test the percentage was determined 12 times by counting the number of germination seeds. ^b The values in parentheses are the mean germination percentages for seeds tested under the same conditions and at the same time, with GR24 as stimulant. ^c Aqueous control containing 0.1, 0.01 and 0.0001% (v/v) acetone.

10 behave quite differently towards seeds of *Striga hermonthica* and *Orobancha crenata*. In the case of *Striga hermonthica*, the biological activity of the substituted GR24 derivatives is relatively little affected as compared to GR24. The activity of amino GR24 **2a** is about one order of magnitude lower than GR24, whereas nitro GR24 **5a** and dansyl GR24 **10** possess comparable concentration-dependent activities. Interestingly, the intrinsic activity of dansyl GR24 **10** is considerably higher than that of GR24, which becomes apparent at the higher concentrations. In contrast, dansyl GR24 **10** is completely inactive in the stimulation of *Orobancha crenata* seeds. Evidently, substituents in the A-part of GR24 have a negative effect on the bioactivity in the case of this parasitic species. The difference in response exerted by A-ring analogues **2a**, **5a** and **10** on seeds of *Striga hermonthica* and *Orobancha crenata* is rather unexpected, since previous studies revealed that structural modifications in the BC-part,⁹ enol ether moiety²⁵ and D-ring²⁶ generally give similar results for both parasitic species. Nevertheless, the prospects of incorporating a tag in the A-ring of GR24 with the aim of identifying the receptor protein is very promising for *Striga hermonthica*. The remarkable activity of the bulky dansyl derivative **10** suggests a large degree of structural freedom in the A-part with retention of full biological activity.

Conclusion

In this synthetic study the preparation of amino GR24 **2a** is described. This compound is a versatile synthon for the preparation of a range of A-ring analogues of GR24. The synthesis of the fluorescent GR24 analogue **10** has been accomplished. Application of the thus developed synthetic strategy provides a feasible approach to the incorporation of radioactive tags and

photoreactive units in the GR24 molecule. The germination stimulatory activity of GR24 analogues **2a**, **5a** and **10** is relatively little affected for seeds of *Striga hermonthica*, whereas it is considerably reduced for seeds of *Orobancha crenata*. Therefore *Striga hermonthica* is an attractive target to perform protein fishing experiments.

Experimental

Synthesis

Nomenclature. We have used the AUTONOM 1.0 program, provided by the Beilstein Institute and Springer Verlag, Weinheim, BRD.

General remarks. 100 MHz ¹H NMR Spectra and 400 MHz ¹H NMR spectra were recorded on a Bruker AC 100 spectrometer and a Bruker AM-400 spectrometer, respectively (Me₄Si as internal standard). All coupling constants are given as ³J in Hz, unless indicated otherwise. IR Spectra were recorded on Perkin-Elmer 298 IR spectrophotometer. For mass spectra a double focussing VG7070E mass spectrometer was used. GC-MS Experiments were run on a Varian Saturn 2 GC-MS ion-trap system. Separation was carried out on a fused-silica capillary column (DB-5, 30 m × 0.25 mm). Helium was used as carrier gas, and electron impact (EI) was used as ionization mode. GLC was conducted with a Hewlett-Packard HP 5890 gas chromatograph, using a capillary column (25 m) of HP-1 and nitrogen (2 ml min⁻¹, 0.5 atm) as the carrier gas. Melting points were measured with a Reichert Thermopan microscope and are uncorrected. Elemental analyses were performed at the Department of Micro-analysis of this laboratory. Fluorescence measurements were performed using a Perkin-Elmer luminescence spectrometer LS50B. Solvents were dried using the following methods: dichloromethane was distilled from P₂O₅; diethyl ether distilled from NaH; hexane was distilled from CaH₂; tetrahydrofuran was distilled from lithium aluminium hydride just before use. All other solvents were of analytical grade. Thin layer chromatography (TLC) was carried out on Merck pre-coated silica gel 60 F254 plates (0.25 mm) using the eluents indicated. Spots were visualized with UV or using molybdate spray. Flash chromatography was carried out at a pressure of ca. 1.5 bar, using Merck Kieselgel 60H. Column chromatography at atmospheric pressure was carried out, using Merck Kieselgel 60.

3,3a,4,8b-Tetrahydroindeno[1,2-*b*]furan-2-one **3** and 5-bromo-3-methylfuran-2(5*H*)-one **7** were prepared according to known procedures.¹¹

7-Nitro-3,3a,4,8b-tetrahydroindeno[1,2-*b*]furan-2-one **4a** and 5-nitro-3,3a,4,8b-tetrahydroindeno[1,2-*b*]furan-2-one **4b**

Sodium nitrate (0.51 g, 6.0 mmol) was added to a solution of the tricyclic lactone **3** (348 mg, 2.00 mmol) in TFA (15 ml) at room temperature. The solution was stirred overnight after which TFA was removed *in vacuo*, and the residue was dissolved in ethyl acetate and saturated aqueous NaHCO₃. The aqueous phase was extracted with ethyl acetate (2×). The combined extracts were dried (MgSO₄), filtered and concentrated *in vacuo* to give a mixture of **4a** and **4b** (399 mg, 91%) as a solid. The ratio of **4a**:**4b** (9:1) was determined by ¹H NMR (100 MHz) analysis. Pure **4a** (319 mg, 75%) was obtained by recrystallization from toluene. The residue, containing **4b**, was purified by flash chromatography (SiO₂, hexane-ethyl acetate, 1:1) to give pure **4b** (40 mg, 9%) as a white solid. An analytically pure sample was obtained by crystallization from hexane-ethyl acetate.

Compound **4a**, *R*_f 0.21 (hexane-ethyl acetate, 1:1), mp 118–119 °C (from hexane-ethyl acetate) (Found: C, 60.12; H, 4.04; N, 6.37. C₁₁H₉NO₄ requires C, 60.28; H, 4.14; N, 6.39); δ_H (400 MHz; CDCl₃) 2.43 (1 H, dd, ²J 18.2, J 5.3, 3-H), 2.96 (1 H, dd, ²J 18.2, J 9.7, 3-H), 3.00 (1 H, dd, ²J 17.2, J 3.4, 4-H), 3.44 (1 H, dd, ²J 17.2, J 8.6, 4 H), 3.52 (1 H, m, 3a-H), 5.94 (1 H, d, J 7.1, 8b-H), 7.45 (1 H, d, J 8.4, 5-H), 8.25 (1 H, dd, ⁴J 2.1, J 8.4, 6-H)

and 8.35 (1 H, d, ⁴J 2.1, 8-H); *m/z* 219 (M⁺, 39%), 175 (100, C₁₀H₉NO₂), 128 (84, C₁₀H₈) and 115 (33, C₉H₇).

Compound **4b**, *R*_f 0.35 (hexane-ethyl acetate, 1:1) mp 137–140 °C (from hexane-ethyl acetate) (Found: C, 59.93; H, 4.02; N, 6.34. C₁₁H₉NO₄ requires C, 60.28; H, 4.14; N, 6.39); δ_H (100 MHz; CDCl₃) 2.46 (1 H, dd, ²J 18.1, J 5.0, 3-H), 2.90 (1 H, dd, ²J 18.1, J 9.5, 3-H), 3.29–4.00 (3 H, m, 4-H and 3a-H), 5.95 (1 H, d, J 7.4, 8b-H), 7.53 (1 H, t, J 7.7, 7-H), 7.83 (1 H, d, J 7.7, 8-H) and 8.25 (1 H, d, J 7.7, 6-H); *m/z* 219 (M⁺, 28%), 128 (100, C₁₀H₈) and 115 (54, C₉H₇).

7-Amino-3,3a,4,8b-tetrahydroindeno[1,2-*b*]furan-2-one **1a**

A mixture containing the 7-nitro tricyclic lactone **4a** (3.00 g, 13.7 mmol) and tin (powdered, 17.7 g, 0.149 mol) in ethanol (63 ml) and 10 M HCl (59 ml) was heated at reflux for 1 h. After cooling of the reaction mixture, it was evaporated *in vacuo* to remove the ethanol and then adjusted to pH 8 by the addition of saturated aqueous NaHCO₃. Insoluble tin salts were removed by filtration over Hyflo. The mixture was extracted with ethyl acetate (3×) and the combined extracts were dried (MgSO₄) and evaporated *in vacuo* to afford crude **1a** as a brownish solid (2.56 g, 99%). Recrystallization of this from ethyl acetate provided analytically pure **1a** (1.99 g, 77%) as pale yellow crystals, mp 128–130 °C (from ethyl acetate) (Found: C, 69.47; H, 5.81; N, 7.30. C₁₁H₁₁NO₂ requires C, 69.83; H, 5.86; N, 7.40); δ_H (100 MHz; CDCl₃) 2.22 (1 H, dd, ²J 17.9, J 5.5, 3-H), 2.54–3.05 (4 H, m, 3-H, 4-H and 3a-H), 3.70 (2 H, br s, NH₂), 5.66 (1 H, d, J 6.8, 8b-H), 6.55 (1 H, dd, ⁴J 2.1, J 7.9, 6-H), 6.63 (1 H, d, ⁴J 2.1, 8-H) and 6.91 (1 H, d, J 7.9, 5-H); *m/z* 189 (M⁺, 100%), 144 (62, C₁₀H₁₀N), 130 (34, C₉H₈N) and 115 (10, C₉H₇).

7-Iodo-3,3a,4,8b-tetrahydroindeno[1,2-*b*]furan-2-one **12a** and 5-iodo-3,3a,4,8b-tetrahydroindeno[1,2-*b*]furan-2-one **12b**

To a solution of the tricyclic lactone **3** (100 mg, 0.575 mmol) and iodine (146 mg, 0.575 mmol) in tetrachloromethane (15 ml) was added [bis(trifluoroacetoxy)iodo]benzene (272 mg, 0.633 mmol) with stirring at room temperature. After 48 h the solvent was removed from the mixture *in vacuo* and the residue purified by flash chromatography (SiO₂, hexane-ethyl acetate, 6:1). Collection of the pure fractions afforded the 7-iodo tricyclic lactone **12a** (65 mg, 38%) and the 5-iodo tricyclic lactone **12b** (48 mg, 28%) both as white solids. Analytical samples of **12a** and **12b** were obtained by recrystallization from dichloromethane-diisopropyl ether.

Compound **12a**, *R*_f 0.30 (hexane-ethyl acetate, 1:1), mp 110–113 °C (from dichloromethane-diisopropyl ether); δ_H (100 MHz; CDCl₃) 2.34 (1 H, dd, ²J 18.0, J 5.4, 3-H), 2.76–3.55 (4 H, m, 3-H, 4-H and 3a-H), 5.83 (1 H, d, J 6.9, 8b-H), 7.03 (1 H, d, J 8.0, 5-H), 7.65 (1 H, dd, ⁴J 1.6, J 8.0, 6-H) and 7.80 (1 H, br s, 8-H); *m/z* 300 (M⁺, 20%), 256 (20, C₁₀H₉I), 129 (100, C₁₀H₉) and 115 (18, C₉H₇) [Found (HRMS): *m/z* 299.9645. Calc. for C₁₁H₉IO₂: 299.9649].

Compound **12b**, *R*_f 0.40 (hexane-ethyl acetate, 1:1), mp 165–167.5 °C (from dichloromethane-diisopropyl ether); δ_H (100 MHz; CDCl₃) 2.42 (1 H, dd, ²J 18.0, J 5.1, 3-H), 2.76–3.38 (4 H, m, 3-H, 4-H and 3a-H), 5.99 (1 H, d, J 6.9, 8b-H), 7.02 (1 H, t, J 7.6, 7-H), 7.45 (1 H, d, J 7.6, 8-H) and 7.74 (1 H, d, J 7.6, 6-H); *m/z* 300 (M⁺, 9%), 256 (19, C₁₀H₉I), 129 (100, C₁₀H₉) and 115 (15, C₉H₇) [Found (HRMS): *m/z* 299.9648. Calc. for C₁₁H₉IO₂: 299.9649].

7-Nitro-3-(4-methyl-5-oxo-2,5-dihydrofuran-2-yloxymethylene)-3,3a,4,8b-tetrahydroindeno[1,2-*b*]furan-2-one **5a**

Potassium *tert*-butoxide (563 mg, 5.03 mmol) was added in small quantities to a solution of the 7-nitro tricyclic lactone **4a** (1.00 g, 4.57 mmol) and methyl formate (0.84 ml, 13.7 mmol) in THF (35 ml) with stirring at –78 °C under nitrogen. The mixture was allowed to warm to room temperature and then stirred for 18 h. After the THF had been removed *in vacuo*, the residue was dissolved in DMF (35 ml) and the solution was cooled to

–60 °C, and treated with the bromo butenolide **7** (0.97 g, 5.5 mmol) in DMF (5 ml), added gradually under nitrogen. The mixture was brought to room temperature and stirred for 18 h after which it was treated with acetic acid (0.60 g, 10 mmol); the suspension was then concentrated *in vacuo*. The residue was dissolved in chloroform and water and the aqueous phase was separated and extracted with chloroform (2×). The combined organic layers were washed with water (1×), dried (MgSO₄) and concentrated *in vacuo*. The crude product was purified by flash chromatography (SiO₂, hexane–ethyl acetate, 1 : 1) to afford two diastereoisomers of **5a** (549 mg, 35%) [*R*_f 0.12 and 0.18 (hexane–ethyl acetate, 1 : 1)] as pale yellow solids. Only the fast-moving diastereoisomer could be obtained in an analytically pure form by recrystallization from ethyl acetate, mp 216–219 °C (from ethyl acetate) (Found: C, 59.21; H, 3.81; N, 4.11. C₁₇H₁₃NO₇ requires C, 59.48; H, 3.82; N, 4.08); δ_H(400 MHz; CDCl₃) 2.06 (3 H, m, CH₃), 3.21 (1 H, dd, ²*J* 18.0, *J* 3.2, 4-H), 3.52 (1 H, dd, ²*J* 18.0, *J* 9.3, 4-H), 4.08 (1 H, m, 3a-H), 5.99 (1 H, d, *J* 8.0, 8b-H), 6.20 (1 H, m, OCHO), 6.97 (1 H, m, =CH), 7.39 (1 H, d, *J* 8.4, 5-H), 7.51 (1 H, d, ⁴*J* 2.3, =CHO), 8.23 (1 H, dd, ⁴*J* 1.9, *J* 8.4, 6-H) and 8.37 (1 H, d, ⁴*J* 1.9, 8-H); *m/z* 343 (M⁺, 1%), 247 (2, C₁₂H₉NO₅), 219 (1, C₁₁H₉NO₄) and 97 (100, C₅H₅O₂).

7-Nitro GR24 **5a** could be prepared in an alternative manner by nitration of GR24 using the procedure described for the synthesis of **4a**. Starting from GR24 (100 mg, 0.336 mmol), **5a** was obtained in 92% yield. ¹H NMR spectroscopic data were in complete agreement with those reported above.

7-Iodo-3-(4-methyl-5-oxo-2,5-dihydrofuran-2-yloxymethylene)-3,3a,4,8b-tetrahydroindeno[1,2-*b*]furan-2-one **11**

Potassium *tert*-butoxide (167 mg, 1.64 mmol) was added in small quantities to a solution of the 7-iodo tricyclic lactone **12a** (447 mg, 1.49 mmol) and ethyl formate (1.20 ml, 14.9 mmol) in THF (25 ml) with stirring at 0 °C under nitrogen. The mixture was allowed to warm to room temperature and then stirred for 18 h. After this an excess of acetic acid (1 ml) was added to the mixture which was then evaporated *in vacuo* to remove the solvent. The mixture was dissolved in ethyl acetate and saturated aqueous NH₄Cl. The aqueous phase was separated and extracted with ethyl acetate (2×). The combined organic layers were dried (MgSO₄) and concentrated *in vacuo* to give the crude formyl tricyclic lactone as a pale yellow solid. Starting **12a** was removed by washing with diethyl ether (2×) to provide pure hydroxymethylene lactone as a tautomeric mixture in 50% yield; δ_H(100 MHz; [²H₆]acetone) 2.85–3.33 (2 H, m, 4-H), 3.82 (1 H, m, 3a-H), 5.69 (1 H, d, *J* 7.5, 8b-H), 6.93 (1 H, d, *J* 7.7, 5-H), 7.38–7.61 (3 H, m, 6-H, 8-H, =CHO) and 9.6 (1 H, br s, OH).

To a solution of thus obtained hydroxymethylene lactone (150 mg, 0.457 mmol) in DMF (10 ml) was added potassium *tert*-butoxide (56.0 mg, 0.503 mmol) at 0 °C under a nitrogen atmosphere. The mixture was cooled to –60 °C and the bromo butenolide **7** (97.0 mg, 0.548 mmol) in DMF (2 ml) was gradually added to it. Work-up was similar to that described for the preparation of 7-nitro GR24 **5a** (*vide supra*). Purification by flash chromatography (SiO₂, hexane–ethyl acetate, 1 : 1) afforded two diastereoisomers in 57% yield. The fast-moving diastereoisomer of **11** (*R*_f 0.35, hexane–ethyl acetate, 1 : 1) was crystallized from ethyl acetate to give **11** as colourless needles, mp 206–209 °C (from ethyl acetate); δ_H(100 MHz; CDCl₃) 2.04 (3 H, m, CH₃), 3.03 (1 H, dd, ²*J* 17.0, *J* 3.5, 4-H), 3.39 (1 H, dd, ²*J* 17.0, *J* 8.8, 4-H), 3.94 (1 H, m, 3a-H), 5.90 (1 H, d, *J* 7.8, 8b-H), 6.19 (1 H, m, OCHO), 6.95 (1 H, m, =CH), 6.97 (1 H, d, *J* 8.0, 5-H), 7.48 (1 H, d, ⁴*J* 2.5, =CHO), 7.64 (1 H, dd, ⁴*J* 1.6, *J* 8.0, 6-H) and 7.83 (1 H, d, ⁴*J* 1.6, 8-H); *m/z* 424 (M⁺, 4%), 327 (26, C₁₂H₈O₃I) and 97 (100, C₅H₅O₂) [Found (HRMS): *m/z* 423.9810. Calc. for C₁₇H₁₃IO₅: 423.9804].

The slow-moving diastereoisomer of **11** (*R*_f 0.26, hexane–ethyl acetate, 1 : 1) was recrystallized from hexane–ethyl acetate

to give colourless crystals, mp 189–191 °C (from hexane–ethyl acetate); the ¹H NMR spectrum (CDCl₃, 100 MHz) was identical with the ¹H NMR spectrum of the fast-moving diastereoisomer of **11** (*vide supra*); *m/z* 424 (M⁺, 6%), 327 (34, C₁₂H₈O₃I) and 97 (100, C₅H₅O₂) [Found (HRMS): *m/z* 423.9810. Calc. for C₁₇H₁₃IO₅: 423.9804].

7-Amino-3-(4-methyl-5-oxo-2,5-dihydrofuran-2-yloxy-methylene)-3,3a,4,8b-tetrahydroindeno[1,2-*b*]furan-2-one **2a**

A solution of the amino tricyclic lactone **1a** (440 mg, 2.33 mmol) and benzaldehyde (247 mg, 2.33 mmol) in ethyl acetate (20 ml), in the presence of molecular sieves 4A, was stirred for 12 h at room temperature. After this MgSO₄ was added to the mixture which was then filtered over Hyflo and then concentrated by removal of the solvent *in vacuo* to give the imine **6** (645 mg, 100%) as a pale yellow solid, which was used immediately in the coupling reaction; δ_H(100 MHz; CDCl₃) 2.36 (1 H, dd, ²*J* 18.1, *J* 5.4, 3-H), 2.70–3.56 (4 H, m, 3-H, 4-H and 3a-H), 5.87 (1 H, d, *J* 6.8, 8b-H), 7.24–7.27 (3 H, m, Ph), 7.43–7.53 (3 H, m, Ph), 7.64–7.94 (2 H, m, Ph) and 8.44 (1 H, s, N=CH).

To a solution of the freshly prepared imine **6** (645 mg, 2.33 mmol) and methyl formate (0.5 ml, 7 mmol) in THF (25 ml) was added potassium *tert*-butoxide (287 mg, 2.56 mmol) in small portions at 0 °C under nitrogen. The mixture was allowed to warm to room temperature after which it was stirred for 18 h and then concentrated by removal of the THF *in vacuo*. The residue was dissolved in DMF (20 ml) and the solution was cooled to –60 °C when it was treated with the bromo butenolide **7** (494 mg, 2.70 mmol) in DMF (3 ml), added gradually under nitrogen. The mixture was brought to room temperature and stirred for 18 h after which it was concentrated by removal of the solvent *in vacuo*. The residue was dissolved in dichloromethane and saturated aqueous NaHCO₃. The aqueous phase was separated and extracted with dichloromethane (2×) and the combined organic layers were washed with saturated aqueous NaHCO₃ (1×), dried (MgSO₄) and concentrated *in vacuo* to provide crude 7-benzalimino GR24 (78%). Flash chromatography (SiO₂, dichloromethane, followed by ethyl acetate–dichloromethane, 3 : 1) gave 7-amino GR24 **2a** (190 mg, 27%) as a mixture of two inseparable diastereoisomers. Crystallization from butyl acetate afforded **2a** as pale yellow crystals (Found: C, 65.16; H, 4.96; N, 4.31. C₁₇H₁₅NO₅ requires C, 65.17; H, 4.82; N, 4.47); δ_H(100 MHz; CDCl₃) 1.94 (3 H, m, CH₃), 2.86 (1 H, dd, ²*J* 16.3, *J* 3.0, 4-H), 3.23 (1 H, dd, ²*J* 16.3, *J* 8.8, 4-H), 3.75 (3 H, m, NH₂ and 3a-H), 5.77 (1 H, d, *J* 7.8, 8b-H), 6.10 (1 H, m, OCHO), 6.59 (1 H, dd, ⁴*J* 2.2, *J* 8.0, 6-H), 6.70 (1 H, d, ⁴*J* 2.2 8-H), 6.88 (1 H, m, =CH), 6.92 (1 H, d, *J* 8.0, 5-H) and 7.39 (1 H, d, ⁴*J* 2.5, =CHO); *m/z* 313 (M⁺, 57%), 216 (40, C₁₂H₁₀NO₃), 188 (3, C₁₁H₁₀NO₂) and 97 (100, C₅H₅O₂).

7-Azido-3-(4-methyl-5-oxo-2,5-dihydrofuran-2-yloxymethylene)-3,3a,4,8b-tetrahydroindeno[1,2-*b*]furan-2-one **8**

Sodium nitrate (33 mg, 0.48 mmol) was added to a solution of a mixture of the diastereoisomers of 7-amino GR24 **2a** (138 mg, 0.44 mmol) in 80% acetic acid (20 ml) at 0 °C with protection from light. After 5 min sodium azide (32 mg, 0.48 mmol) was added to the mixture and stirring was continued for 2 h. The mixture was then concentrated *in vacuo* and the residue was dissolved in ethyl acetate and saturated aqueous NaHCO₃. The aqueous phase was separated and extracted with ethyl acetate (2×) and the combined organic layers were washed with saturated aqueous NaHCO₃ (1×), dried (MgSO₄) and concentrated *in vacuo*. Purification of the residue by flash chromatography (SiO₂, hexane–ethyl acetate 1 : 1) afforded two diastereoisomers of **8** (131 mg, 88%) as white solids.

Fast-moving diastereoisomer of **8**, *R*_f 0.29 (hexane–ethyl acetate, 1 : 1), mp 151–154 °C; ν_{max}(CCl₄)/cm^{–1} 2120 (N₃), 1795 (C=O), 1765 (C=O) and 1685 (C=C, enol ether); δ_H(100 MHz; CDCl₃) 2.04 (3 H, m, CH₃), 3.06 (1 H, dd, ²*J* 16.7, *J* 3.6, 4-H), 3.41 (1 H, dd, ²*J* 16.7, *J* 8.8, 4-H), 3.96 (1 H, m, 3a-H), 5.91

(1 H, d, J 7.8, 8b-H), 6.19 (1 H, m, OCHO), 6.93–7.25 (4 H, m, Ph and =CH) and 7.48 (1 H, d, 4J 2.5, =CHO); m/z 339 (M^+ , 2%), 313 (1, $C_{17}H_{15}NO_5$), 214 (2, $C_{12}H_8NO_3$) and 97 (100, $C_5H_5O_2$) [Found (HRMS): m/z 339.0856. Calc. for $C_{17}H_{13}N_3O_5$: 339.0855].

Slow-moving diastereoisomer of **8**, R_f 0.19 (hexane–ethyl acetate, 1:1), mp 164–167 °C; the 1H NMR spectrum ($CDCl_3$, 100 MHz) was identical with the 1H NMR spectrum of the fast-moving diastereoisomer of **8**; m/z 339 (M^+ , 2%), 311 (3, $C_{17}H_{13}NO_5$), 215 (3, $C_{12}H_8NO_3$) and 97 (100, $C_5H_5O_2$) [Found (HRMS): m/z 339.0856. Calc. for $C_{17}H_{13}N_3O_5$: 339.0855].

N*-[3-(4-Methyl-5-oxo-2,5-dihydrofuran-2-yloxymethylene)-2-oxo-3,3a,4,8b-tetrahydro-2*H*-indeno[1,2-*b*]furan-7-yl]-5-dimethylaminonaphthalene-1-sulfonamide **10*

A mixture of slow- and fast-moving 7-amino GR24 **2a** (170 mg, 0.543 mmol) and dansyl chloride (146 mg, 0.543 mmol) were dissolved in pyridine (10 ml) and the solution was stirred for 65 h with protection from light. After this, the mixture was concentrated by removal of the solvent *in vacuo* and the residue was dissolved in ethyl acetate and 5% aqueous oxalic acid. The aqueous phase was separated and extracted with ethyl acetate (2 \times), and the combined organic layers were washed with 5% aqueous oxalic acid (1 \times), dried ($MgSO_4$) and concentrated *in vacuo*. The crude product was purified by flash chromatography (SiO_2 , hexane–ethyl acetate, 1:1) to provide two diastereoisomers of **10** (264 mg, 89%) as pale yellow solids with R_f 0.2 and 0.17 (hexane–ethyl acetate, 1:1). An analytically pure sample of the fast-moving diastereoisomer of **10** was obtained by recrystallization from diisopropyl ether–dichloromethane, mp 186–188 °C (from diisopropyl ether–dichloromethane) (Found: C, 63.50; H, 4.83; N, 5.10; S, 5.83. $C_{29}H_{26}N_2O_7S$ requires C, 63.73; H, 4.79; N, 5.13; S, 5.83); δ_{H^1} (400 MHz; $CDCl_3$) 2.03 (3 H, m, CH_3 D-ring), 2.88 [6 H, s, $N(CH_3)_2$], 2.96 (1 H, dd, 2J 16.4, J 3.5, 4-H), 3.28 (1 H, dd, 2J 16.4, J 9.4, 4-H), 3.86 (1 H, m, 3a-H), 5.72 (1 H, d, J 8.2, 8b-H), 6.14 (1 H, m, OCHO), 6.81 (1 H, s, NH), 6.92 (1 H, m, =CH), 6.96 (1 H, s, 8-H), 7.00 (2 H, s, 5-H and 6-H), 7.19 (1 H, d, J 7.6, Ph dansyl), 7.42 (1 H, t, J 7.6 Ph dansyl), 7.43 (1 H, d, 4J 2.4, =CHO), 7.59 (1 H, t, J 8.4, Ph dansyl), 8.14 (1 H, d, J 7.6, Ph dansyl), 8.31 (1 H, d, J 8.4, Ph dansyl) and 8.50 (1 H, d, J 8.4, Ph dansyl); m/z 450 ($M^+ + 1 - C_5H_5O_2$, 1%), 422 (3, $C_{23}H_{22}N_2O_4S$), 217 (11, $C_{12}H_{11}NO_3$), 171 (2, $C_{12}H_{13}N$), 97 (12, $C_5H_5O_2$) and 28 (100, CO); fluorescence (c 2.0 mM, MeOH): λ_{exc} 350 nm (ϵ 4.12×10^3), λ_{em} 525 nm.

N*-[3-(4-Methyl-5-oxo-2,5-dihydrofuran-2-yloxymethylene)-2-oxo-3,3a,4,8b-tetrahydro-2*H*-indeno[1,2-*b*]furan-7-yl]-methanesulfonamide **9*

The procedure described for the preparation of **10** was followed using amino GR24 **2a** (100 mg, 0.320 mmol), methanesulfonyl chloride (36.6 mg, 0.320 mmol) and pyridine (10 ml). Work-up followed by purification by flash chromatography (SiO_2 , dichloromethane–ethyl acetate, 3:1) gave methanesulfonyl GR24 **9** (98 mg, 78%) as a mixture of inseparable diastereoisomers. Recrystallization of the residue from ethanol afforded **9** as a white solid (Found: C, 54.89; H, 4.24; N, 3.72; S, 8.08. $C_{18}H_{17}NO_5S$ requires C, 55.24; H, 4.38; N, 3.58; S, 8.19); δ_{H^1} (100 MHz; $CDCl_3$) 2.05 (3 H, m, = CH_3), 2.97 (3 H, s, SCH_3) 3.05 (1 H, dd, 2J 16.9, J 3.0 4-H), 3.39 (1 H, dd, 2J 16.9, J 8.8, 4-H), 3.96 (1 H, m, 3a-H), 5.92 (1 H, d, J 7.8, 8b-H), 6.21 (1 H, m, OCHO), 7.00 (1 H, m, =CH), 7.14–7.27 (4 H, m, Ph and NH) and 7.54 (1 H, d, 4J 2.5, =CHO); m/z 391 (M^+ , 1%), 295 (3, $C_{13}H_{13}NO_5S$), 216 (1, $C_{12}H_{10}NO_3$), 97 (12, $C_5H_5O_2$) and 28 (100, CO).

Biological activity

Seeds. Seeds of *Striga hermonthica* and *Orobanche crenata* were harvested in Sudan in 1987 and in Egypt in 1991, respectively and were stored in the dark at room temperature until used in germination tests.

Preparation of test solutions. A compound (10 mg) to be tested was weighed out very accurately, dissolved in acetone p.a. (10 ml) and diluted with demineralized water to 100 ml. Aliquots of this stock solution were further diluted with water to obtain test solutions containing 1, 0.1, 0.01 and 0.001 mg l^{-1} test compound and 0.1, 0.01, 0.001 and 0.0001% (v/v) of acetone, respectively.

Bioassays. For sterilization, seeds of *Striga hermonthica* and *Orobanche crenata* were exposed to an aqueous sodium hypochlorite (2% active chlorine) for 5 min with agitation. The seeds were then thoroughly rinsed with water and dried overnight.

For conditioning the sterilized seeds were spread on glass fibre filter paper disks (8 mm diameter; approximately 30–70 seeds per disk) in Petri dishes, moistened with water and stored in the dark for 14 days at 20 °C for *Orobanche* seeds and at 30 °C for *Striga* seeds. The conditioning water was then removed and replaced by 100 μ l of test solution per disk. After incubation for 24 h (*Striga*) and 5 days (*Orobanche*) in the dark at the indicated temperatures, the germination percentage was determined under a microscope. Seeds were considered to be germinated if the radical protruded through the seed coat.

In each test series aqueous solutions with 0.1, 0.01, 0.001 and 0.0001% (v/v) of acetone were used as negative control. Test solutions of the stimulant GR24 (concentrations of 1, 0.1, 0.01 and 0.001 mg l^{-1}) were used as positive controls. All tests were performed in duplicates, and in each test the germination percentages were determined on 12 disks. For full details of the bioassay, see ref. 27.

Acknowledgements

We thank Dr A. G. T. Babiker and Dr F. M. F. Zaitoun for supplying *Striga* and *Orobanche* seeds. We thank H. Amattajais, P. van Galen and A. Swolfs for conducting elemental analysis, mass and 400 MHz 1H NMR measurements, respectively. These investigations were supported by the Netherlands Foundation of Chemical Research (SON) with financial aid from the Netherlands Organization for the Advancement of Research (NWO).

References

- 1 L. J. Musselman, Ed. *Parasitic Weeds in Agriculture, Vol. I, Striga*, CRC Press, Boca Raton, FL., 1987, 317.
- 2 C. Parker, *Scope of the agronomic problems caused by Orobanche species*, in *Proceedings of a Workshop on Biology and Control of Orobanche*, ed. S. J. ter Borg, LH/VPO, Wageningen, The Netherlands, 1986, p. 11.
- 3 C. E. Cook, L. P. Whichard, M. E. Wall, G. H. Egley, P. Coggan, P. A. Luhan and A. T. McPhail, *J. Am. Chem. Soc.*, 1972, **94**, 6198.
- 4 D. W. Brooks, H. S. Bevinakatti and D. R. Powell, *J. Org. Chem.*, 1985, **50**, 3779.
- 5 A. W. Johnson, G. Roseberry and C. Parker, *Weed Res.*, 1976, **16**, 233.
- 6 A. W. Johnson, G. Gowda, A. Hassanali, J. Knox, S. Monaco, Z. Razawi and G. Roseberry, *J. Chem. Soc., Perkin Trans. 1*, 1981, 1734.
- 7 S. L. Vail, O. D. Dailey, E. J. Blanchard, A. B. Pepperman and J. L. Riopel, *J. Plant Growth Regul.*, 1990, **9**, 77.
- 8 E. M. Mangnus and B. Zwanenburg, *J. Agric. Food Chem.*, 1992, **40**, 1066.
- 9 E. M. Mangnus, L. A. van Vliet, D. A. L. Vandenput and B. Zwanenburg, *J. Agric. Food Chem.*, 1992, **40**, 1222.
- 10 E. M. Mangnus and B. Zwanenburg, *Recl. Trav. Chim. Pays-Bays*, 1992, **111**, 155.
- 11 E. M. Mangnus, F. J. Dommerholt, R. L. P. de Jong and B. Zwanenburg, *J. Agric. Food Chem.*, 1992, **40**, 1230.
- 12 R. J. Weselake and J. C. Jain, *Physiol. Plant.*, 1992, **84**, 301.
- 13 H. Bayley and J. R. Knowles, *Methods Enzymol.*, 1977, **46**, 69.
- 14 For a recent review of chemical reagents in photoaffinity labelling, see S. A. Fleming, *Tetrahedron*, 1995, **51**, 12 479.
- 15 A. M. Jones and M. A. Venis, *Proc. Natl. Acad. Sci. USA*, 1986, **86**, 6153.

- 16 R. P. Walker, W. M. Waterworth, M. H. Beale and R. Hooley, *Plant Growth Regul.*, 1994, **15**, 271.
- 17 A. Hassanali, *Strigol analogues: Synthetic achievements and prospects*, in *Striga: Biology and Control*, ed. E. S. Ayensu, H. Doggett, R. D. Keynes, J. Marton-Lefevre, L. J. Musselman and C. Parker, ICSU Press, Paris, 1984, pp. 125-132.
- 18 S. Uemura, A. Toshimitsu and M. Okano, *J. Chem. Soc., Perkin Trans. 1*, 1978, 1076.
- 19 J. A. Cowan, *Tetrahedron Lett.*, 1986, **27**, 1205.
- 20 K. M. Doxsee, M. Fiegel, K. D. Stewart, J. W. Canary, C. B. Knobler and D. J. Cram, *J. Am. Chem. Soc.*, 1987, **109**, 3098.
- 21 K. G. Pinney and J. A. Katzenellenbogen, *J. Org. Chem.*, 1991, **56**, 3125.
- 22 C. M. Himmel, W. G. Aboul-Saad and S. Uk, *J. Agric. Food Chem.*, 1971, **19**, 1175.
- 23 For experimental details see: J. W. J. F. Thuring, Thesis, 1996, University of Nijmegen, The Netherlands.
- 24 E. B. Merkushev, N. D. Simakhina and G. M. Koveshnikova, *Synthesis*, 1980, 486.
- 25 J. W. J. F. Thuring, A. A. M. A. van Gaal, S. J. Hornes, M. M. K. de Kok, G. H. L. Nefkens and B. Zwanenburg, *J. Chem. Soc., Perkin Trans. 1*, 1997, 767.
- 26 J. W. J. F. Thuring, H. H. Bitter, M. M. K. de Kok, G. H. L. Nefkens, A. M. D. A. van Riel and B. Zwanenburg, *J. Agric. Food Chem.*, in the press.
- 27 E. M. Magnus, P. L. A. Stommen and B. Zwanenburg, *J. Plant Growth Regul.*, 1992, **11**, 91.

Paper 6/04685A
Received 4th July 1996
Accepted 16th October 1996