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

J an W illem J . F. Thuring, Rolf K eltjens, Gerard H. L. N efkens and Binne Z. w anenburg*

N S R-Centre for M olecular Structure, D esign and S ynthesis, D epartment of O rganic C hemistry, U niversity of N ijmegen, T oernooiveld, 6525 ED N ijmegen, T he N etherlands

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 photoactive 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. 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. 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), several structure-bioactivity studies have been conducted, which revealed that the bioactive site resides in the CD-fragment and the vinyl ether moiety.

In particular, GR24 (Fig. 1) turned out to be a highly potent synthetic strigol analogue. Based on the structural requirements for retaining stimulatory activity a tentative molecular mechanism for germination has been proposed which is depicted in 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. 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. 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 photolabile ligand-binding site. A radioactively or fluorescent tag can be introduced in the photoreactive ligand in order to allow detection during isolation and enable the characterization of the protein.

It has been demonstrated that this technique is a useful tool in plant hormone research, exemplified by the photoaffinity labelling of auxin binding proteins and gibberellin binding proteins. 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 bioactive site 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 performed 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. Since the CD-part as well as the connecting enol ether unit are
essential for full biological activity. 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 \(3\) in a regiocontrolled manner (Scheme 3).

The amino group in \(1\) was first protected as a Schiff base by reaction with benzaldehyde, then followed by formylation, coupling with bromo butenolide \(7\), similar to that described for GR24, and finally deprotection. Crude 7-benzalimino GR24 was isolated in an overall yield of 78%, based on \(1\). 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.

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

Diazotation and substitution in the presence of NaN\(_3\) 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. The alternative procedure for the preparation of the labelled GR24 analogues \(8, 9\) and \(10\), involving modification of the amino function of \(1\) into the azido, mesylamino and dansylamino group, respectively, fol-
of because the response of seeds of parasitic weeds, in particular results obtained in different test series, which is important, assay. This reference to GR24 enables a comparison between obtained for GR24 under the same conditions in the same bio-

moving diastereoisomers were used, whereas amino GR24 less active diastereoisomer. GR24 that the activity of the most active (fast-moving) was tested as a mixture of diastereoisomers. It was shown for azido GR24 that the substitution of the diazo moiety by the presence of the GR24, whereas nitro GR24 amino GR24 is not seriously influenced by the presence of the more diastereoisomer is not seriously influenced by the presence of the diastereoisomer that is not seriously influenced by the presence of the

The stimulatory activity of nitro GR24 was tested as a mixture of diastereoisomers. It was shown for azido GR24 since the substitution of the diazo moiety by iodide in a similar procedure to that used for the preparation of azido GR24 (vide supra), was unsuccessful. Therefore, the tricyclic lactone 3 was iodinated (cf. route I in Scheme 2) employing iodine in the presence of bis(trimfluoroacetoxyl)iodo-benzene (Scheme 3) by adopting the procedure of M. Kushnir et al. 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 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. 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 hydroxymethyleno 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. Akylation 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 Orobanche 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. The data in Tables 1 and 2 reveal that compounds 2a, 5a and

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<th>Entry</th>
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<th>0.01 mg l⁻¹</th>
<th>0.001 mg l⁻¹</th>
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<td>19.4 ± 1.3</td>
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<td>(21.4 ± 1.7)</td>
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<td>59.3 ± 4.6</td>
<td>44.1 ± 2.2</td>
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<td></td>
<td></td>
<td>(51.1 ± 2.4)</td>
<td>(60.1 ± 3.0)</td>
<td>(32.4 ± 2.0)</td>
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<tr>
<td>3</td>
<td>2a</td>
<td>46.9 ± 3.8</td>
<td>17.5 ± 2.2</td>
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<td></td>
<td></td>
<td>(50.5 ± 1.2)</td>
<td>(54.8 ± 1.4)</td>
<td>(29.0 ± 5.5)</td>
</tr>
<tr>
<td>4</td>
<td>Control</td>
<td>9.8 ± 0.5</td>
<td>11.2 ± 1.3</td>
<td>9.8 ± 0.8</td>
</tr>
</tbody>
</table>

* Germination percentages are the mean of two replicate tests. In each test the percentage was determined 12 times by counting the number of germination seeds. 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. A aqueous control containing 0.1, 0.001 and 0.0001% (v/v) acetone.

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

<table>
<thead>
<tr>
<th>Entry</th>
<th>Compound</th>
<th>1 mg l⁻¹</th>
<th>0.1 mg l⁻¹</th>
<th>0.01 mg l⁻¹</th>
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<tr>
<td>1</td>
<td>10</td>
<td>1.5 ± 0.6</td>
<td>0.0 ± 0.0</td>
<td>0.2 ± 0.2</td>
</tr>
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<td></td>
<td></td>
<td>(60.7 ± 2.8)</td>
<td>(27.3 ± 3.6)</td>
<td>(1.2 ± 0.9)</td>
</tr>
<tr>
<td>2</td>
<td>5a</td>
<td>56.7 ± 3.5</td>
<td>9.3 ± 1.6</td>
<td>1.2 ± 0.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(60.0 ± 3.1)</td>
<td>(27.3 ± 3.6)</td>
<td>(1.6 ± 0.4)</td>
</tr>
<tr>
<td>3</td>
<td>2a</td>
<td>33.6 ± 3.5</td>
<td>2.7 ± 1.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(58.1 ± 3.8)</td>
<td>(24.7 ± 2.4)</td>
<td>(0.1 ± 0.1)</td>
</tr>
<tr>
<td>4</td>
<td>Control</td>
<td>0.0 ± 0.0</td>
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10 behave quite differently towards seeds of Striga hermonthica and Orobanche 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 that of 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 Orobanche 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 Orobanche crenata is rather unexpected, since previous studies revealed that structural modifications in the BC-part, enol ether moiety 25 and D-ring 26 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 G R24 molecule. The germination stimulatory activity of GR24 analogues 2a, 5a and 10 is relatively little affected for seeds of Striga hermonitica, whereas it is considerably reduced for seeds of Orobanche crenata. Therefore Striga hermonitica is an attractive target to perform protein fishing experiments.

**E Experimental**

**Synthesis**

**Nomenclature.** We have used the AUTONOM 1.0 program, provided by the Belstein Institute and Springer Verlag, Weinheim, B.R.D.

**General remarks.** 100 M Hz; 1 H NMR spectra were recorded on a Bruker AC 100 spectrometer and a Bruker AM-400 spectrometer, respectively (M eSi as internal standard). All coupling constants are given as in Hz, unless indicated otherwise. IR Spectra were recorded on Perkin-Elmer 298 IR spectrophotometer. For mass spectra a double focussing VG 7070E mass spectrometer was used. GC-M S Experiments were run on a Varian Saturn 2 GC-M S ion-trap system. Separation was carried out on a fused-silica capillary column (DB-5, 30 m x 0.25 mm). H elium was used as carrier gas, and electron impact (EI) was used as ionization method. GLC was conducted on a Carlo Erba 3040 GC using Merck Kieselgel 60. Thin layer chromatography (TLC) was carried out on glass plates coated with Merck Kieselgel 60H. Column chromatography at atmospheric pressure was carried out, using eluents indicated. Spots were visualized with UV or using specific spraying reagents.

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7-iodo-3-(4-methyl-5-oxo-2,5-dihydrofuruan-2-yl)oxy-methylene)-3,3a,4,8b-tetrahydroindeno[1,2-b]fururan-2-one 12a

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 concentrated in vacuo to give the crude formyl tricyclic lactone as a pale yellow solid. Starting 12a was removed by washing with diethyl ether (2x) to provide pure hydroxymethylene lactone as a tautomeric mixture in 50% yield (70%) (δ (CDCl3) 1H 3.65 (2H, d, J 7.5, 100 Hz), 3.25 (2H, d, J 7.5, 100 Hz), 1.78 (H, d, J 7.5, 100 Hz). The aqueous phase was separated and extracted with ethyl acetate (2x) and the combined organic layers were washed with saturated aqueous NaHCO3, dried (MgSO4) and concentrated in vacuo to give the crude 7-benzaldehyde 7 (513 mg, 100%) as a white solid. The crude product was purified by flash chromatography (SiO2, hexane-ethyl acetate 1:2) to provide 7-iodo GR 24 a 12a (190 mg, 27%) as a white solid. The product was then used as a precursor for the preparation of 7-amino GR 24 2a.

7-Azido-3-(4-methyl-5-oxo-2,5-dihydrofuruan-2-yl)oxy-methylene)-3,3a,4,8b-tetrahydroindeno[1,2-b]fururan-2-one 8

Sodium nitrate (33 mg, 0.48 mmol) was added to a solution of the mixture of the diastereoisomers of 7-azido GR 24 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 and the mixture was stirred 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 (2x) and the combined organic layers were washed with saturated aqueous NaHCO3 (1x), dried (MgSO4) and concentrated in vacuo to give the crude 7-azido GR 24 2a (183 mg, 57%) as a white solid. The product was used as a precursor for the preparation of 7-amino GR 24 2a.
(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, J = 2.5, 7-H); m/z 339 (M + H, 2%), 313 (1, C_{12}H_{13}NO_{3}), 214 (2, C_{6}H_{4}NO) and 97 (100, C_{4}H_{8}O). [Found (HR M S): m/z 339.0856. Calc. for C_{12}H_{13}NO_{3}: 339.0855].

Slow-moving diastereoisomer of 8, R_{p} 0.19 (hexane-ethyle 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 393 (M + H, 2%), 313 (1, C_{12}H_{13}NO_{3}), 214 (2, C_{6}H_{4}NO) and 97 (100, C_{4}H_{8}O). [Found (HR M S): m/z 393.0856. Calc. for C_{12}H_{13}NO_{3}: 393.0855].

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

A mixture of slow- and fast-moving 7-amino GR 24 2a (170 mg, 0.543 mmol) and dansyl chloride (36.6 mg, 0.320 mmol) and pyridine (10 ml). Work-up of the mixture was followed 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 GR 24 (concentrations of 1, 0.1, 0.01 and 0.001 mg l\(^{-1}\)) were prepared by serial dilution with water to form duplicated, and in each test the germination percentages were determined 12 disks. For full details of the bioassay, see ref. 27.

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References