Chemical Studies on Germination Stimulants for Seeds of the Parasitic Weeds *Striga* and *Orobanche*

Een wetenschappelijke proeve op het gebied van
de Natuurwetenschappen, Wiskunde en Informatica

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"Si non fecundas vertentes vomere glebas
terraique solum subigentes cimus ad ortus"
Lucretius, De Rerum Natura, Liber V, 210.

"Als wij de vruchtbare kluiten niet keeren gaan
met de ploegschaar, en omleggend het bouwland,
de zaadkiemen prikk'len tot groeien, kunnen zij
niet uit zichzelf naar den klaren hemel zich tillen"

Aan mijn ouders

Paranimfen

Linda Ballijns

Sander Hornes

Voorwoord

"Als wij de vruchtbare kluiten niet keeren gaan met de ploegschaar, en omleggend het bouwland, de zaadkiemen prikk'len tot groeien, kunnen zij niet uit zichzelf naar den klaren hemel zich tillen." Dit citaat van Lucretius is zowel van toepassing op het onderwerp van dit proefschrift, de parasitaire onkruidzaden als op mijzelf: zonder stimulans komen zij niet tot bloei. Daarom wil ik hier aan aantal mensen bedanken, zonder wie dit proefschrift niet geschreven had kunnen worden. Ten eerste professor Zwanenburg, voor de kansen die hij mij heeft gegeven om mij te ontwikkelen op wetenschappelijk en persoonlijk gebied. Ten tweede Jan Willem Thuring, mijn voorganger op het *Striga* project. Jouw belangstelling en betrokkenheid bij het onderzoek waren van onschatbare waarde. I owe a special word of thanks to Yukihiro Sugimoto for achieving the synthesis of sorgolactone. I am most grateful for the pleasant collaboration and I feel privileged that these important synthetic results can be included in my thesis.

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Contents

1

Introduction

Background

Semiochemicals are biologically active molecules that are used to disseminate information between individuals. The word is derived from the Greek 'semio' = sign and is synonymous with signal substances. Semiochemicals are divided in two groups: pheromones, that are used for communication between individuals within the same species, and allelochemicals, which are responsible for communication among individuals belonging to different species.[1,2] Several hundred different organic compounds released from animals, plants, insects, and microbes are known to affect the growth, development and distribution of the receiving organisms.[3,4]

Important examples of plants that have become completely dependent on allelochemicals are the parasitic weeds witchweed (*Striga* spp., Scrophulariaceae) and broomrape (*Orobanche* spp., Orobanchaceae).[5,6,7,8] The seeds of these weeds only germinate in response to specific chemicals (germination stimulants) present in the rhizosphere of host plants and some non-host plants.[9,10] For these parasitic flowering plants, which are totally dependent on a specific association with a host that provides them with nutrients and water, this system ensures that germination only starts when suitable host roots are available in the immediate vicinity. Other allelochemicals are required to effect attachment of the germinated seeds to the roots of a host plant via a specialized organ, the haustorium. Once a parasitic weed is attached to a host root, the vascular connections between host and parasite obviate any further need for host-parasite communication via chemical signals exuded into the soil.

Host plants for *Striga* and *Orobanche* include many agriculturally important crops. *Striga hermonthica* (Del.) Benth and *Striga asiatica* (L.) Kuntze are among the economically most devastating parasites, which preferentially attack monocotyledonous crops like maize, sorghum, millet and rice in tropical and subtropical areas in Africa and Asia (especially India). Another important species, *Striga gesnerioides* (Willd.) Vatke mainly parasitizes on

the legume crop cowpea and is widely distributed in Africa. The most common *Orobanche* species, *viz. O. crenata* Forsk and *O. aegyptiaca* Pers, predominantly occur in the Mediterranean area, Eastern Europe and the Middle East. *O. crenata* has a rather narrow host range from which faba bean is the most seriously affected, whereas *O. aegyptiaca* has a wide host range, including several dicotyledonous crops such as tomato, tobacco and sunflower.

Striga and *Orobanche* parasitism often results in a significant yield damage; yield losses of cereals of 50-90% have been reported.[11] Each *Striga* and *Orobanche* plant produces thousands of tiny seeds, that can remain viable in the soil for may years[12]; this results in a dramatic increase of the parasite seedbank. Levels of infestation are frequently so great, that sufficient cereal production becomes impossible and farmers abandon these fields in search of less infested areas. In West Africa, it was estimated that about 40 million hectares in cereal production are severely infested by *Striga* spp., while nearly 70 million hectares have moderate levels of infestation.[13] As a result, the Food and Agriculture Organization of the United Nations (FAO) estimated that annual yield losses due to *Striga* spp. in the savannah regions alone account for US $ 700 billion and are detrimental to the lives of over 100 million African people.[14] Also in the USA, *Striga* infestations have occurred, in North and South Carolina in the 1950's, and even though an extensive quarantine and eradication program was successfully implemented, the problem was very persistent.[15] Recently, it was reported that *Orobanche* infestations have spread to tobacco crops in Germany and France.[16] In Eastern Europe, predominantly sunflower production is threatened by *Orobanche* parasitism.[16]

Parasitic Weed Control

Finding adequate methods to control parasitic weeds has been the subject of extensive research and several reviews have been published on this topic.[11,17,18,19,20,21] The most important control methods are briefly discussed below:

Hand weeding: Especially in fields with a low infestation level, this is an effective method to control *Striga* and *Orobanche*. The removal of mature plants prevents the increase of the parasitic weed seedbank. However, when the parasite emerges from the soil, most of the damage to the host crop has already occurred. Furthermore, hand weeding is a very labour intensive method, and therefore not very practical. Nevertheless, in poor agricultural areas, this is the only control method in use, as more sophisticated techniques are out of reach for financial reasons.

Agricultural measures: Several possibilities for controlling parasitic weeds using strict agricultural regimes can be envisaged. *Crop rotation* involves growing a susceptible crop one year, followed by a fallow period or the cultivation of crops that are not susceptible to *Striga* or *Orobanche*. *Catch crops,* that induce the germination of parasitic weed seeds and are also parasitized, are used to reduce the number of *Striga* seeds in the soil. Also *trap crops*, that are crops which stimulate the germination of *Striga* and *Orobanche* seeds, but are not attacked

themselves (*e.g.* cotton), are used for this purpose. The last three mentioned techniques all have a negative effect on the agricultural production of staple food, which is often highly undesirable. The growth of the human population and the increasing demand for production of food led to intensified use of land, with more monocropping and little or no fallow, which holds the danger of enlarging the numbers of parasite seeds in the soil of infested areas.

Resistant crops: Breeding of resistant crops by the conventional selection techniques requires long term investments. It takes several years before a resistant crop can be introduced for general use by the farmers. Although it is quite difficult, this approach has good promise and recently some success has been achieved.[22,23] Unfortunately, the problem with most resistant varieties is that they are not as high yielding as the original crops. In addition, there is a risk of breakdown of resistance with the result that these species may suffer from parasitic weed attack in the same way as susceptible crops after a certain period of time.[24] Genetic manipulation has received only little attention so far; it is particularly applied to introduce genes that confer resistance to various herbicides in the host crop, and not to make them resistant to the parasitic weeds themselves.[25,26]

Biological Control: Biological control of *Striga* in particular, has mainly focused on weevils (*Smicronyx* spp.) and phytopathogenic fungi (*Fusarium* spp.). *Smicronyx* forms galls in the fruits of *Striga* and in West Africa it has been shown, that seed production of *S. hermonthica* can be reduced by more than 80% due to gall formation.[19,27] Usually, reductions are however much lower.[28,29] It has been estimated that more than 95% of the seeds needs to be destroyed to achieve a significant reduction of the *Striga* population. Therefore, *Smicronyx* alone will not be capable of a high degree of control.[30] *Fusarium* spp. attack both *Striga* seeds and parasitic plants, whereas they do not affect the cereal hosts. Large reductions in *Striga* emergence have been observed after inoculation of the soil with these fungi.[31,32] The main problem associated with the use of *Fusarium* spp. as biological control agents is their ability to produce mycotoxins affecting humans.[33]

Chemical Control: Several herbicides and in particular combinations of herbicides have been shown to give good control of parasitic weeds in some cases.[19,34,35] Dicamba (3,6-dichloro-2-methoxy-benzoic acid) and 2,4-D (2,4-dichlorophenoxy-acetic acid) as well as Round-up (glyphosate, *N*-(phosphoromethyl)glycine) are widely used to control *Striga* and *Orobanche*. These agrochemicals are usually applied after emergence of the parasitic weed, and at that stage of the parasitic process substantial damage to the host crop has already occurred. A disadvantage of these compounds is that they are also, to a certain extent, harmful to the host crop. Hence, the application of the aforementioned herbicides requires a strict timing, as well as controlled use of fertilizer. Their main advantage is that they prevent the production of more parasitic weed seeds, which could lead to a significant decrease in the dispersal of the parasite at the long run.

Recently, the use of sulfonylureas and imidazolinone herbicides has received much attention in the control of *Striga* and *Orobanche*.[36,37] In addition to pre- and post emergence treatment of parasitized crops, imidazolinones can be applied to the seeds of the host crop, which results in good control of broomrape before attachment.[38] Imidazolinone herbicides specifically inhibit the biosynthesis of branched chain amino acids.[39] Many crops have been developed which are resistant to imidazolinones, due to metabolic detoxification of these compounds. This feature could make this group of herbicides very useful in parasitic weed control. In general, major restraints to the use of herbicides in the developing countries are their availability, their cost and the necessity for expensive equipment and technology to apply them. Furthermore, the use of systemic herbicides is not desirable from an environmental point of view. An integrated approach, that combines several control methods is the best way to tackle the problem of parasitic weeds.[21]

Suicidal germination: In contrast with the aforementioned non-specific herbicides, an alternative approach to control *Striga* and *Orobanche* infestations may be the use of allelochemicals that specifically induce the germination of parasitic weed seeds. In fact, the use of catch- and trap crops is based on the principle of untimely germination of parasite seeds. A (synthetic) germination stimulant, applied to the soil in the absence of the desired host crop, would induce suicidal germination of the *Striga* or *Orobanche* seeds, because the germinated seeds cannot survive without a suitable host. Thus, a considerable reduction of the amount of parasite seeds in the soil could be achieved.[40,41] This method should generally be applicable for all *Striga* and *Orobanche* infestations, provided a suitable germinating agent is available. The naturally occurring semiochemicals, that elicit the germination of parasitic weed seeds, would be an excellent starting point for the development of such a very specific herbicide.

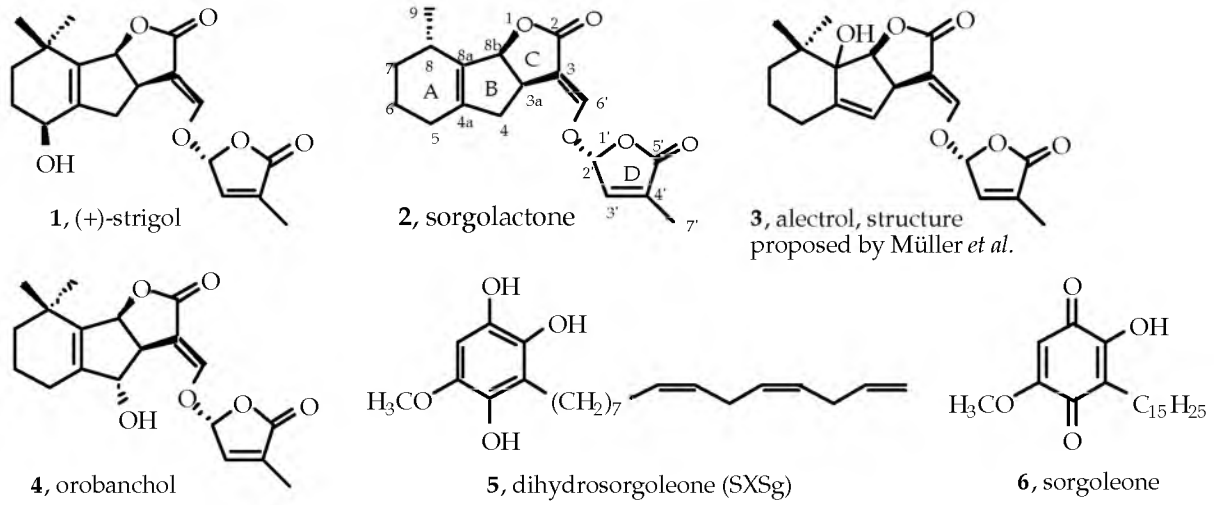
Naturally Occurring Germination Stimulants

Strigolactones

The first naturally occurring germination stimulant for *Striga* was isolated in 1966 from the root exudate of cotton *(Gossypium hirsutum* L.), which is not a host for *Striga* or *Orobanche*.[42] The gross structure of strigol (**1**) was elucidated in 1972 and its absolute stereochemistry was established by X-ray diffraction analysis in 1985.[43,44]

It was not until 1992 that sorgolactone (**2**), a compound with a structure similar to strigol, was isolated from the roots of a true host for *Striga,* sorghum *(Sorghum bicolor* L. Moench).[45] Soon thereafter, the same authors reported the isolation of a germination stimulant, alectrol (**3**) that was obtained from the root exudate of cowpea *(Vigna unguiculata)*, which is a host for *S. gesnerioides*.[46] Strigol itself was shown to be the major *Striga* germination stimulant produced by maize (*Zea mays* L.) and proso millet (*Panicum miliaceum* L.).[47] The collective name 'strigolactones' was proposed for this class of molecules.[8]

Figure 1



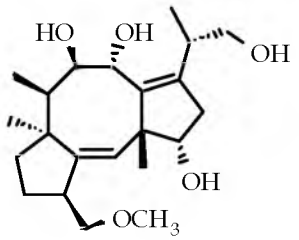
In addition, it has been demonstrated that root exudates of *Striga* hosts generally contain a mixture of these strigolactones, albeit in different ratios.[47] Recently, orobanchol (**4**), which is also a strigolactone, has been isolated from the *Orobanche* host red clover *(Trifolium pratense)*.[48]

Other germination stimulants

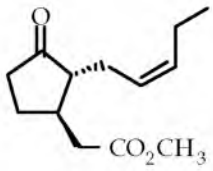
Strigol was isolated from a non-host for *Striga* and consequently its significance in the host parasite interaction was uncertain for a long time. In 1986, dihydrosorgoleone (**5**), which is also named SXSg, has been claimed as the actual stimulant exuded by sorghum roots.[49] Fate *et al.* suggested that the ready oxidation of **5** to the corresponding quinone, sorgoleone (**6**), explains the distance dependence observed in the germination of *Striga* seeds.[50] Sorgoleone, which constitutes >90% of sorghum root exudate[51], exhibits no germinating activity, but several papers have appeared on the phytotoxicity of this allelochemical.[52,53,54,55] It was shown that **6** is a selective natural herbicide, inhibiting photosynthetic electron transport, thus reducing growth of broadleaf and grass weeds. Notably, neither SXSg (**5**) nor sorgoleone (**6**) was ever detected in the root exudate of maize, which is highly susceptible to *Striga*.[56] An extended discussion on the role of SXSg in the inception of *Striga* seed germination is given in chapter 2.

Ethene, a known plant growth regulator, is able to stimulate the germination of *S. asiatica* and *S. hermonthica*.[57,58] In fact, ethene gas has been used to eradicate *S. asiatica* in the USA.[15] For this purpose, ethene was injected into the soil under high pressure using highly specialized equipment, which prevents the application of this control method in the developing countries.

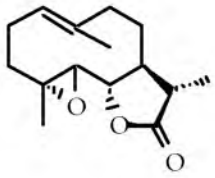
Figure 2. Structures of natural compounds that are claimed to induce germination of Striga and Orobanche spp. Between brackets are the concentrations of these compounds at which germinating activity towards S. hermonthica was observed.



7, fusicoccin deacetyl aglycone (10^{-4}, 10^{-5}M)



8, methyl jasmonate (10^{-3}-10^{-4}M)



9, 11β,13-dihydroparthenolide (10^{-2}-10^{-21}M)

The precursor of the biosynthesis of ethene, *viz.* 1-aminocyclopropane-1-carboxylic acid (ACC), also induces germination of certain *Striga* species.[59] It has been suggested that strigol and its analogues may stimulate *Striga* seed germination by eliciting the biosynthesis of ethene.[57,58,60,61] However, from recent experiments it was concluded that the synthetic strigol analogue GR 24 exerts its effect prior to ACC in the biochemical cascade leading to ethene production. Furthermore, a concerted mode of action of ethene and GR 24 in the germination of *S. hermonthica* seeds was suggested.[62,63] The oxidation of endogenous ACC to ethene might also be an explanation for the germination stimulating activity of some oxidizing agents such as sulfuric acid and sodium hypochlorite.[64,65] *Orobanche* on the other hand, is not responsive to ethene.[66]

Several reports have appeared claiming that seed germination of *Striga* and *Orobanche* is induced by other natural compounds including sesquiterpene lactones (which are not strigolactones), cytokinins, auxins, giberellins, cotylenins, fusicoccins and jasmonates (Figure 2).[12,67,68,69,70,71] It is important to note that, in general, these other germination stimulants exert their effect at much higher concentrations than the strigolactones and thus have a much less specific mode of action. In addition, the results of the germination assays conducted with these compounds are not always unambiguous, *e.g.* the activity of 11β, 13-dihydroparthenolide (**9**) as claimed by Fischer *et al.*[67] and Rugutt & Rugutt[69] was not found when this compound was tested under the standardized bioassay conditions developed by Mangnus *et al.*[72]

Synthetic germination stimulants

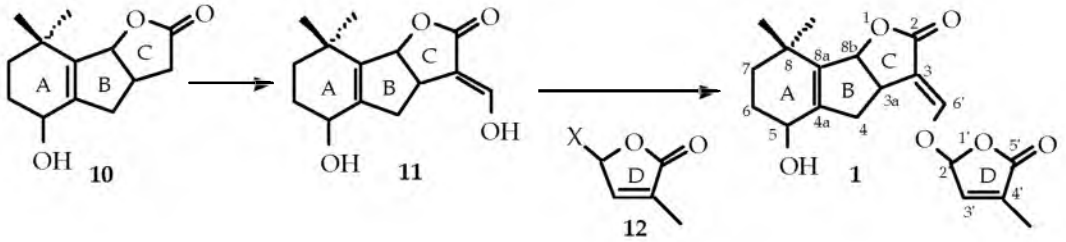
Total synthesis of naturally occurring strigolactones

The first naturally occurring strigolactone that was discovered, (+)-strigol (**1**), has received much attention in synthetic organic chemistry. Seven total syntheses have been reported so far.[73,74,75,76,77,78,79,80] In all of these synthetic routes, the final step involves the coupling of the two key building blocks **10** and **12** as is depicted in scheme 1. For this purpose tricyclic lactone **10** is formylated α to the lactone carbonyl group and the resulting hydroxymethylene

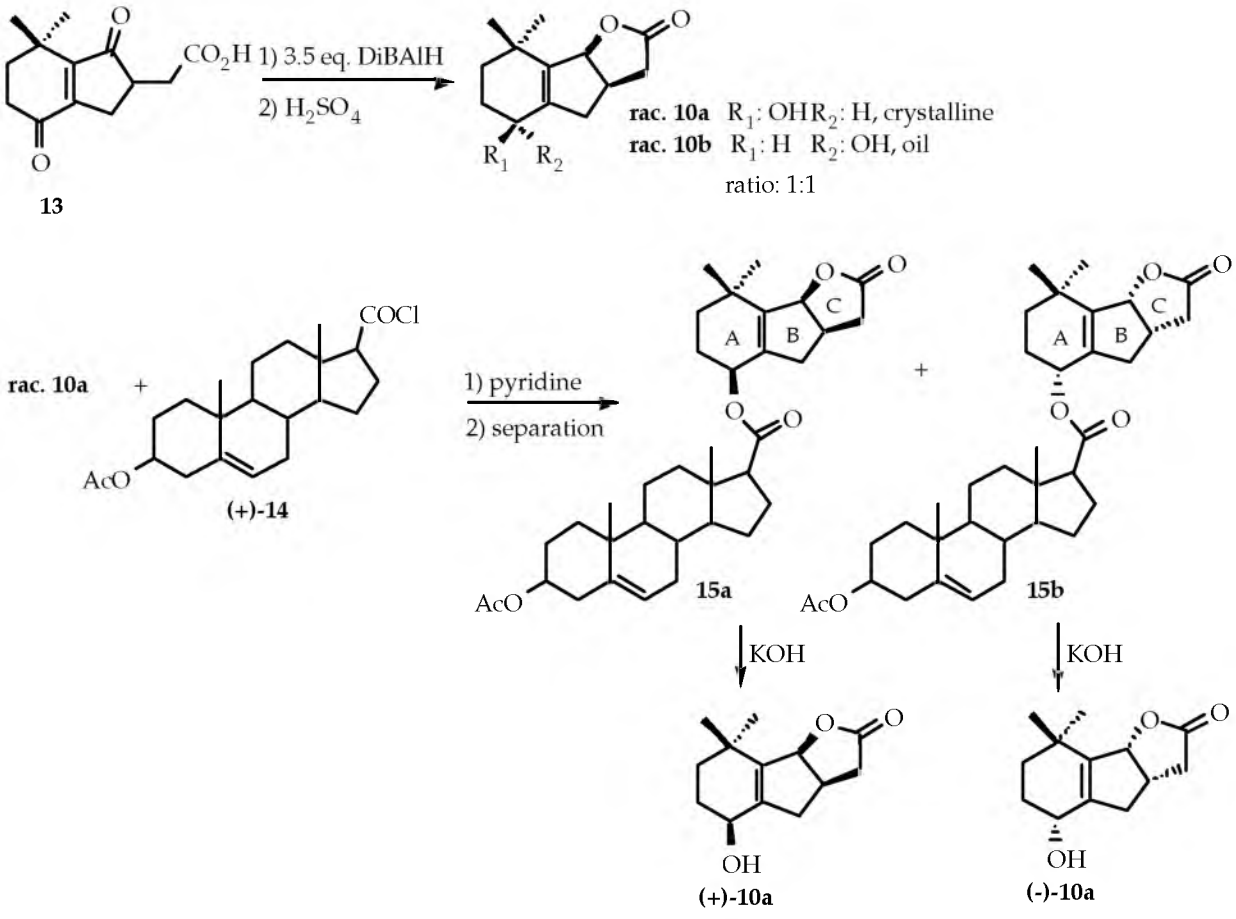
compound **11** is coupled with butenolide **12**, with concurrent expulsion of the leaving group X, which is usually a bromide. The resulting product has the desired *E*-geometry of the enol ether olefinic bond as has been shown by ^{1}H NMR analysis.[75,76] The first four synthetic routes to strigol were adequately reviewed by Dailey *et al.*[81]

Strigol effectively contains three stereogenic centers, *viz.* C3a/C8b, C5 and C2', and can therefore exist in eight stereoisomeric forms. Thus far, the synthesis of four separate stereoisomers has been disclosed. Introduction of the hydroxy moiety in the ABC-part resulted in the formation of two pairs of racemic ABC-diastereomers, that could be separated by crystallisation. Coupling of diastereomerically pure ABC-lactone with the D-ring (**12**) consequently leads to the formation of two pairs of racemic diastereomers of strigol.

Scheme 1.



Scheme 2

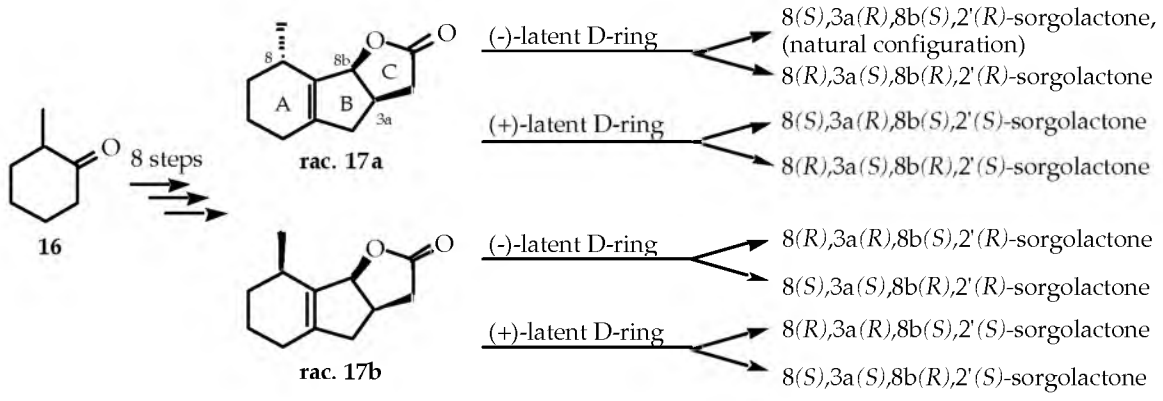


Enantiopure stereoisomers of strigol were prepared by resolution of diastereomerically pure racemic ABC-hydroxy lactone **rac. 10a** with a resolving agent (Scheme 2).[74] Coupling of the thus obtained optically pure ABC-part **(+)-10a** with **12** gave a mixture of (+)-strigol (**1**) and the corresponding C2' epimer, (+)-2'-epistrigol, that could be separated by column chromatography to give these two diastereoisomers in enantiopure form. Similarly, **(-)-10a** was converted into (-)-strigol and (-)-2'-epistrigol. In theory, resolution of **rac. 10b** in the same manner would give access to the remaining four strigol stereoisomers; however this work has not been reported. An alternative approach to obtain **(+)-10a** and **(-)-10a** was followed by Samson *et al.*, who employed chiral chromatography over cellulose triacetate to separate **rac. 10a** in its enantiomers.[80]

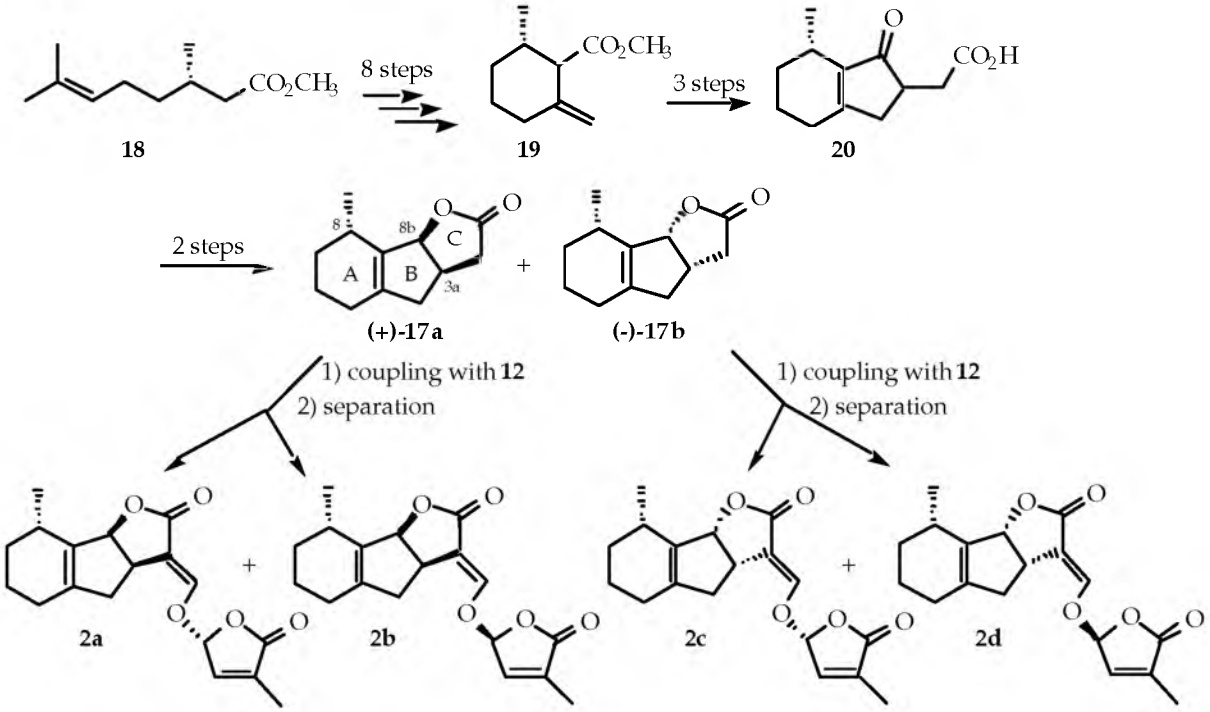
The synthesis of sorgolactone (**2**) was accomplished in 1998 in the Nijmegen research group. This synthetic effort, which is described in chapter 3 of this thesis, involved the preparation of all eight possible diastereomers of this germination stimulant as single stereoisomers.[82,83] The strategy involved the stereoselective coupling of two enantiopure D-ring precursors with the diastereomerically pure racemic sorgolactone ABC-parts **rac. 17a** and **rac. 17b**, respectively (Scheme 3). In this manner, four diastereomeric mixtures of sorgolactone precursors were obtained, that could be separated over silicagel. Deprotection then furnished the eight single stereoisomers of sorgolactone. The proposed structure for the natural germination stimulant was confirmed by this total synthesis.

At approximately the same time, Mori *et al.* described the synthesis of the four racemic diastereoisomers of sorgolactone, employing a similar synthetic strategy, although the final coupling step was the conventional reaction with bromobutenolide (see scheme 1), leading to racemic sorgolactone mixtures.[84] In addition, Mori and Matsui reported the preparation of four enantiopure sorgolactone stereoisomers.[85] The use of methyl *(S)*-(-)-citronellate (**18**) as a chiral starting material secured the correct stereochemistry at C8, and consequently only two ABC-lactones *viz.* **(+)-17a** and **(-)-17b**, were obtained, both in optically pure form (Scheme 4). Reaction with racemic bromobutenolide, followed by chromatography, furnished the naturally occurring sorgolactone and three of its stereoisomers, all having the *S* configuration at C8.

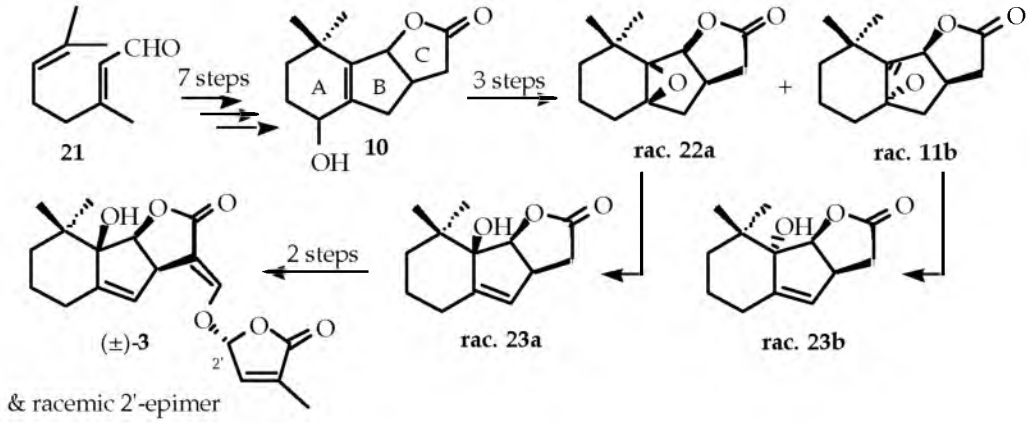
Scheme 3



Scheme 4

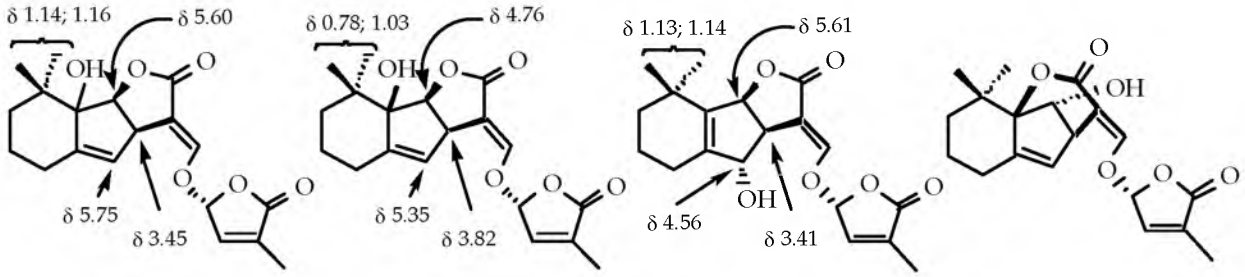


Scheme 5



The structure proposed for the third naturally occurring strigolactone, alectrol (**3**), was synthesized by the Mori group in racemic form starting from citral (**21**), via the strigol ABC-lactone (**10**), that was subsequently dehydroxylated and epoxidized.[86] Reaction of the epoxides **rac. 22a** and **rac. 22b** with aluminium isopropoxide afforded the desired alectrol ABC-lactones **rac. 23a** and **rac. 23b**, respectively (Scheme 5). Formylation of hydroxylactone **rac. 23a** followed by coupling with bromobutenolide **12**, resulted in the formation of two pairs of racemic diastereomers of alectrol, *viz.* (±)-**3** and its C2' epimer. Subsequent ^{1}H NMR analysis of this material revealed that several chemical shift values differed significantly from those reported for the naturally occurring compound (Figure 3).[46] The Japanese group concluded that the structure proposed for alectrol was not correct.

Figure 3



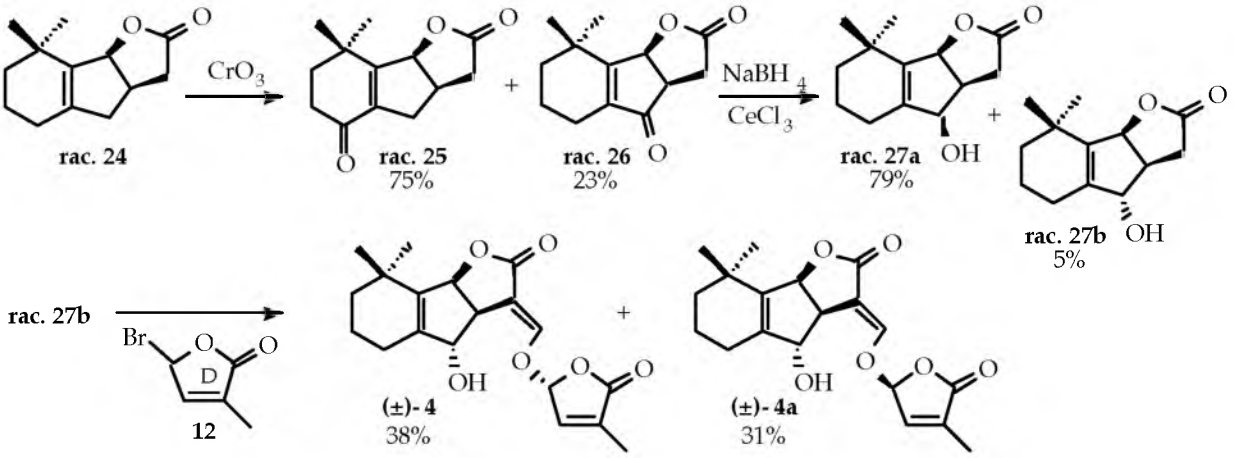
3, alectrol, structure originally proposed by Müller *et al.* , 1992.

(±)-3, structure synthesized by Mori *et al.*, 1998.; not the natural product.

(±)-4, alternative structure for alectrol (Mori et al., 1998); not the natural product.

28, newly proposed structure for alectrol

Scheme 6



An alternative structure, *viz.* (±)-**4**, was synthesized[86,87] (Scheme 6), but its spectral data were also not in accordance with the ^{1}H NMR spectrum of the natural germinating agent from *Vigna unguiculata*. (Figure 3). In a later stage, this alternative structure proved to be identical with that of orobanchol (**4**), the germination stimulant isolated from *Trifolium pratense*, a host for *Orobanche minor*.[48,87]

The results of Mori *et al.*, summarized in figure 3, imply that the true structure of alectrol is not known. On the basis of these NMR data and the original spectral data of naturally occurring alectrol, structure **28** for the germination stimulant isolated from cowpea is proposed here (Figure 3). In this structure, the tertiary alcohol in the structure originally proposed for alectrol (**3**) is now part of the C-ring lactone. It is suggested, that the stereochemistry of the CD-part of **28** is analogous to that of strigol and sorgolactone, and that the carbon atom attached to the secondary OH has the sterically least congested configuration.

Biological activity of naturally occurring strigolactones

The activity of (+)-strigol as a seed germination stimulant toward *Striga* species is very high. Half maximal effects at concentrations of 10^{-11}-10^{-10} M have been reported.[45,46,88] Interestingly, seeds of the related root parasite *Alectra vogelii* are more responsive to (-)-strigol. (+)-Strigol concentrations that induce half maximal germination of *Orobanche* species are generally in the order of 10^{-8} M.[88,89] Bioassays of all eight stereoisomers of sorgolactone revealed that the naturally occurring stereoisomer was indeed the most active one in the stimulation of *S. hermonthica* and *O. crenata*.[83] Half maximal activity toward *S. hermonthica* and *S. asiatica* was induced by concentrations of 10^{-10} M and 10^{-8} M, respectively.[83,90,91] For *O. crenata* half maximal effects were observed at 10^{-7} M; see also chapter 3 and 4.

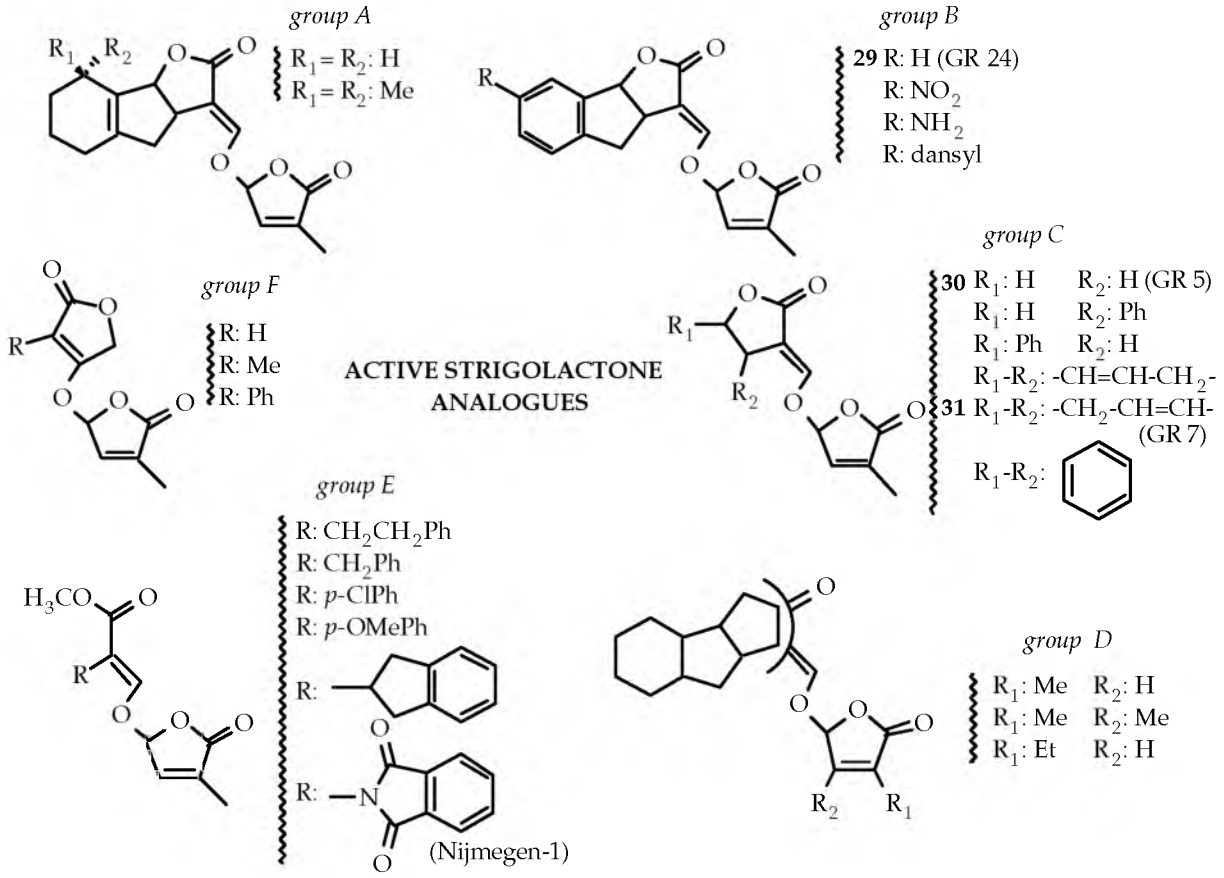
Bioassays with racemic mixtures of the sorgolactone stereoisomers led to the conclusion that the racemate of the naturally occurring stereoisomer was not the most active one in the case of *Orobanche minor*, but the racemic mixture of its C2' epimer.[84] For alectrol, only bioassay data for the isolated natural compound are available. These show that the stimulant from *Vigna unguiculata* was very active in germinating seeds of *Alectra vogelii* and *Striga gesnerioides* at concentrations in the order of 10^{-11}-10^{-10} M. Seeds of *S. asiatica, S. hermonthica* and *O. aegyptiaca* also germinated in response to alectrol, albeit at somewhat higher concentrations.[46] For orobanchol (**4**), bioassay data have not yet been reported.

Synthesis and biological activity of strigolactone analogues

Since the elucidation of the structure of strigol, much effort has been put in the synthesis of analogues, with the aim to obtain a simplified structure that might be used to induce suicidal germination of parasitic weed seeds (among others, Johnson *et al.*[40,92]; Pepperman *et al.*[93], Mangnus[94], Zwanenburg *et al.*[95,96,97]). A summary of all active analogues is given in figure 4 and a similar representation of inactive synthetic strigolactone analogues is shown in figure 5.

The group *A* of active analogues (Figure 4) contains compounds with a relatively small variation in the substituents of the A-ring, when compared with the naturally occurring stimulants strigol and sorgolactone. In group *B*, the typical feature is the aromatic A-ring. The A-ring is lacking in group *C*. Some members of this group, *e.g.* GR 7 (**31**) still contain the B-ring, while others e.g. GR 5 (**30**), lack both the A- and B-ring. In the three groups *A*, *B* and *C* the butenolide D-ring is still present. In group *D*, various substituted D-rings are included. It should be emphasized, that the bioactivity is strongly dependent on the nature of the substitution pattern in the D-ring, whereby the natural D-ring has the optimal activity for the various ABC-units in this test series of stimulants. Group *E* contains an α,β–unsaturated ester as the ABC-unit with various substituents at the olefinic bond. Among these stimulants is also Nijmegen-1.

Figure 4

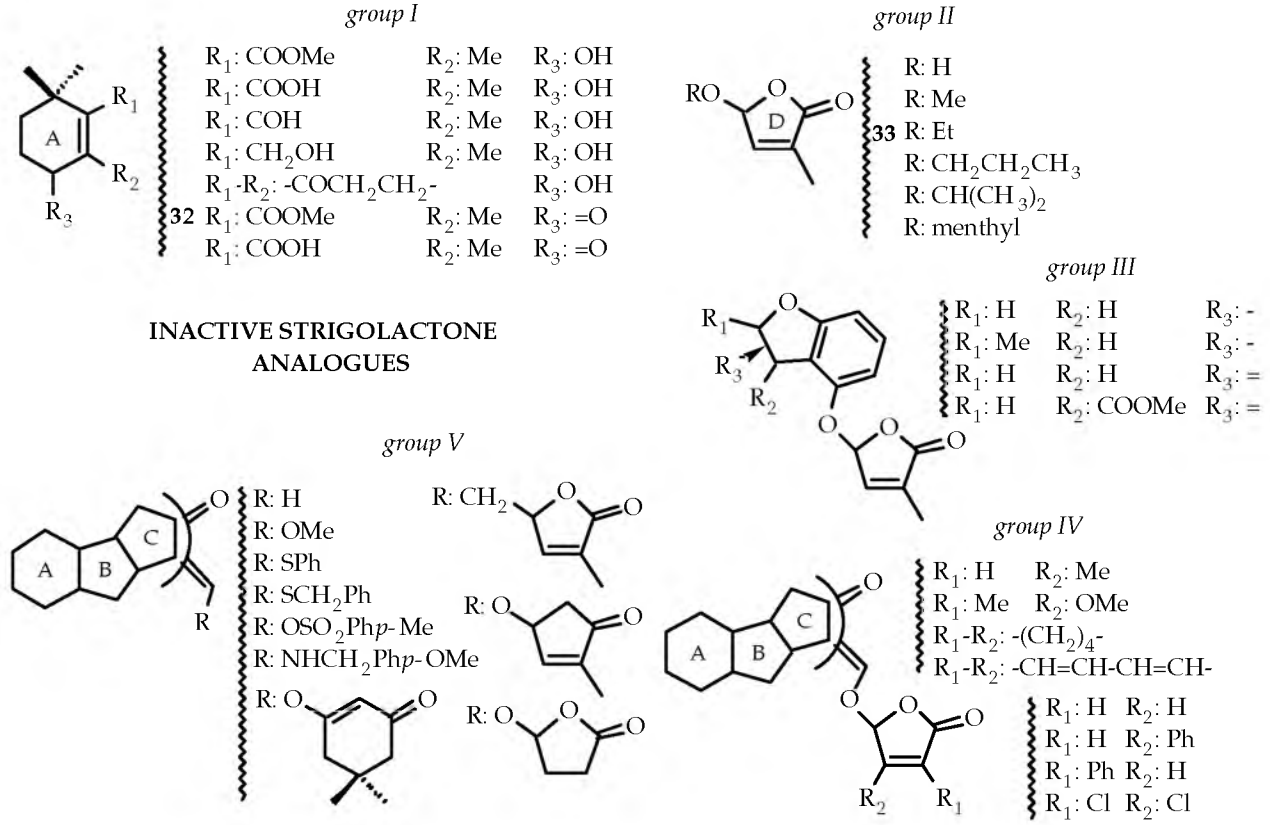


In group *F* the ABC-unit is replaced by a butenolide group, which is derived from tetronic acid. All these stimulants contain the enol ether moiety connecting the ABC-part with the D-ring.

In the inactive series of analogues (Figure 5), group *I* is derived from the A-ring, in all cases the D-ring is lacking. In group *II,* the D-ring is taken as the basis, however the enol ether moiety as well as the ABC-unit is absent.[98] The annelated phenol group is taken as the ABC-unit in group *III*. In group *IV,* an ABC-unit is combined with a D-ring having a strongly deviant substitution pattern. These compounds are inactive, demonstrating that the substituents on the D-ring have a profound effect on the bioactivity. The compounds in group *V* have in common a modified structure of the substituent at the olefinic bond.

The development of a standardized bioassay provided an important tool to obtain reliable biological activity data.[72] Until then, inconsistencies in the bioactivities of strigol analogues were frequently encountered in the literature, for example A-ring analogue **32** was first reported to be active, however this result could not be reproduced.[93] Especially the use of dimethyl sulfoxide (DMSO) as a co-solvent to prepare the aqueous stimulant solutions should be avoided, as it leads to unreliable bioassays. The reason probably is that DMSO may cause the oxidation of endogenous ACC to ethene *(vide supra)* which may exert synergistic effects with the stimulant under investigation. Furthermore, DMSO may influence membrane permeability, which might also affect the results of the bioassay.[72,93]

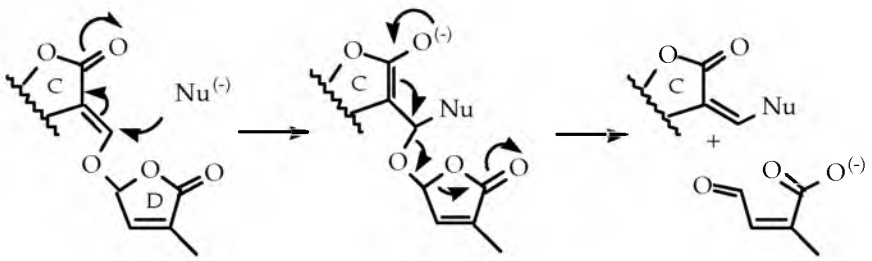
Figure 5



Another important aspect is the use of the very potent synthetic germination stimulant GR 24 (**29**) as a positive control, which enables the comparison of results obtained in different test series. An analysis of these results regarding the structure-activity relationship, allowed the identification of the bioactiphore, *i.e.* the part of the strigol molecule which is primarily responsible for biological activity.[94,99] It appeared that the CD-part of the molecule including the enol ether linkage are the minimum structural requirements for biological activity. Mangnus & Zwanenburg devised a tentative molecular mechanism for the initial triggering of the germination of parasitic weeds seeds (Scheme 7).[99] This receptor mediated mechanism involves addition of a nucleophilic species, present at the receptor site, in a Michael fashion, followed by elimination of the D-ring. The ultimate result is that the ABC-part of the stimulant is covalently bound to the receptor, a chemical change that may be responsible for triggering germination.

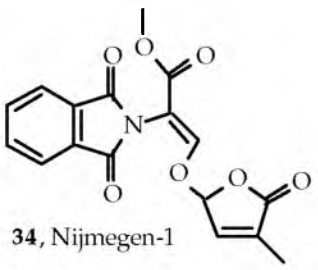
Support for this mechanism was derived from the fact that replacement of the vinyl ether linkage by a single bond resulted in complete loss of activity. Substitution of the vinyl ether oxygen by a carbon atom also led to an inactive molecule, due to the fact that the D-ring can no longer function as a leaving group.[100] In addition, reactions with some nucleophiles, such as benzenethiolate and *p*-methoxybenzyl amine led to displacement of the D-ring by the nucleophile. Further structure-activity studies showed that the presence of the D-ring is essential for biological activity; it can not be replaced by another leaving group.[89,99]

Scheme 7



The molecular mechanism for the induction of germination of parasitic weed seeds, was used to design new simplified strigol analogues, *e.g.* Nijmegen-1 (Figure 6.). This molecule contains the essential structural features required for bioactivity, *viz.* the D-ring, the enol ether linkage and the α,β-unsaturated system of ring C.[101] Nijmegen-1 can easily be prepared in multigram quantities and is an attractive candidate for parasitic weed control by the suicidal germination approach, as is further elaborated on in chapter 4 of this thesis.[91]

Figure 6

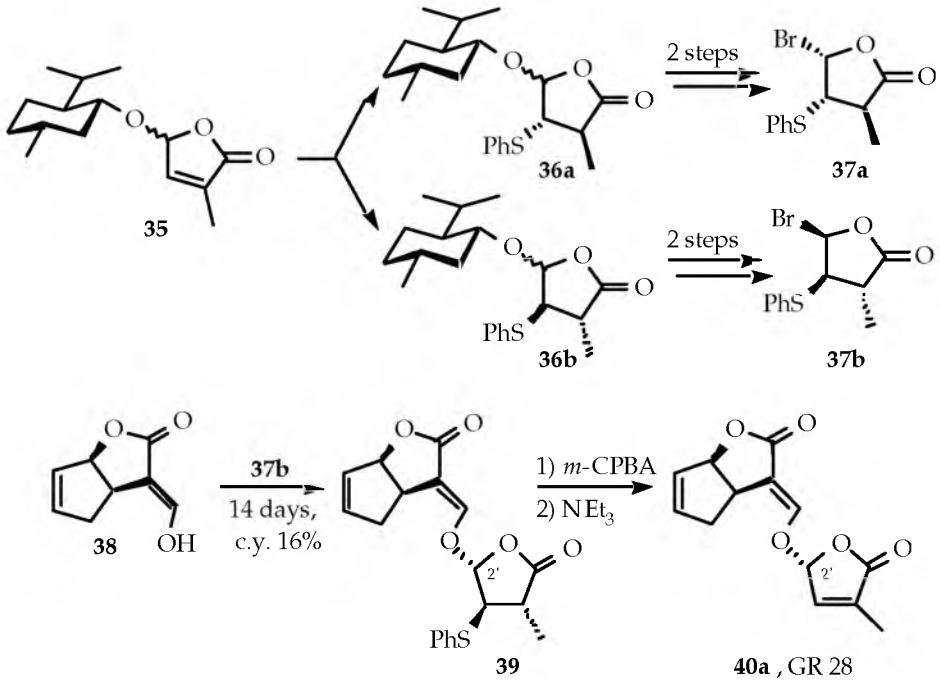


34, Nijmegen-1

Asymmetric synthesis of strigolactone analogues

Already at the time of the first total synthesis of strigol, the importance of stereochemistry in strigolactones was recognized.[74] Optically active strigol has been obtained by resolution of racemic strigol[44,88], by resolution of the ABC-part[74,80] and by asymmetric synthesis via the chiral pool approach.[79] The stereoselective introduction of the D-ring however, remained a problem for a long time.[102] The first synthetic approach that allows control of the C2' configuration was reported by Frischmuth *et al.*[103] This method involved Michael addition of thiophenol to menthyloxy butenolide **35**, analogous to the methodology developed by Feringa *et al.*[104] The addition proceeds *trans* to the menthyloxy group, but protonation of the intermediate anion is then almost stereo random. Therefore mixtures of adducts **36** are formed, which are difficult to separate and in addition, it is difficult to assign their stereostructures. However, it was possible to obtain bromo derivatives **37** and coupling with hydroxy lactone **38** then proceeded stereoselectively to give **39** in the case of D-ring precursor **37b** and its C2' epimer with **37a** (Scheme 8). Oxidative removal of the phenylsulphanyl group provided the desired GR 28 stereoisomers (**40**). It should be noted, that the assignment of the configuration at C2', relative to the ABC-part, cannot be performed by means of NMR spectroscopy.

Scheme 8

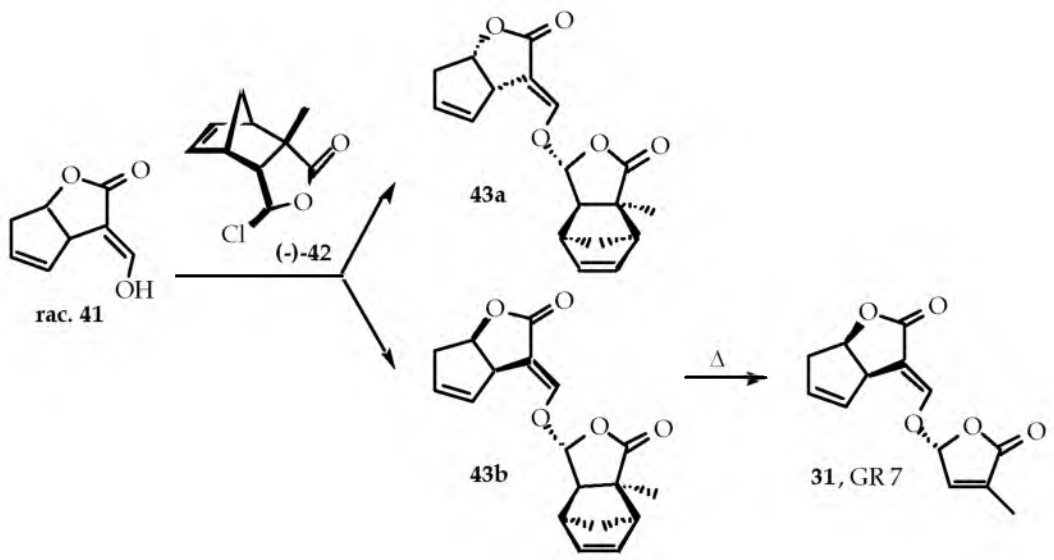


Circular dichroism however, turned out to be a reliable method for this assignment, thereby taking strigolactones, *e.g.* (+)-strigol (**1**), GR 28 (**40**) and GR 7 (**31**), of which the absolute configuration was determined by X-ray diffraction as references.[105] Two recent publications on the Michael addition/elimination approach *(vide supra)* did not elaborate on the scope of this method.[106,107]

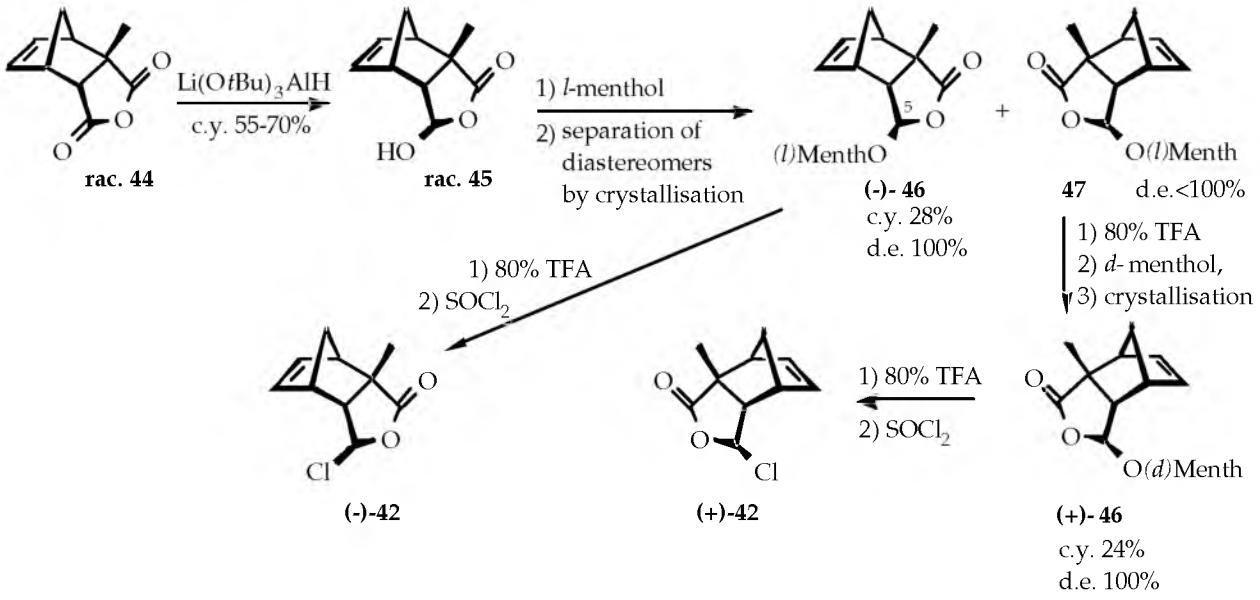
Superior methodology for the stereoselective introduction of the D-ring was described by Thuring *et al.*[108,109] Coupling of an ABC-precursor with an enantiopure adduct of the strigolactone D-ring and cyclopentadiene *e.g.* **(-)-42** proceeds with complete exo-selectivity with respect to the norbornene bridge (Scheme 9). In the case of a racemic lactone, such as **rac. 41**, a mixture of two diastereomeric products is obtained, namely **43a** and **43b**. Separation of these diastereoisomers and subsequent retro Diels-Alder reaction, which also proceeds without racemization, furnishes the optically pure strigol analogues; *e.g.* GR 7 (**31**) was obtained from **43b**.[110] This method was also successfully employed in the stereoselective synthesis of enantiopure GR 24[111], desmethylsorgolactone[112] and Nijmegen-1.[101] This method will be elaborated on in chapter 3 for the synthesis of sorgolactone.[83]

Enantiopure latent D-ring synthons **(-)-42** and **(+)-42** were prepared as depicted in scheme 10.[109] The adduct of citraconic anhydride and cyclopentadiene, **rac. 44**, was regioselectively reduced to yield racemic hydroxy lactone **rac. 45**. Resolution of the *l*-menthol adduct of **rac. 45** was achieved by careful crystallisation. In this manner **(-)-46** could be obtained in 100% d.e. Removal of the chiral auxiliary, followed by chlorination afforded enantiopure latent D-ring precursor **(-)-42.** In order to obtain the precursor of **(+)-42** in diastereomerically pure form, **47** was converted to the *d*-menthol derivative **(+)-46**, which crystallized readily. This derivative **(+)-46** was then hydrolyzed and reacted with thionyl chloride to give chlorolactone **(+)-42**.

Scheme 9



Scheme 10

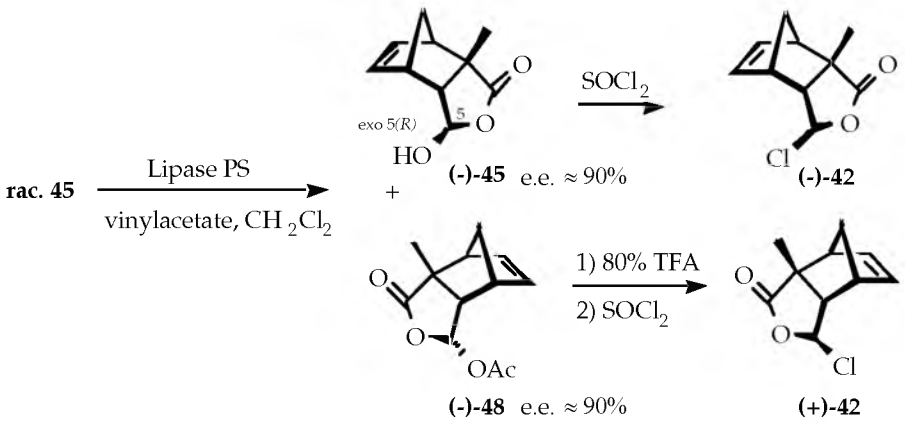


In a later stage, the resolution step was considerably improved by the use of Lipase PS (scheme 11).[113] The enzyme selectively acetylates the endo 5(*R*)-isomer of **45**, which is in equilibrium with exo 5(*S*)-isomer **(+)-45**, through mutarotation of the labile acetal bond. Exo 5(*R*)-isomer **(-)-45**, is not touched by the enzyme. In this manner, both **(-)-45** and **(-)-48** were obtained in high optical purity and they were easily converted to the homochiral D-ring synthons **(-)-42** and **(+)-42**, respectively. The methodology depicted in schemes 10 and 11 makes use of the concept of transient chirality, which in this case involves the conversion of achiral citraconic anhydride into a chiral compound by means of the cycloaddition with cyclopentadiene.[114] After synthetic elaboration of this adduct, the synthetic auxiliary, *i.e.*

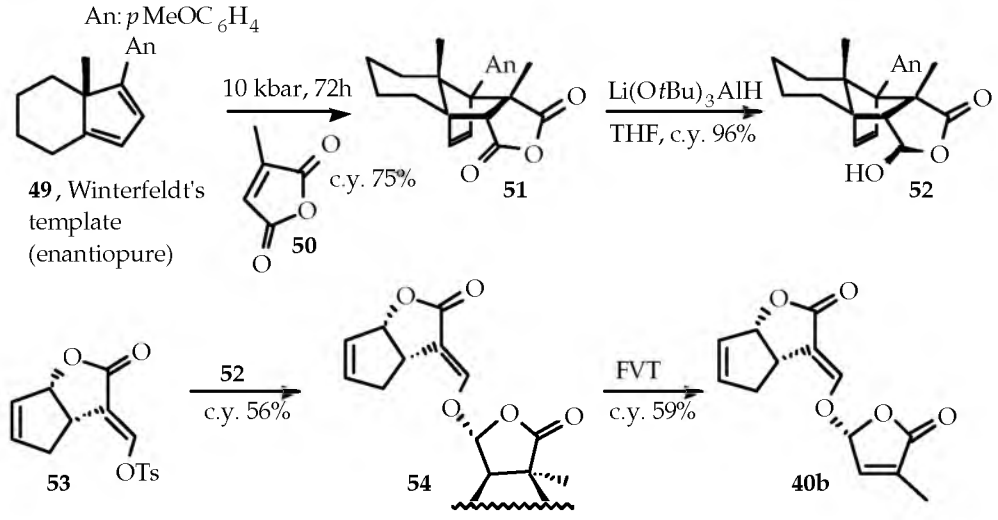
cyclopentadiene, is removed, leaving an optically active species, derived essentially from an achiral compound.

The concept of transient chirality to direct the stereochemistry of C2' in strigolactone analogues, was also employed by Welzel *et al.*, who used Winterfeldt's template (**49**) as a chiral auxiliary.[115] The Diels-Alder adduct of diene **49** and citraconic anhydride (**50**) was regioselectively reduced to the homochiral latent D-ring synthon **52**, which was subsequently coupled to lactone **53**. The chiral template was removed employing a thermal retro Diels-Alder reaction, analogous to the method of Zwanenburg & Thuring.[108,110] The difference between the two methods is that Winterfeldt's auxiliary is derived from a chiral substrate, *viz.* the Hajos-Wiechert ketone[116], whereas in the approach of the Nijmegen group, the D-ring precursor was synthesized from racemic anhydride **44**, allowing the synthesis of both C2' strigolactone epimers. Winterfeldt's template is only known as one stereoisomer. As a consequence Welzel *et al.* only could prepare strigol analogues with the C2'*(R)* configuration.

Scheme 11



Scheme 12



Outline of this thesis

In chapter 1 the background of the research is described; special attention is given to the stereoselective synthesis of strigolactones and their analogues. In chapter 2 a critical account on the inception of *Striga* seed germination is presented. It is shown that the mechanistic connection between SXSg (**5**) and the strigolactone D-ring, as was suggested by Lynn *et al.*[117] is not justified. This study supports the hypothesis, that the germination of *Striga* and *Orobanche* seeds proceeds via a receptor-mediated mechanism. Chapter 3 is devoted to the synthesis and biological activity of all eight stereoisomers of the naturally occurring germination stimulant sorgolactone (**2**). In Chapter 4, dose-response curves of sorgolactone and the synthetic analogues GR24 (**29**) and Nijmegen-1 (**34**) are presented. It was shown that both synthetic analogues show appreciable activity at low concentrations towards a variety of parasite seeds, and have good promise in the application as suicidal germination agents for parasitic weed control. Chapter 5 describes an expeditious synthesis of enantiopure strigolactone analogues. Separation of racemic germination stimulants by chiral HPLC over cellulose carbamate, allows the relatively fast collection of enough optically pure material for analysis and biological testing. All stereoisomers of 8-methyl GR 24, which is the aromatic analogue of sorgolactone, and its regioisomer 6-methyl GR 24 were synthesized in this manner. In the final chapter, the synthesis of all four stereoisomers of amino-GR 24 is described. The amino function was used for the attachment of various labels, that can be used to study the interaction between these strigolactone analogues and their hypothesized protein receptor. Preliminary results of tetramethyl rhodamine-labelled GR 24 and protein extracts of *S. hermonthica*, studied with Fluorescence Correlation Spectroscopy (FCS) and conventional fluorescence microscopy are described. This thesis is concluded with summaries in English and Dutch.

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2

A Critical Account on the Inception of *Striga* Seed Germination

Abstract The seeds of the parasitic weed *Striga* germinate in response to stimulants exuded by the roots of host plants and some non-host plants. Literature data are summarized that support the view that strigolactones induce germination of parasitic weed seeds via a receptor-mediated mechanism. The suggestion by Lynn *et al.* that the strigol D-ring is solely responsible for germinating *Striga* seeds via a redox reaction, was based on hypothesized structural similarities between the natural compound dihydrosorgoleone (SXSg) and the strigol D-ring. Experiments have shown that the mechanistic connection between SXSg and the strigol D-ring is not valid, and therefore the proposed redox mechanism for the induction of *Striga* seed germination by strigolactones does not hold.

Introduction

Parasitic weeds belonging to the genera *Striga* and *Orobanche* severely reduce the yields of economically important crops in tropical and semitropical areas of the eastern hemisphere and in the Mediterranean region. Germination of the weeds is induced by stimulants present in the root exudates of host plants and some non-host plants.[1,2,3] The first naturally occurring germination stimulant, (+)-strigol (**1**), was isolated in 1966 from the root exudate of cotton.[4] The significance of strigol in the host parasite interaction was uncertain for a long time, because cotton is not a host for *Striga* or *Orobanche*. A breakthrough was achieved when Hauck *et al.* identified sorgolactone (**2**), a structural analogue of strigol, as the major *Striga* germination stimulant produced by sorghum - a cereal crop which is severely affected by *Striga*. [5] At about the same time, these authors also reported the isolation of another germination stimulant, *viz.* alectrol (**3**), from the root exudate of cowpea (Figure 1).[6] Soon thereafter Siame *et al.* showed that strigol itself is the major germination stimulant produced by the *Striga* hosts maize and millet.[7]

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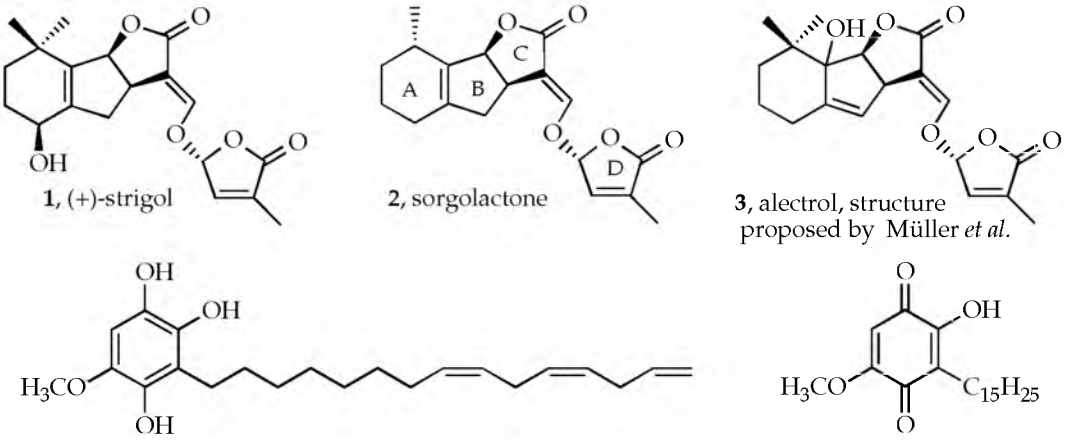
Figure 1

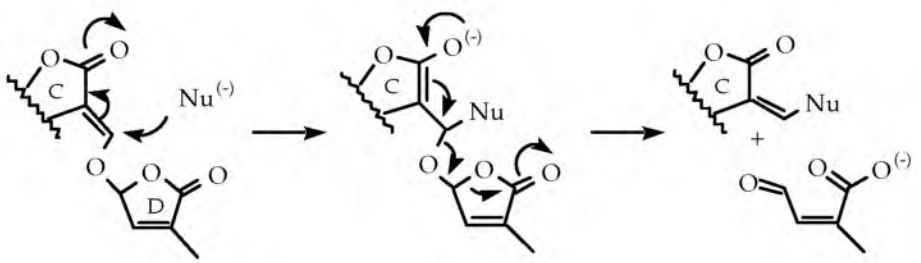


The collective name "strigolactones" was proposed for this class of compounds.[3] In addition, it has been demonstrated that root exudates of *Striga* hosts generally contain a mixture of these strigolactones, albeit in different ratios.[7]

Structure activity studies of a series of synthetic strigol analogues[8,9,10,11,12,13] revealed that the bioactiphore of the stimulants resides in the CD-part of the molecule and a tentative molecular mechanism for the induction of germination has been proposed.[9] This molecular mechanism involves addition of a nucleophilic species, present at the receptor site, in a Michael fashion, followed by elimination of the D-ring. The ultimate result is that the ABC-part of the stimulant is covalently bound to the receptor, a chemical change that may be responsible for triggering germination (Scheme 1).

Furthermore, the synthesis and biological evaluation of all possible diastereomers of sorgolactone[14,15,16] and its analogues DMSL[17], GR7[18] and GR 24[19] has clearly demonstrated the effect of the stereochemistry of the stimulant molecules on the biological activity. Only those isomers possessing the same absolute stereochemical configuration as the natural germinating agents exhibit high germination stimulatory activity. The concentration range in which strigolactones express half maximal germination inducing activity is generally in the order of 10^{-9} M. This concentration range and the critical dependence of the bioactivity on the absolute stereochemistry, are strong indications that the inception of germination of parasitic weed seeds is mediated by a receptor.

Scheme 1



In contrast, there is the explicit claim that the hydroquinone derivative, dihydrosorgoleone (**4**), also named SXSg, plays the key role during the inception of the seed germination process.[20] Moreover, Lynn and Boone make a connection between the functions of SXSg and strigolactones in the germination process.[21] In this chapter a critical account on the initial stages of *Striga* germination is given, with the aim to oppose the different views on this matter. The identity of the true stimulant is essential for the design of mimics with high potential to control these parasites selectively using the concept of 'suicidal germination'. This approach involves the application of a (synthetic) germination stimulant to the soil before planting the desired crop.[22,23] The *Striga* seeds will germinate, but in the absence of a suitable host plant they can not survive.

Results and Discussion

Prior to the identification of the strigolactones as the major stimulatory constituents produced by *Striga* hosts, Chang *et al.* suggested that dihydrosorgoleone (SXSg) (**4**) is the actual stimulant exuded from sorghum roots.[20] The authors attributed significance to SXSg because it is readily oxidized to the corresponding inactive quinone, sorgoleone (**5**). This might explain the distance dependence observed in the germination of *Striga* seeds. Only those seeds less than 5 mm away from a host root germinate and can subsequently attach themselves to the host plant. On the basis of experiments in agar, Chang *et al.* claim that strigol is too stable to explain this distance dependence.[20] It is relevant to mention however, that it was established that strigol and its synthetic analogues are short-lived in soil, presumably due to hydrolytic degradation.[24,25] Notably, neither SXSg nor sorgoleone was ever detected in the root exudate of maize, which is highly susceptible to *Striga*.[26]

Evidence has been accumulated, which demonstrates that strigolactones *(vide supra)* are the true stimulants for *Striga* germination. On the other hand, a series of articles continued to appear, elaborating on the presumed prominent role of SXSg in the germination process.[21,27,28,29,30] The concentration at which SXSg induces half maximal germination of *Striga asiatica* is reported to be 10^{-6} M. This is at least a thousand fold higher than the concentration range in which the strigolactones exhibit half maximal activity. Moreover, the variation in amounts of SXSg produced by different sorghum cultivars is at most 10 fold and shows little, if any, correlation between reported field resistance/susceptibility to *Striga* infection.[31] The variation in the amount of strigolactones exuded by these sorghum species on the other hand, is a billion fold: high producers are almost always susceptible to *Striga* infection whereas the lowest producers are invariably resistant.[31,32] In other words, there is a good correlation between strigolactone exudation and field susceptibility for *Striga* attack. Hess *et al.* concluded that SXSg plays only a minor role, if any, in controlling germination of *Striga* seeds.[31]

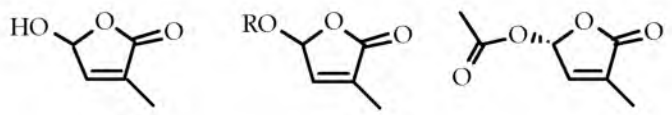
Despite these contradicting reports, Fate and Lynn stated that 'SXSg is the only component exuded from sorghum roots capable of inducing *Striga* germination'.[30] It should be noted here that Hauck *et al.* isolated sorgolactone from the same sorghum root exudate.[5] Inconsistent with their recurring view that strigolactones are not relevant for the induction of *Striga* germination,

Boone *et al.* reported that 'the active portion of the strigol molecule is restricted to the D-ring', *i.e.* 5-hydroxy-3-methyl-2-(5H)-furanone (**6**) (also named hydroxy butenolide, Figure 2).[29] According to Lynn and Boone, the stimulatory activity of strigolactones can be explained, because their D-ring is structurally similar to SXSg and can be oxidized in the same manner.[21] Therefore, these authors proposed a mechanistic connection between SXSg and strigolactones in the germination process.

In order to confirm the suggestion that the butenolide is the active part of strigolactones, Lynn and Boone synthesized several furanone derivatives, such as hydroxy butenolide **6**, 5-ethoxy-3-methyl-2-(5H)-furanone (**7**) and 5-octyloxy-3-methyl-2-(5H)-furanone (**8**) (Figure 2) and evaluated them for their capacity to induce germination of *S. asiatica* seeds.[21] Their respective ED_{50} values were 10^{-5}, 10^{-6} and 10^{-7} M. The difference in biological activity is explained by a difference in the ability of the inducer to permeate the seeds: the more hydrophobic alkyl acetals of the furanones have been shown to have activity comparable to that of strigol. The claim by Lynn *et al.* that only the strigol D-ring is responsible for the inception of *Striga* germination, would imply that the ABC-part of a strigolactone is just a lipophilic carrier, which is in sharp contrast with the results from Zwanenburg's research team, that clearly indicate the importance of the stereochemistry on the biological activity of strigol and its analogues.[15,17,18,19] However, Lynn and co-workers do not offer any explanation about how the substituent group on the butenolide is removed inside the seeds, in order to release the supposedly active species **6**. Interestingly, the views of these authors on the role of strigolactones are ambivalent as on the one hand these compounds are too stable to explain the distance dependence observed in the germination of *Striga* seeds[20], whereas on the other hand their D-ring is readily split off to serve as a substrate in the redox reaction *(vide infra)*.[29]

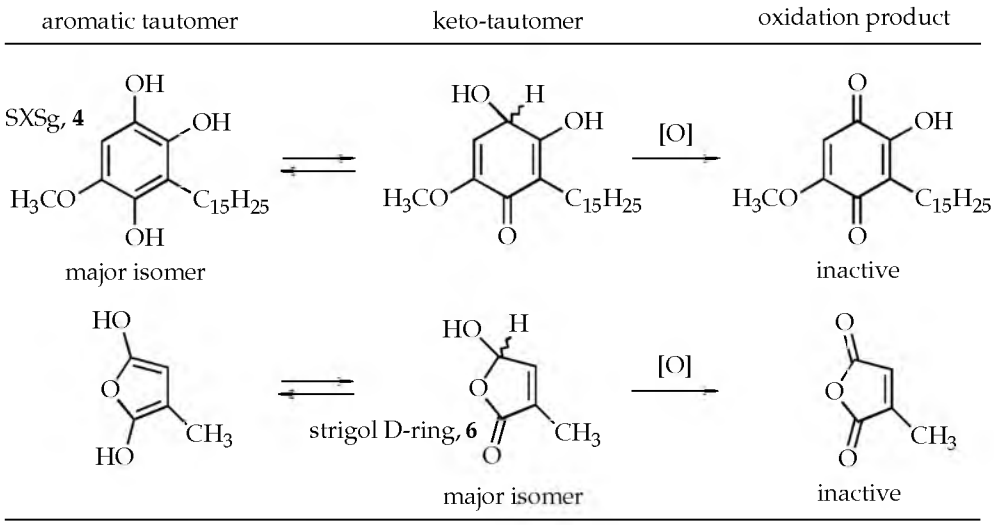
Lynn's claim that the D-ring is the only relevant part of the strigol molecule is also based on the results of Pepperman *et al.*, who reported that ethoxy furanone (**7**) was active in inducing germination of *Striga asiatica*.[33] Notably, hydroxy butenolide (**6**), methoxy-, propoxy- and isopropoxy furanone were not active according to Pepperman. This in itself is inconsistent with Lynn's statement that the more lipophilic alkyl furanones show enhanced biological activity.

Figure 2



6 **7**, R = C_2H_5 **8**, R = C_8H_{17} **9**

Scheme 2. Comparison of the structural domains of SXSg and strigol that are responsible for germination inducing activity according to Boone et al.[29] Two different tautomeric forms of each are shown, together with their oxidation products.

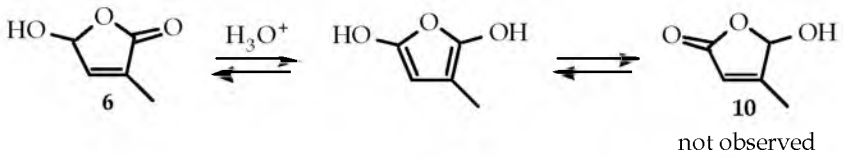


In tests with *Striga hermonthica* and *Orobanche crenata* seeds in the Nijmegen laboratory, neither hydroxy butenolide, nor ethoxy furanone ever showed any stimulatory activity. Furthermore, Pepperman admits in the very same publication that the biological activity of ethoxy furanone was not reproducible and that also with other compounds tested there are 'inconsistencies'. He concludes that 'the butenolide ring alone is not a structural feature capable of inducing germination of witchweed seed'. In all of Pepperman's experiments, DMSO was used as a cosolvent. We know now that DMSO affects the permeability of the seed coats; it is therefore impossible to draw reliable conclusions about compounds tested for germination inducing activity in the presence of DMSO.[34] It is important to note that Lynn does not give any information about the reagents and conditions used in his bioassays with the various furanones.

In an attempt to provide a mechanistic explanation of the induction of germination, Boone and Lynn draw a parallel between SXSg and hydroxy butenolide (D-ring).[21,29] It was hypothesized that the induction of germination of *Striga* seeds proceeds via a redox reaction in which the somehow eliminated D-ring or SXSg is oxidized. This reaction is considered to trigger the germination process (Scheme 2).

Lynn's hypothesis is mainly based on the suggested structural similarity between SXSg and the D-ring (*i.e.* hydroxy butenolide (**6**)), especially because of the presumed existence of an equilibrium with its 2,5-dihydroxy furan tautomer. If such an equilibrium does indeed exist, stirring of furanone **6** under acidic conditions should result in the formation of its regio isomer **10** (Scheme 3). The formation of this isomer **10** was not observed, even after 5 weeks of stirring in a 5% H_2SO_4 solution the only compound detected in the solution was the original hydroxy furanone **6**. This experiment clearly demonstrates that the actual occurrence of this tautomeric equilibrium is very unlikely.

Scheme 3. Presumed tautomeric equilibrium of the strigolactone D-ring.[21]

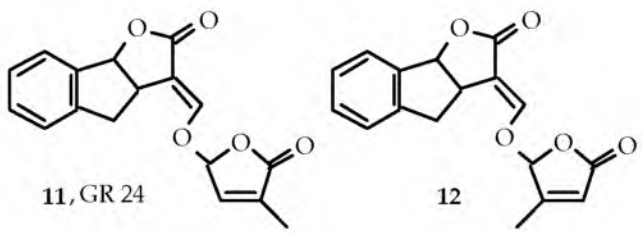


This conclusion is further supported by Mangnus' results with GR 24 (**11**)[35] and its D-ring isomer **12** (Figure 3).[34] Upon liberation of the D-ring, which is a prerequisite for the oxidative mechanism, compounds **6** and **10** (see scheme 3) should be obtained. According to the suggested tautomeric equilibrium structures **6** and **10** are interchangeable and therefore they should both be active in stimulating *Striga* seed germination. Even when this structural interchange does not take place, oxidation of both **6** and **10** results in the same product, implying biological activity of both butenolides **6** and **10**. A substituent effect on the oxidation behaviour of **6** and **10** is highly unlikely. However, in actual practice only compound **11** (GR 24) exhibited considerable germination stimulatory activity toward both *S. hermonthica* and *O. crenata*, whereas its isomer **12** is virtually inactive. This observation raises serious doubts concerning the redox mechanism shown in scheme 2.

In addition, the assumption of a tautomeric equilibrium between furanone and hydroxy furan, would imply that the stereogenic center present in alkoxy substituted furanones is labile. According to this presumed intrinsic lability of the D-ring chiral center, the stereochemistry of the strigolactone D-ring would not have an effect on the biological activity. As mentioned before, the chirality of the stimulants does have a profound influence on their biological activity.

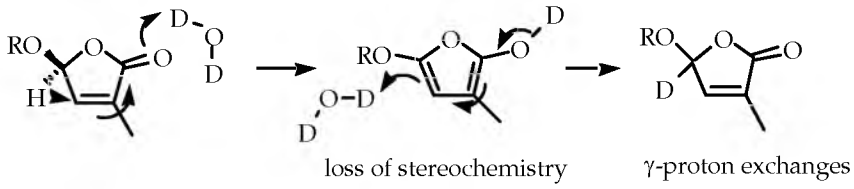
To study the stability of the furanone stereogenic center in more detail, ethoxy furanone (**7**) was prepared in optically active form and used as a model compound.[36] When stirring optically active **7** under the conditions applied in the standardized bioassay[34] (acetone in water) ethoxy furanone did, however, racemize completely within 20 hours. However, when enantiopure acetoxy butenolide (**9**) (readily obtained by enzymatic resolution[37]) was treated similarly the optical activity was retained. These observations could be supportive for Lynn's views. However, racemization of alkoxy butenolides can be explained by two possible reaction mechanisms which are depicted in scheme 4.

Figure 3

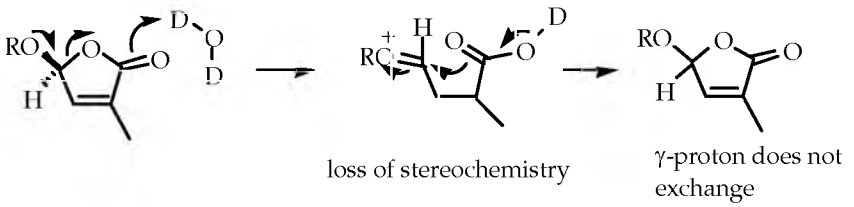


Scheme 4

keto-enol mechanism:



ring-opening/ring closure mechanism:



If the racemization is explained by the keto-enol equilibrium, as is proposed by Lynn *et al.*, then the γ-proton will be exchanged for a deuterium atom by performing the racemization experiment in D_2O. When the racemization proceeds via ring opening/ring closure, the γ-proton will not be exchangeable. In order to investigate which mechanism is operative, racemic octyloxy butenolide **8** was stirred in a mixture of acetone-D_6 and D_2O and the signal of the γ-proton (δ 5.78 ppm) was monitored by ^{1}H-NMR. Even after 3 days, no H/D exchange was detected. Optically active ethoxy butenolide **7** was also stirred in a mixture of acetone-D_6 and D_2O and the racemization of the compound was monitored with chiral GC analysis. After 5 min: e.e. 48%, 1h: e.e. 44%, 5h: e.e. 25%, 8h: e.e. 18%. After 5 days the mixture had racemized completely. NMR analysis of the same aliquots showed that the signal of the γ-proton (δ 5.78 ppm) remained unchanged. This proton did not at all exchange for a deuterium atom during the racemization. The fact that in none of the cases any exchange of the γ-proton for deuterium was observed, while the optically active starting material had become completely racemic, is compelling evidence that the racemization proceeds via a ring opening/ring closure mechanism. The keto-enol equilibrium does not play a role. On the basis of these results, it is clear that Lynn's suggestion that the stimulatory activity of butenolides can be rationalized by the structural similarity of these compounds to SXSg does not hold. If R is an electron withdrawing group, as in **9**, the formation of an oxonium ion intermediate is much less likely, explaining the stability of acetoxy butenolide **9** towards racemization.

Finally, for the postulated redox mechanism to take place, the lipophilic part of the stimulant has to be detached from the furanone. In the molecular mechanism proposed for the induction of germination, the D-ring is split off in the key step of the process (see scheme 1).[9] Theoretically, Lynn's proposal cannot be excluded entirely. However, another very significant point is the concentration range in which Lynn *et al.* observed germination inducing activity of hydroxy butenolide **6**: 50% germination at a concentration of 10^{-5} mol

L^{-1}. If this is compared with the concentrations of strigol and GR 24 that induce half maximum germination ([stimulant]=10^{-11} - 10^{-9} mol L^{-1}), there is a difference of at least a factor 10^4. In the concentration range in which strigol and GR 24 induce germination of *Striga* seeds the D-ring (*i.e.* **6**) is not active. For this reason, Lynn's claim can not be correct, especially if one assumes that the D-ring is originally derived from a strigolactone molecule in the root exudate.

The considerations given above are strong evidence that germination of *Striga* seeds is induced by strigolactones and proceeds via a receptor-mediated mechanism. The hypothesized mechanistic connection between the strigol D-ring **6** and SXSg (**4**) lacks credence. Therefore, the conclusion is justified that Lynn's redox mechanism for the induction of *Striga* germination by oxidation of the strigolactone D-ring can not be valid. Whatever role SXSg may have in the germination process, it is surely very different from that of the strigolactones.

Experimental

General. ^{1}H NMR (100 MHz) and ^{1}H NMR (400 MHz) spectra were recorded on Bruker AC 100 and Bruker AM-400 spectrometers, respectively (Me_4Si as internal standard). All coupling constants (^{3}J) are given in Hertz, unless indicated otherwise. GC was conducted with a Hewlett-Packard HP 5890 gas chromatograph, using a capillary column (25 m) of HP17 and nitrogen (2 mL/min) as the carrier gas. The temperature programme was as follows: initial temperature: 100°C, during 5 minutes; then heating: 5°C/min. for 10 minutes followed by 5 minutes at the final temperature of 150°C. Total run time was 20 minutes. Enantiomeric excesses were determined using a chiral Beta-dex^{TM} 120 fused silica capillary column (60m x 0.25 mm ID, 0.25 μm film thickness) (Supelco) and nitrogen (1 mL/min) as the carrier gas. Solvents were of analytical grade and used as purchased.

5*(R)*-Ethoxy-3-methyl-2-(5H)-furanone (7). Optically active ethoxy butenolide (**7**) was prepared according to van Oeveren[36] and obtained as a colourless oil in 50-70% yield with e.e.'s varying between 48-83%. Retention times of both enantiomers of **7** after separation on a Beta-dex^{TM} 120 GC column, at 130°C isotherm: 34.4 and 35.6 min. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.27 (t, 3H, J=7.0 Hz, CH_3 ethyl), 1.94 (s, 3H, =CCH_3), 3.74 and 3.93 (2 x m, 2 x 1H, CH_2O), 5.79 (s, 1H, OCHO), 6.80 (s, 1H, H-C=).

3-Methyl-5-octyloxy-2-(5H)-furanone (8). Hydroxy furanone (**6**) (0.5 g; 4.4 mmol) was dissolved in a mixture of benzene (15 mL) and 1.1 equiv. (0.63 g) of n-octanol. A catalytic amount of *p*-TsOH was added and the mixture was refluxed under Dean-Stark conditions for 1 hour. Subsequently benzene was evaporated and the residue was dissolved in ethyl acetate and washed with water. Drying ($MgSO_4$) and concentration under reduced pressure followed by column chromatography over silica gel (hexane/ethyl acetate 9/1) afforded **8** (0.72 g, 72%)

as a colourless oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.88 (t, 3H, J=7.0 Hz, CH_3 octyl), 1.27-1.35 (m, 10H, CH_2 octyl), 1.60 (m, 2H, $C\underline{H}_2CH_2O$), 1.94 (s, 3H, $=CCH_3$), 3.65 and 3.85 (2 x m, 2 x 1H, CH_2O), 5.78 (s, 1H, $OC\underline{H}O$), 6.80 (s, 1H, H-C=).

Equilibration experiment: Hydroxy butenolide (**6**) (50 mg) was dissolved in a 5% aqueous solution of sulfuric acid (1 mL). The mixture was stirred at room temperature for 5 weeks. During this period, aliquots were assayed by gas chromatography for the formation of compound **10**. Retention time of **6**: 14.5 min. Retention time of **10**: 17.3 min.

Racemization experiments: Optically active ethoxy furanone (**7**) (20 mg; e.e. 48%) was dissolved in 1 mL of acetone and added to 15 mL of water or phosphate buffer of pH 6,7 and 8 respectively. The mixtures were stirred at room temperature. Aliquots were taken and extracted with ethyl acetate and e.e.'s were determined with chiral GC. In all cases the mixture had become completely racemic within 24 hours. Acetoxy butenolide (**9**) retained its e.e. when subjected to identical conditions. Retention time of both enantiomers of **9** after separation on a Beta-dex^{TM} 120 GC column, at 160°C isotherm: 25.9 and 26.4 min.

Deuterium exchange experiments: Optically active ethoxy butenolide (**7**) (15 mg; e.e. 48%) was dissolved in 0.5 mL of acetone-D_6 and 4.5 mL of D_2O and stirred at room temperature. Aliquots were taken after 5 minutes, 1 hour, 5 hours, 8 hours and 5 days and assayed by ^{1}H NMR and chiral GC. Racemic octyloxy butenolide (**8**) was dissolved in a mixture of acetone-D_6 and D_2O (see above) in an NMR tube and NMR spectra were recorded during 4 successive days.

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3

Synthesis of All Eight Stereoisomers of the Germination Stimulant Sorgolactone

Abstract The naturally occurring sesquiterpene sorgolactone **2** belongs to the class of "strigolactones", which are highly potent germination stimulants for seeds of the parasitic weeds *Striga* and *Orobanche.* The aim of the present work was to synthesize all eight stereoisomers of sorgolactone and to evaluate their activities in the stimulation of germination of *S. hermonthica* and *O. crenata.* Two racemic diastereomers of the ABC-part of sorgolactone, **rac. 10a** and **rac. 10b** respectively, were prepared and coupled with homochiral latent D-ring synthons **12** and **ent. 12**. In this manner, 4 mixtures of 2 separable (protected) sorgolactone diastereomers were obtained. Deprotection gave all eight target compounds as single isomers. Bioassays revealed that only those isomers possessing the same stereochemistry as natural sorgolactone at two adjacent chiral centers exhibit high biological activities.

Introduction

Parasitic weeds of the genera *Striga*, *Orobanche* and *Alectra* have an extremely devastating impact on several graminaceous and leguminous crops in tropical and semi tropical areas of the eastern hemi-sphere.[1,2] These root parasitic weeds specifically interact with their hosts at four levels: 1) germination of the parasitic seed; 2) initiation of haustorial development ; 3) transfer of water and minerals and 4) host responses to infection.[3] The first two events mentioned require host-derived signals as recognition cues. Especially the stimulation of germination and the compounds that trigger this process have attracted much attention. The first naturally occurring germination stimulant, strigol (**1**) (Figure 1), was isolated from the root exudate of the false host cotton (*Gossypium hirsutum* L.)[4] and its structure was elucidated in 1972.[5] The absolute configuration was unambiguously assigned several years later.[6]

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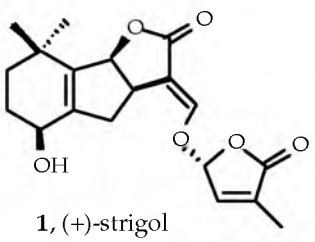
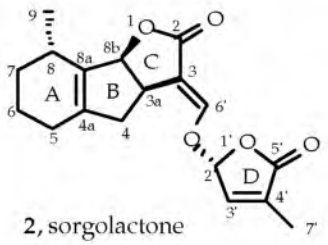
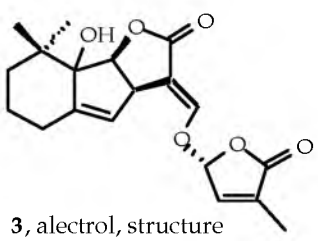
Figure 1



1, (+)-strigol



2, sorgolactone



3, alectrol, structure proposed by Müller *et al.*

It was not until 1992 that some germination stimulants closely related to strigol were isolated from the root exudates of true hosts, *viz.* sorgolactone (**2**)[7] from sorghum (*Sorghum bicolor* (L.) Moench) and alectrol (**3**)[8] from cowpea (*Vigna unguiculata* (L.). Walp) (Figure 1; see also chapter 1). Strigol itself was shown to be the major *Striga* germination stimulant produced by maize (*Zea mays* L.) and proso millet (*Panicum miliaceum* L.).[9] The structure of sorgolactone (**2**) has been tentatively assigned as depicted in figure 1. On the basis of spectroscopic evidence its gross structure was elucidated. The absolute configuration of the stereogenic centers C3a, C8b and C2' was deduced from the similar circular dichroic (CD) data by comparison with those of (+)-strigol (**1**).

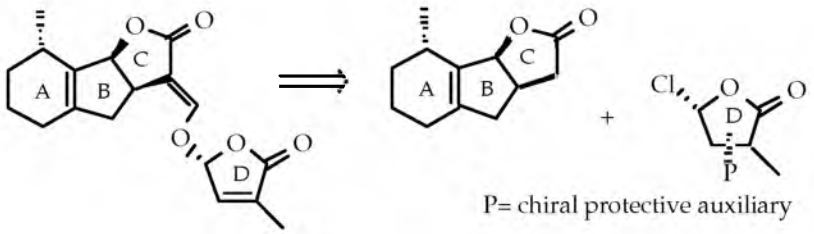
Because of lack of spectroscopic detail due to the small amount of sorgolactone obtained (5 µg from 300,000 sorghum plants) and the extremely laborious isolation procedure, the proposed structure could not be ascertained. Therefore, the total synthesis of **2** was undertaken to verify this tentative structure and to unambiguously establish the absolute stereochemical configuration. The results are reported in this chapter. Shortly after the preliminary publication of this synthetic effort had appeared[10], the synthesis of four racemic stereoisomers of sorgolactone was reported by Mori *et al.*, supporting the gross structure proposed by Hauck.[11] The aim of the research described in this chapter, is also to develop a strategy that allows the synthesis of all eight possible sorgolactone diastereoisomers as single isomers. Evaluation of their respective bioactivities will provide more insight in the structure-activity relationships of these germination stimulants. In this chapter the synthesis and biological evaluation of these eight sorgolactones is presented.

Results and Discussion

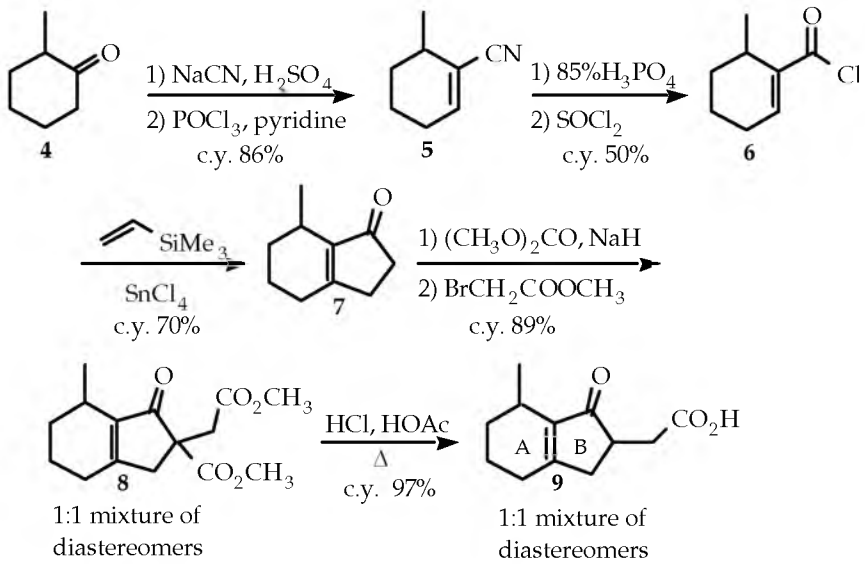
Synthesis

Retrosynthesis of the target compounds involves coupling of the D-ring to the ABC-part of the molecule. (Scheme 1). Stereoselective coupling of the butenolide to diastereomerically pure tricyclic ABC-lactone **10** furnishes a separable mixture of two protected sorgolactone diastereomers. A method to introduce the D-ring of a strigol analogue stereoselectively was recently developed in the Nijmegen laboratory[12,13] and applied in the stereoselective synthesis of strigol analogues GR 7[12], GR 24[14] and demethylsorgolactone (DMSL), the sorgolactone analogue lacking the A-ring methyl group.[15]

Scheme 1



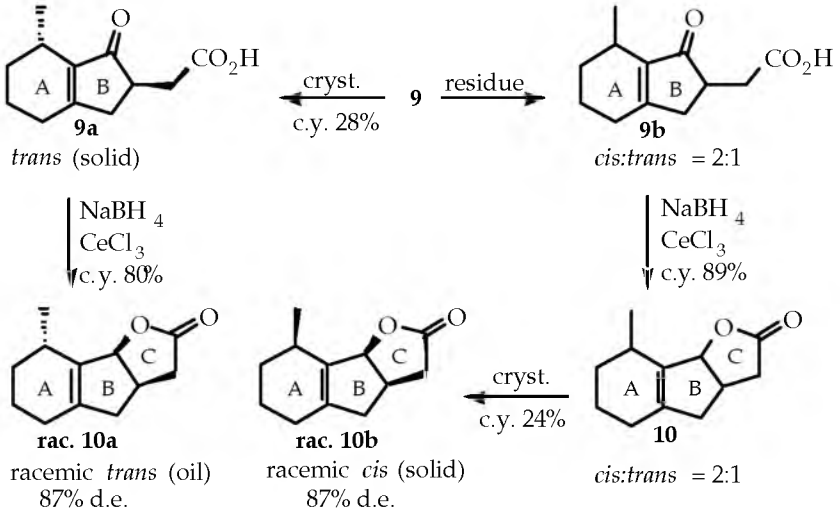
Scheme 2



Application of this coupling methodology in the synthesis of **2** necessitates the preparation of *trans*- and *cis*-tricyclic lactones **rac. 10a** and **rac. 10b** as individual diastereomers. To this end, acid chloride **6** was prepared via slightly modified literature procedures[16,17,18,19] and was then subjected to a Friedel-Crafts reaction in the presence of trimethylvinylsilane, followed by a Nazarov cyclization of the *in situ* formed cyclopentadienyl cation to give AB-fragment **7**.[20] (Scheme 2). Thus obtained bicyclopentenone **7** was then converted via standard procedures[21] into keto acid **9** as a 1:1 mixture of diastereomers from which the *trans*-isomer **9a** remained as a precipitate when **9** was dissolved in diisopropyl ether. Repeated crystallizations afforded the *trans*-isomer of **9** in a d.e. of 87% as was deduced from a detailed analysis of the ^{13}C NMR spectra.

The residue **9b**, enriched with the *cis*-diastereomer, was subjected to a reduction employing sodium borohydride in the presence of cerium trichloride[22], which resulted in a mixture of the sorgolactone ABC-fragments **10** (Scheme 3). The *cis*-tricyclic lactone **rac. 10b** crystallized from the mixture and could be obtained in 87% d.e. Reduction of *trans*-AB-intermediate **9a** with sodium borohydride proceeded with complete retention of stereochemistry, leading to the *trans*-sorgolactone ABC-part **rac. 10a** in a purity of 93.5%; the contaminant was identified as the *cis*-ABC-fragment **10b**, consequently the d.e. of **10a** is 87%.

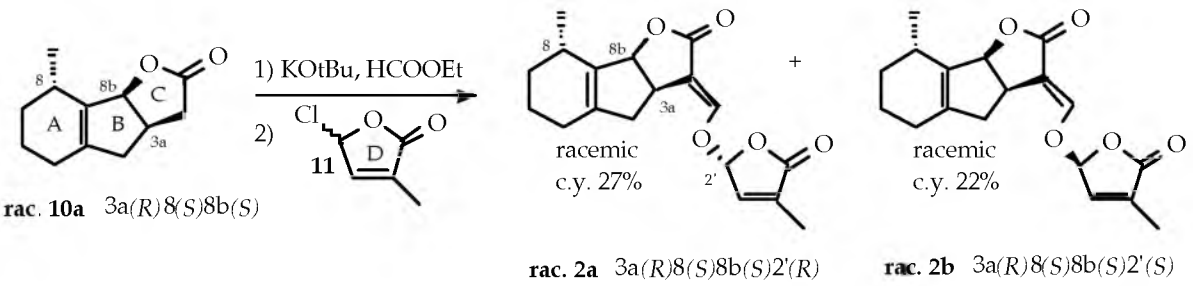
Scheme 3



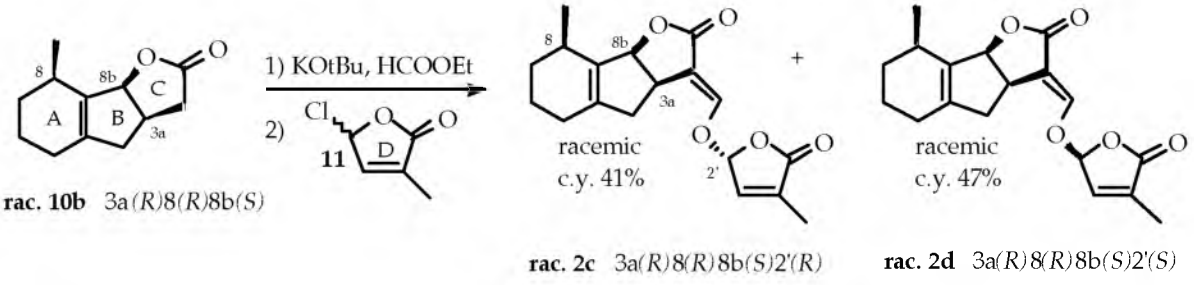
Using 2D-NOESY experiments, the relative configurations of **rac. 10a** and **rac. 10b** were assigned. The oily diastereomer **rac. 10a** showed its characteristic doublet, which was attributed to H8b, at 5.5 ppm, whereas the corresponding proton of crystalline **rac. 10b** resonates at 5.3 ppm.

In order to obtain four racemic mixtures of the sorgolactone stereoisomers, both racemic ABC-diastereomers **rac. 10a** and **rac. 10b** were coupled with chlorobutenolide (**11**)[23,24] The reaction of **rac. 10a** (of which only the 3a*(R)*8*(S)*8b*(S)* enantiomer is drawn) with **11** gave a mixture of two diastereomeric racemates of sorgolactone, **rac. 2a** and **rac. 2b** respectively (yields not optimized), in a ratio of about 1:1 (Scheme 4). They were readily separated by flash chromatography. In the same manner **rac. 2c** and **rac. 2d** were obtained starting from **rac. 10b**, of which only the 3a*(R)*8*(R)*8b*(S)* enantiomer is depicted (Scheme 5).

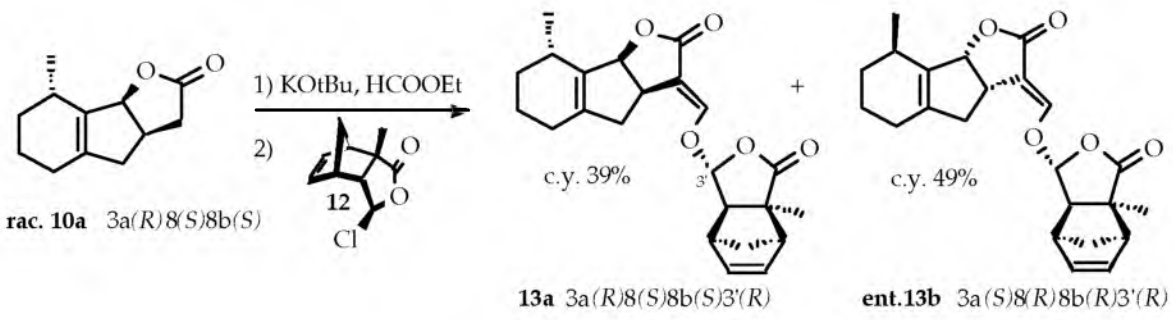
Scheme 4



Scheme 5



Scheme 6



The chemical shifts of the proton H8b of diastereoisomers **rac. 2a-d** were compared with the corresponding value reported by Hauck.[7] In the *trans*-ABC-sorgolactone diastereomers **rac. 2a** and **rac. 2b** the H8b doublet resonates at the same frequency as in the natural compound, *viz.* 5.5 ppm, whereas in the *cis*-ABC-diastereoisomers **rac. 2c** and **rac. 2d** H8b resonates at 5.3 ppm. This comparison allows the conclusion that the original tentative assignment of a *trans*-relationship between the A-ring methyl moiety and ring C is correct. As was known from previous syntheses of strigolactone analogues, deduction of the relative stereochemistry at C2' is not feasible using NMR techniques.[25] Consequently, the NMR signals that can be distinguished in Hauck's ^{1}H NMR spectra match with both diastereomers **rac. 2a** and **rac. 2b**. Circular dichroism spectrometry is necessary for the determination of the absolute configuration at C2'.

Recently, both enantiopure latent D-ring synthons **12** and its enantiomer **ent. 12** were prepared.[12,13] *Trans* tricyclic lactone **rac. 10a** was coupled via its potassium enolate with chlorolactone **12** to give diastereomers **13a** and **ent. 13b**, which were separated by flash chromatography (Scheme 6). Similarly, **rac. 10a** was coupled with chlorolactone **ent. 12** to give diastereomers **13b** and **ent. 13a** (structures not shown). In the same manner, reaction of *cis*-ABC-lactone **rac. 10b** with **12** provided **13c** and **ent. 13d** respectively (structures not shown). Identically, coupling of *cis*-ABC-lactone **rac. 10b** with **ent. 12** provided **13d** and **ent. 13c**, respectively (Scheme 7). These reactions proceeded with complete *exo* selectivity, as judged from ^{1}H NMR analysis.

Scheme 7

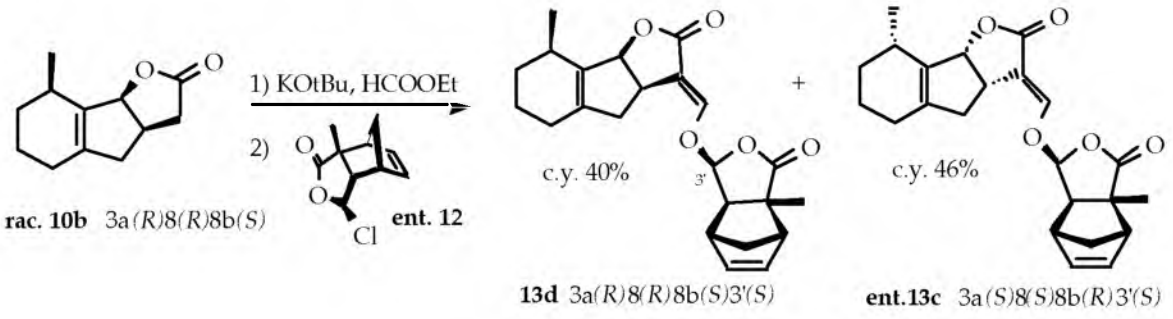
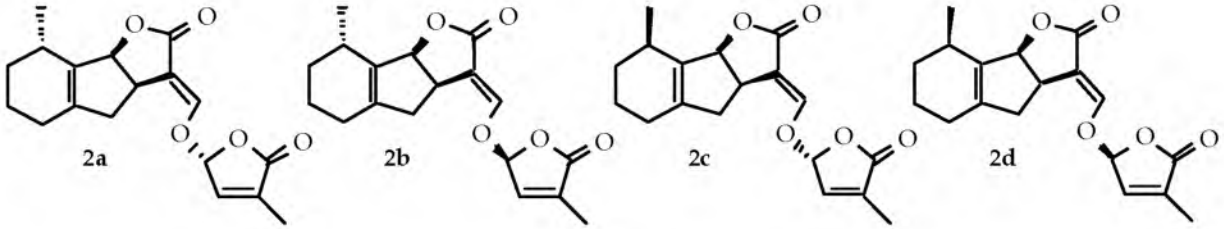


Figure 2



The thermal retro-Diels-Alder reaction of homochiral adducts **13a-d** and **ent. 13a-d** was accomplished by heating the compounds in *o*-dichlorobenzene. In this way all single sorgolactone stereoisomers **2a-d** and **ent. 2a-d** were obtained (Figure 2, **ent. 2a-d** not shown).

The results obtained for the cycloreversions are collected in Table 1. It should be noted that in none of the reactions any epimerization was observed. Due to the fact that the d.e. of tricyclic compounds **rac. 10a** and **rac. 10b** was not 100%, sorgolactones **2a-d** and **ent. 2a-d** contained a small diastereomeric impurity, *viz.* their C8 epimer. These diastereomeric impurities could be largely removed by recrystallization and in the case of **ent. 2a**, **2b** and **ent. 2b** by preparative HPLC.

*Table 1. Cycloreversion of adducts **13a-d** and their enantiomers **ent. 13a-d**.*

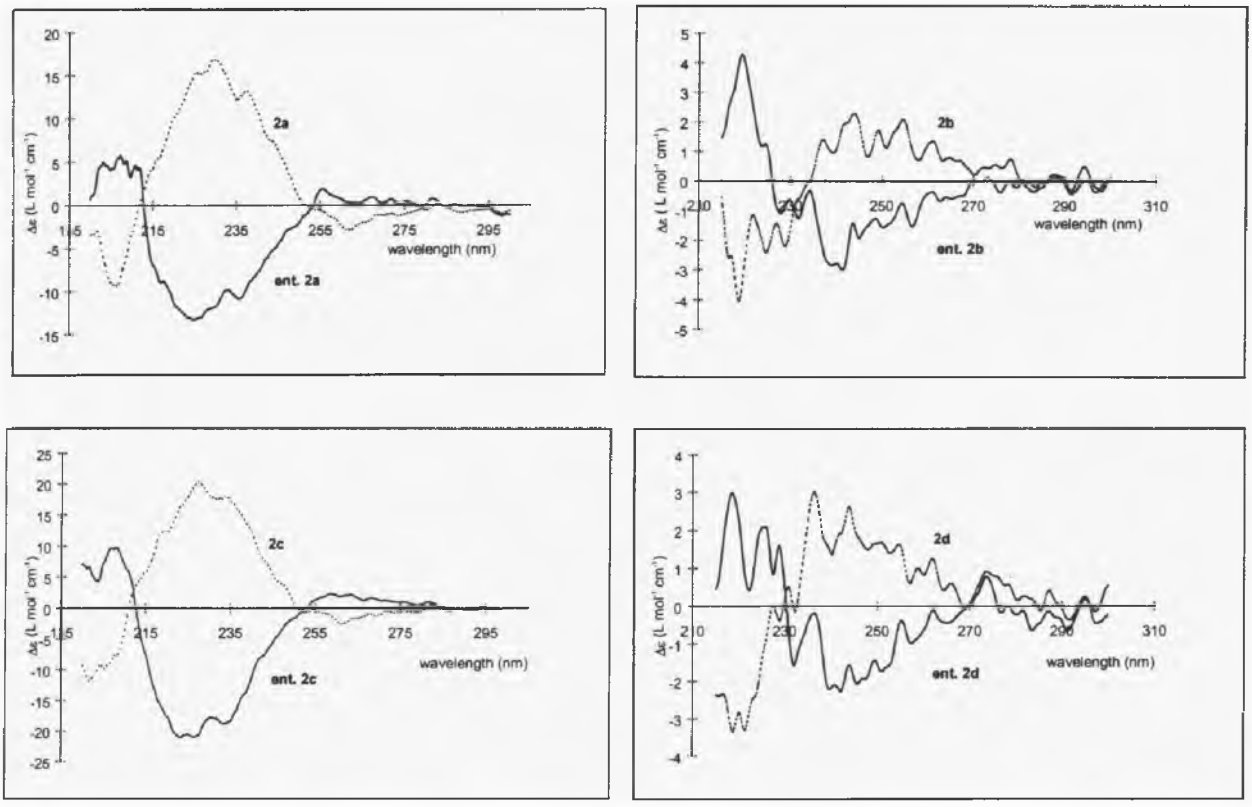
entry	adduct	product	yield(%)	purity(%)[a]	e.e.(%)[b]	$[\alpha]_D$
1	**13a**	**2a**	49	>99	>99	+271.2
2	**ent. 13a**	**ent. 2a**	52	97.4	>99	-278.9
3	**13b**	**2b**	77	98.6	>99	+138.6
4	**ent. 13b**	**ent. 2b**	65	94.3	>99	-137.5
5	**13c**	**2c**	40	>99	>99	+283.4
6	**ent. 13c**	**ent. 2c**	46	98.9	>99	-286.5
7	**13d**	**2d**	58	96.7	>99	+170.8
8	**ent. 13d**	**ent. 2d**	67	97.4	>99	-167.2

[a]Purities were determined by chiral HPLC. The impurities present were identified as diastereomers of the major products. [b]Enantiomeric excesses were determined by chiral HPLC

The absolute configurations of all eight sorgolactone stereoisomers were determined from their ^{1}H NMR spectra in combination with comparison of their CD spectra (Figure 3) with those of the corresponding isomers of demethylsorgolactone and (+)- and (-)-strigol.[15,25,26] From these previously reported data it was inferred that the sign of the Cotton effect around 270 nm could be directly correlated with the stereochemistry at C2', a negative sign corresponding with the C2'*(R)* configuration. The configuration at C2' as deduced from the CD sign around 270 nm was in all cases in complete agreement with the expected stereochemistry based on the chirality of the latent D-ring synthon. Furthermore, a large Cotton effect around 240 nm in combination with a small Cotton effect of opposite sign at 270 nm proved to be indicative of a "transoid"

relationship between the C-ring and the stereocenter C2' [*i.e.* 3a*(R)*8b*(S)*2'*(R)* and 3a*(S)*8b*(R)*2'*(S)*]. In contrast, a small Cotton effect at 240 nm and no change of sign at 270 nm corresponds with a "cisoid" relationship between ring C and C2' [*i.e.* 3a*(R)*8b*(S)*2'*(S)* and 3a*(S)*8b*(R)*2'*(R)*]. A positive Cotton effect at 240 nm corresponds with the 3a*(R)*8b*(S)* configuration, whereas a negative Cotton effect at this wavelength indicates 3a*(S)*8b*(R)* stereochemistry. In this manner, all absolute configurations were unambiguously assigned. The CD spectrum of (+)-sorgolactone (**2a**) (Figure 2) was identical to that reported for the naturally occurring germination stimulant, thus proving that the proposed absolute configuration is indeed correct.

Figure 3. CD spectra of sorgolactone stereoisomers ***2a-d*** *and their enantiomers* ***ent. 2a-d*** *using acetonitrile as the solvent.*



Biological Activity

The germination stimulatory activity of all eight stereoisomers of sorgolactone, **2a-d** and **ent. 2a-d,** was assayed using seeds of *Striga hermonthica* and *Orobanche crenata.*[27] In preliminary experiments the concentration dependent activity range (GR 24 and desmethylsorgolactone) of seeds of *S. hermonthica* has been established.

*Table 2. Germination percentages for seeds of S. hermonthica after exposure to solutions (0.01, 0.001 and 0.0001 mg/L)[a] of sorgolactone diastereoisomers **2a-d** and **ent. 2a-d** and the control GR 24.[b]*

		configuration		% germination ± SE at a concentration of		
entry	compound	at C3aC8C8b	at C2'	10^{-2} mg/L	10^{-3} mg/L	10^{-4} mg/L
1	**2a**	*RSS*	*R*	58.1 ± 6.0	60.6 ± 1.3	48.6 ± 3.7
2	**ent. 2a**	*SRR*	*S*	47.6 ± 0.5	16.2 ± 5.3	7.7 ± 3.3[c]
3	**2b**	*RSS*	*S*	57.7 ± 2.1	30.5 ± 1.4	21.1 ± 5.3
4	**ent. 2b**	*SRR*	*R*	40.5 ± 3.9	8.9 ± 1.9	0.2 ± 0.2[c]
5	**2c**	*RRS*	*R*	57.1 ± 4.6	50.6 ± 4.1	33.5 ± 6.9
6	**ent. 2c**	*SSR*	*S*	38.4 ± 4.8	14.3 ± 3.9	1.4 ± 1.4[c]
7	**2d**	*RRS*	*S*	53.3 ± 0.8	9.2 ± 2.4	0.3 ± 0.3[c]
8	**ent. 2d**	*SSR*	*R*	50.8 ± 1.1	22.9 ± 0.4	5.8 ± 1.3[c]
9	**GR 24**[d]	racemic		62.4 ± 2.9	55.6 ± 2.1	38.8 ± 4.9

[a] 3.16 10^{-8} mol/L, 3.16 10^{-9} mol/L and 3.16 10^{-10} mol/L respectively for sorgolactone, 3.35 10^{-8} mol/L, 3.35 10^{-9} mol/L and 3.35 10^{-10} mol/L respectively for GR 24.[b] Data presented are the mean ± SE of one representative experiment. [c] Not significantly different from aqueous control (without stimulant). [d] Equimolar mixture of two racemic diastereomers.

Figure 3. Bar representation of the percentages of germinated seeds of S. hermonthica after exposure to various concentrations of sorgolactone stereoisomers 2 and the control GR 24.

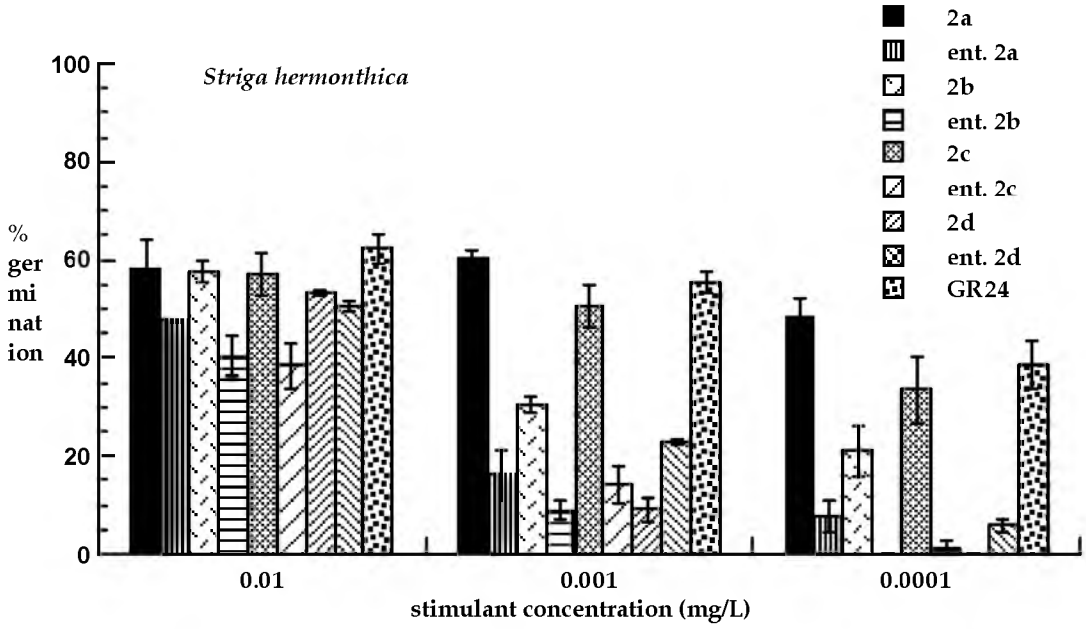
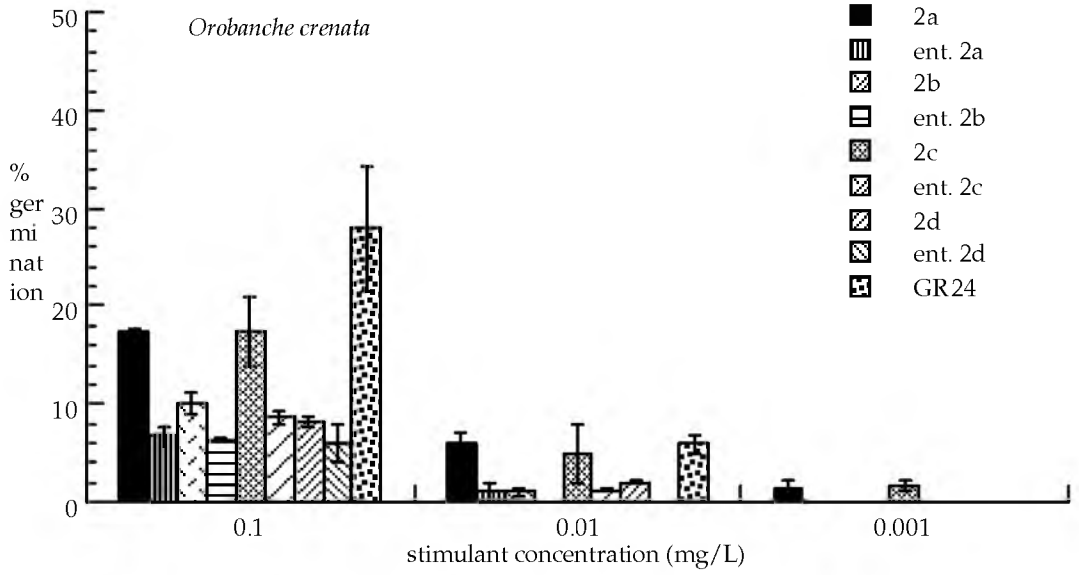


Table 3. Germination percentages for seeds of O. crenata after exposure to solutions (0.1, 0.01 and 0.001 mg/L)[a] of sorgolactone diastereoisomers ***2a-d*** *and* ***ent. 2a-d*** *and the control GR 24.[b]*

		configuration		% germination ± SE at a concentration of		
entry	compound	at C3aC8C8b	at C2'	10^{-1} mg/L	10^{-2} mg/L	10^{-3} mg/L
1	**2a**	*RSS*	*R*	17.3 ± 0.5	5.9 ± 1.2	1.5 ± 0.8[c]
2	**ent. 2a**	*SRR*	*S*	6.7 ± 1.0	1.0 ± 1.0[c]	0[c]
3	**2b**	*RSS*	*S*	10.0 ± 1.1	1.0 ± 0.5[c]	0[c]
4	**ent. 2b**	*SRR*	*R*	6.4 ± 0.1	0[c]	0[c]
5	**2c**	*RRS*	*R*	17.4 ± 3.5	4.9 ± 2.9	1.7 ± 0.5
6	**ent. 2c**	*SSR*	*S*	8.6 ± 0.6	1.2 ± 0.1[c]	0[c]
7	**2d**	*RRS*	*S*	8.2 ± 0.5	2.0 ± 0.1	0[c]
8	**ent. 2d**	*SSR*	*R*	5.9 ± 1.9	0[c]	0[c]
9	**GR 24**[d]	racemic		27.9 ± 6.3	5.9 ± 1.0	n.d.[e]

[a] 3.16 10^{-7} mol/L, 3.16 10^{-8} mol/L and 3.16 10^{-9} mol/L respectively for sorgolactone, 3.35 10^{-7} mol/L, 3.35 10^{-8} mol/L and 3.35 10^{-9} mol/L respectively for GR 24.[b] Data presented are the mean ± SE of one representative experiment. [c] Not significantly different from aqueous control (without stimulant). [d] Equimolar mixture of two racemic diastereomers.[e] Germination percentage not determined.

Figure 4. Bar representation of the percentages of germinated seeds of O. crenata after exposure to different concentrations of sorgolactone stereoisomers ***2*** *and the control GR 24.*



Maximal germination percentages were obtained within the concentration range of 1 and 0.01 mg/L. Half maximal activity was observed at approximately 0.001mg/L. Assessment of the relative bioactivity of the individual stereoisomers of sorgolactone was therefore established at concentrations of 0.01 and 0.001 mg/L and at the sensitive concentration of 0.0001mg/L. It was expected that the lower concentration should exhibit more profound differences. Relevant data are collected in table 2 and a bar representation of these results is given in figure 3.

The data presented in table 2 and figure 3 reveal that there is a significant difference in germination stimulatory activity between the eight stereoisomers. For *Striga*, sorgolactone diastereomer **2a**, possessing the "natural" absolute stereochemistry, is the most active one. For *S. hermonthica* the difference in activity between **2a** and most other diastereomers amounts at least a factor of 100; *i.e.* that the activity of about 50%, which is exhibited by all sorgolactone isomers at a concentration of 10^{-2} mg/L, is at a concentration of 10^{-4} mg/L only exhibited by compound **2a**. At this sensitive concentration, the activity of almost all other sorgolactones is strongly reduced. Interestingly **2c,** which only differs from **2a** in the configuration at C8, also exhibits a relatively high germinating activity. Apparently, the absolute configuration at C8 is much less critical for the bioactivity than that of the stereocenter at C3aC8b. Remarkably, isomer **2b**, which is the C2' epimer of "natural" sorgolactone (**2a**) also is appreciably active. The receptor for the germination stimulant still can accommodate this epimer in such a manner that induction of germination can take place. Extended bioassays involving a series of strigolactones will be needed however, to allow the development of a model for the interaction of receptor and stimulant.

It is well documented[28,29] that seeds of *Orobanche* also respond to strigolactones. Therefore, for the sake of comparison, bioassays were also performed with seeds of *O. crenata*, using the same concentration range of stimulant. The results are collected in table 3 and represented in figure 4. These data clearly indicate that the sorgolactones are considerably less active in germinating *Orobanche* seeds (however, the dose-response curve for *O. crenata* is evidently different from that for *S. hermonthica*). As far as the absolute stereochemistry is concerned, the trend is the same as for *Striga* seeds. It should be noted that Sorghum, from which sorgolactone was isolated, is not a natural host for *Orobanche crenata*.

Conclusions

All eight stereoisomers of the germination stimulant sorgolactone were synthesized. Combination of the relative *trans*-configuration of the natural sorgolactone ABC-part (determined by NMR) with the absolute stereochemistry of the C-ring stereogenic centers C8bC3a and that of C2' of naturally occurring sorgolactone (determined by CD spectrometry) leads to the conclusion that the proposed absolute structure of natural sorgolactone is correct. The bioactivity of all eight stereoisomers was determined and only those isomers containing the same absolute stereochemistry as "natural" sorgolactone (**2a**) at 2 adjacent stereocenters exhibit significant germination stimulatory activity at sensitive concentrations.

Experimental

Synthesis

General. For general methods and instrumentation see chapter 5. Final purification of sorgolactone diastereomers **ent. 2a**, **2b** and **ent. 2b** was accomplished by preparative HPLC using a Lichrosorb Si60 (7 mm) column (Merck, Hibar, 250 x 25 mm) and hexane/2-propanol 95/5 as the eluent. Enantiomeric excess and purities of stereoisomers **2a-d** and **ent. 2a-d** were determined by analytical HPLC using a Chiralcel OD (10 mm) cellulose carbamate column (Baker, 250 x 4.6 mm) and hexane/2-propanol 80/20 as the eluent.

8*(SR)*-Methyl-3-[4'-methyl-5'-oxo-2',5'-dihydro-furan-2'*(RS)*-yloxymethylene]-3,3a*(RS)*,4,5,6,7,8,8b*(SR)*-octahydro-indeno[1,2-b]-furan-2-one (rac. 2a) and its 2'*(SR)* diastereomer: **8*(SR)*-Methyl-3-[4'-methyl-5'-oxo-2',5'-dihydro-furan-2'*(SR)*-yloxymethylene]-3,3a*(RS)*,4,5,6,7,8,8b*(S)R)*-octahydro-indeno[1,2-b]-furan-2-one (rac. 2b).** These products were synthesized in the same manner as **rac. 2c** and **rac. 2d** starting from *trans*-tricyclic lactone **rac. 10a** and chlorobutenolide **11** *(vide infra)*. Yields were 27% of fast moving diastereomer **rac. 2a** and 22% of slow moving diastereomer **rac. 2b**, that were both obtained as colourless crystals by recrystallisation from hexane/ethyl acetate.

rac. 2a: mp: 114-115°C. 400MHz ^{1}H NMR ($CDCl_3$): δ 1.06 (d, 3H, J=6.9Hz, 3xH9); 1.25 (m, 1H, H7); 1.55 (m, 1H, H6); 1.70 (m, 1H, H6); 1.78 (m, 1H, H7); 1.93 (m, 2H. 2xH5); 2.03 (s, 3H, 3xH7'); 2.34 (bd, 1H, J=15.9Hz, H4); 2.36 (m, 1H, H8); 2.75 (dd, 1H, J=8.5Hz,15.9Hz, H4); 3.61 (m, 1H, H3a); 5.49 (d, 1H, J=7.6Hz, H8b); 6.15 (s, 1H, H2'); 6.93 (s, 1H, H3'); 7.43 (d, 1H, J=2.6Hz, H6'). MS [EI *m/z*, rel. intensity (%)]: 316 ([M]$^+$, 6.9); 219 ([$C_{13}H_{15}O_3$]$^+$, 21.8); 201 ([$C_{13}H_{13}O_2$]$^+$, 52.8); 173 ([$C_{12}H_{13}O$]$^+$, 24.7); 97 ([$C_5H_5O_2$]$^+$, 92.7); 91 ([C_7H_7]$^+$, 27.2). Anal. calcd. for $C_{18}H_{20}O_5$: C, 68.34; H, 6.37 found: C, 68.25; H, 6.37.

rac. 2b: mp: 112-114°C. 400MHz ^{1}H NMR ($CDCl_3$): δ 1.06 (d, 3H, J=6.9Hz, 3xH9); 1.25 (m, 1H, H7); 1.55 (m, 1H, H6); 1.70 (m, 1H, H6); 1.77 (m, 1H, H7); 1.93 (m, 2H. 2xH5); 2.03 (s, 3H, 3xH7'); 2.33 (bd, 1H, J=15.9Hz, H4); 2.36 (m, 1H, H8); 2.73 (dd, 1H, J=8.5Hz,15.9Hz, H4); 3.60 (m, 1H, H3a); 5.49 (d, 1H, J=7.7Hz, H8b); 6.14 (s, 1H, H2'); 6.94 (s, 1H, H3'); 7.43 (d, 1H, J=2.6Hz, H6'). MS [EI *m/z*, rel. intensity (%)]: 316 ([M]$^+$, 8.5); 219 ([$C_{13}H_{15}O_3$]$^+$, 26.7); 201 ([$C_{13}H_{13}O_2$]$^+$, 55.7); 173 ([$C_{12}H_{13}O$]$^+$, 22.8); 97 ([$C_5H_5O_2$]$^+$, 100); 91 ([C_7H_7]$^+$, 17.0). Anal. calcd. for $C_{18}H_{20}O_5$: C, 68.34; H, 6.37 found: C, 68.28; H, 6.36.

8*(RS)*-Methyl-3-[4'-methyl-5'-oxo-2',5'-dihydro-furan-2'*(RS)*-yloxymethylene]-3,3a*(RS)*,4,5,6,7,8,8b*(SR)*-octahydro-indeno[1,2-b]-furan-2-one (rac. 2c) and its 2'*(SR)* diastereomer: **8*(RS)*-Methyl-3-[4'-methyl-5'-oxo-2',5'-dihydro-furan-2'*(SR)*-yloxymethylene]-3,3a*(RS)*,4,5,6,7,8,8b*(SR)*-octahydro-indeno[1,2-b]-furan-2-one (rac. 2d).** To a cooled (0°C) and stirred solution of racemic *cis*-tricyclic lactone **rac. 10b** (380 mg, 2.0 mmol) in diethyl ether (10 mL) were added, under a continuous stream of nitrogen, 3 equiv. of ethyl formate (444 mg, 6.0 mmol) and 1.1 equiv. of potassium *tert*-butoxide (247 mg, 2.2 mmol). The mixture was stirred overnight at room

temperature. Ether was removed using a stream of nitrogen gas and the thus obtained potassium salt of formylated **rac. 10b** was dissolved in DMF (15 mL) and cooled to -50°C. Then a solution of chlorobutenolide 11 (265 mg, 2 mmol) in DMF (3mL) was gradually added at -50°C under nitrogen. After overnight stirring at room temperature the mixture was quenched with acetic acid (0.5 mL) and the solvent was removed *in vacuo*. The residue was dissolved in a mixture of water and ethyl acetate. The aqueous phase was extracted with ethyl acetate (2 times) and the combined organic layers were washed with brine (2 times), dried ($MgSO_4$) and concentrated under reduced pressure. The crude product was purified using flash chromatography (SiO_2, hexane/ethyl acetate 3/1) to afford two diastereomeric products. Fast moving diastereomer **rac. 2c** (253 mg, 41%) and slow moving diastereomer **rac. 2d** (294 mg, 47%) were obtained as colourless crystals. by recrystallisation from hexane/ethyl acetate.

rac. 2c: mp: 142-144°C. 400MHz ^{1}H NMR ($CDCl_3$): δ 1.13 (d, 3H, J=7.0Hz, 3xH9); 1.34 (m, 1H, H7); 1.54 (m, 1H, H6); 1.72 (m, 2H, H6+H7); 1.98 (m, 2H. 2xH5); 2.03 (s, 3H, 3xH7'); 2.31 (m, 1H, H8); 2.33 (bd, 1H, J=16.9Hz, H4); 2.71 (ddd, 1H, J=3.1Hz, 9.2Hz, 16.8Hz, H4); 3.62 (m, 1H, H3a); 5.35 (d, 1H, J=7.6Hz, H8b); 6.15 (s, 1H, H2'); 6.92 (s, 1H, H3'); 7.42 (d, 1H, J=2.5Hz, H6'). MS [EI *m/z*, rel. intensity (%)]: 316 ([M]$^+$, 9.8); 219 ([$C_{13}H_{15}O_3$]$^+$, 43.9); 201 ([$C_{13}H_{13}O_2$]$^+$, 67.7); 173 ([$C_{12}H_{13}O$]$^+$, 36.6); 97 ([$C_5H_5O_2$]$^+$, 100); 91 ([C_7H_7]$^+$, 28.7). Anal. calcd. for $C_{18}H_{20}O_5$: C, 68.34; H, 6.37 found: C, 68.40; H, 6.37.

rac. 2d: mp: 138-139°C.400MHz ^{1}H NMR ($CDCl_3$): δ 1.12 (d, 3H, J=7.0Hz, 3xH9); 1.36 (m, 1H, H7); 1.54 (m, 1H, H6); 1.72 (m, 2H, H6+H7); 1.95 (m, 2H. 2xH5); 2.03 (s, 3H, 3xH7'); 2.31 (m, 1H, H8); 2.33 (bd, 1H, J=16.9Hz, H4); 2.69 (ddd, 1H, J=2.9Hz, 12.1Hz, 16.9Hz, H4); 3.61 (m, 1H, H3a); 5.35 (d, 1H, J=7.7Hz, H8b); 6.13 (s, 1H, H2'); 6.93 (s, 1H, H3'); 7.42 (d, 1H, J=2.5Hz, H6'). MS [EI *m/z*, rel. intensity (%)]: 316 ([M]$^+$, 5.7); 219 ([$C_{13}H_{15}O_3$]$^+$, 43.9); 201 ([$C_{13}H_{13}O_2$]$^+$, 76.9); 173 ([$C_{12}H_{13}O$]$^+$, 40.2); 97 ([$C_5H_5O_2$]$^+$, 100); 91 ([C_7H_7]$^+$, 28.9). Elemental analysis was done for the enantiopure compounds, **2d** and **ent. 2d**.

8*(S)*-Methyl-3-[4'-methyl-5'-oxo-2',5'-dihydro-furan-2'*(R)*-yloxymethylene]-3,3a*(R)*,4,5,6,7,8,8b *(S)*-octahydro-indeno[1,2-b]-furan-2-one (2a). Deprotection of Diels Alder adduct **13a** (229 mg, 0.6 mmol) was accomplished by boiling in *o*-dichlorobenzene (50 mL) under reflux cooling for 7 hours as was previously described by Thuring *et al.*[15] Sorgolactone stereoisomer **2a** was obtained as off white crystals in 49% yield. An analytically pure sample was obtained by recrystallisation from ethyl acetate/diisopropyl ether. mp: 139.5-142°C. $[\alpha]^{21}_D$=+271.2° (c=0.25, $CDCl_3$). purity 99.7%, e.e.>99% (determined by HPLC). 400MHz ^{1}H NMR ($CDCl_3$): δ 1.06 (d, 3H, J=6.9Hz, 3xH9); 1.26 (m, 1H, H7); 1.55 (m, 1H, H6); 1.70 (m, 1H, H6); 1.77 (m, 1H, H7); 1.94 (m, 2H. 2xH5); 2.03 (s, 3H, 3xH7'); 2.33 (bd, 1H, J=15.4Hz, H4); 2.36 (m, 1H, H8); 2.75 (dd, 1H, J=9.0Hz,15.4Hz, H4); 3.61 (m, 1H, H3a); 5.49 (d, 1H, J=7.4Hz, H8b); 6.15 (s, 1H, H2'); 6.92 (s, 1H, H3'); 7.41 (d, 1H, J=2.6Hz, H6'). ^{13}C NMR ($CDCl_3$): δ 10.8; 18.6; 20.7; 26.1; 27.8; 31.2; 36.5; 41.4; 88.0; 100.4; 114.5; 136.0; 137.3; 140.9; 141.3; 150.0; 170.2; 171.8. MS [EI *m/z*, rel. intensity (%)]: 316 ([M]$^+$, 8.1); 219 ([$C_{13}H_{15}O_3$]$^+$, 25.2); 201 ([$C_{13}H_{13}O_2$]$^+$, 48.2); 173 ([$C_{12}H_{13}O$]$^+$, 18.0); 97 ([$C_5H_5O_2$]$^+$, 100); 91

($[C_7H_7]^+$, 14.2). Anal. calcd. for $C_{18}H_{20}O_5$: C, 68.34; H, 6.37 found: C, 68.15; H, 6.32. CD (CH_3CN, c=35.9mM): λ_{max}=230nm, $\Delta\varepsilon$=+20 L mol^{-1} cm^{-1}.

8*(R)*-Methyl-3-[4'-methyl-5'-oxo-2',5'-dihydro-furan-2'*(S)*-yloxymethylene]-3,3a*(S)*,4,5,6,7,8,8b *(R)*-octahydro-indeno[1,2-b]-furan-2-one (ent. 2a). Sorgolactone stereoisomer **ent. 2a** was prepared in the same manner as reported for isomer **2a** starting from **ent. 13a** (229 mg, 0.6 mmol). **ent. 2a** was obtained as a yellowish oil in 52% yield. Final purification by HPLC afforded off white crystals, that were recrystallized from hexane/ethyl acetate. mp: 145-146°C. $[\alpha]^{21}_D$=-278.9° (c=0.10, CH_2Cl_2). purity 97.4%, e.e.>99% (determined by HPLC). ^{1}H NMR, ^{13}C NMR and mass data were identical to those of its enantiomer **2a** CD (CH_3CN, c=40.5mM): λ_{max}=226nm, $\Delta\varepsilon$=-13.5 L mol^{-1} cm^{-1}.

8*(S)*-Methyl-3-[4'-methyl-5'-oxo-2',5'-dihydro-furan-2'*(S)*-yloxymethylene]-3,3a*(R)*,4,5,6,7,8,8b *(S)*-octahydro-indeno[1,2-b]-furan-2-one (2b). Sorgolactone stereoisomer **2b** was synthesized in the same way as described for isomer **2a** starting from **13b** (153 mg, 0.4 mmol). **2b** was obtained as a yellowish oil in 77% yield. Further purification by HPLC gave off white crystals, that were recrystallized from ethyl acetate/diisopropyl ether. mp: 111-114°C. $[\alpha]^{21}_D$=+138.6° (c=0.17, $CHCl_3$). purity 98.6%, e.e.>99% (determined by HPLC). ^{1}H NMR, ^{13}C NMR and mass data were identical to those of its enantiomer **ent. 2b**. CD (CH_3CN, c=31.6mM): λ_{max}=243nm, $\Delta\varepsilon$=+2.5 L mol^{-1} cm^{-1}.

8*(R)*-Methyl-3-[4'-methyl-5'-oxo-2',5'-dihydro-furan-2'*(R)*-yloxymethylene]-3,3a*(S)*,4,5,6,7,8,8b *(R)*-octahydro-indeno[1,2-b]-furan-2-one (ent. 2b). Sorgolactone stereoisomer **ent. 2b** was synthesized in the same manner as described for isomer **2a** starting from **ent. 13b** (229 mg, 0.6 mmol). **ent. 2b** was obtained as a yellowish oil in 65% yield. Chromatographic purification by HPLC, followed by recrystallisation from ethyl acetate/diisopropyl ether afforded off white crystals. mp: 109-112°C. $[\alpha]^{21}_D$=-137.5° (c=0.10, CH_2Cl_2). purity 94.3%, e.e.>99% (determined by HPLC). 400MHz ^{1}H NMR ($CDCl_3$): δ 0.99 (d, 3H, J=6.9Hz, 3xH9); 1.17 (m, 1H, H7); 1.49 (m, 1H, H6); 1.63 (m, 1H, H6); 1.70 (m, 1H, H7); 1.87 (m, 2H. 2xH5); 1.96 (s, 3H, 3xH7'); 2.26 (bd, 1H, J=15.9Hz, H4); 2.29 (m, 1H, H8); 2.66 (dd, 1H, J=8.6Hz,15.9Hz, H4); 3.53 (m, 1H, H3a); 5.43 (d, 1H, J=7.7Hz, H8b); 6.07 (s, 1H, H2'); 6.87 (s, 1H, H3'); 7.36 (d, 1H, J=2.6Hz, H6'). ^{13}C NMR ($CDCl_3$): δ 10.5; 18.6; 20.7; 26.0; 27.8; 31.6; 36.5; 41.4; 88.0; 100.6; 114.6; 135.8; 137.2; 141.0; 141.5; 150.3; 170.3; 171.8. MS [EI *m/z*, rel. intensity (%)]: 316 ($[M]^+$, 8.9); 219 ($[C_{13}H_{15}O_3]^+$, 29.1); 201 ($[C_{13}H_{13}O_2]^+$, 53.6); 173 ($[C_{12}H_{13}O]^+$, 20.2); 97 ($[C_5H_5O_2]^+$, 96.4); 91 ($[C_7H_7]^+$, 3.1). HRMS/EI, *m/z* calcd for $C_{18}H_{20}O_5$: 316.13107, found: 316.13098 ± 0.00088. CD (CH_3CN, c=29.1mM): λ_{max}=240nm, $\Delta\varepsilon$=-3 L mol^{-1} cm^{-1}.

8*(R)*-Methyl-3-[4'-methyl-5'-oxo-2',5'-dihydrofuran-2'*(R)*-yloxymethylene]-3,3a*(R)*,4,5,6,7,8,8b *(S)*-octahydro-indeno[1,2-b]-furan-2-one (2c). Sorgolactone stereoisomer **2c** was prepared in the same manner as described for isomer **2a** starting from adduct **13c** (191 mg, 0.5 mmol). **2c** was obtained as colourless crystals in 40% yield. An analytically pure sample was obtained by recrystallisation from ethyl acetate/diisopropyl ether. mp: 178-181°C. $[\alpha]^{21}_D$=+284.4° (c=0.21, $CHCl_3$). purity 100%, e.e.>99% (determined by HPLC). 400MHz ^{1}H NMR ($CDCl_3$): δ 1.13 (d, 3H, J=7.0Hz, 3xH9); 1.34 (m, 1H, H7); 1.54 (m, 1H, H6); 1.72 (m, 2H, H6+H7); 1.98 (m, 2H. 2xH5); 2.03 (s, 3H, 3xH7'); 2.31 (m, 1H, H8); 2.33 (bd, 1H, J=16.9Hz, H4); 2.71 (ddd, 1H, J=3.1Hz, 9.2Hz, 16.8Hz, H4); 3.62 (m, 1H, H3a); 5.35 (d, 1H, J=7.6Hz, H8b); 6.15 (s, 1H, H2'); 6.92 (s, 1H, H3'); 7.42 (d, 1H, J=2.5Hz, H6'). ^{13}C NMR ($CDCl_3$): δ 10.8; 20.0; 20.3; 26.2; 30.1; 31.6; 36.9; 41.4; 90.9; 100.4; 114.6; 136.0; 136.6; 140.9; 142.8; 149.6; 170.2; 171.8. MS [EI *m/z*, rel. intensity (%)]: 316 ($[M]^+$, 7.5); 219 ($[C_{13}H_{15}O_3]^+$, 32.6); 201 ($[C_{13}H_{13}O_2]^+$, 48.8); 173 ($[C_{12}H_{13}O]^+$, 20.7); 97 ($[C_5H_5O_2]^+$, 100); 91 ($[C_7H_7]^+$, 15.6). Anal. calcd. for $C_{18}H_{20}O_5$: C, 68.34; H, 6.37 found: C, 68.63; H, 6.36. CD (CH_3CN, c=34.2mM): λ_{max}=227nm, Δε=+22 L mol^{-1} cm^{-1}.

8*(S)*-Methyl-3-[4'-methyl-5'-oxo-2',5'-dihydrofuran-2'*(S)*-yloxymethylene]-3,3a*(S)*,4,5,6,7,8,8b *(R)*-octahydro indeno[1,2-b]-furan-2-one (ent. 2c). Sorgolactone stereoisomer **ent. 2c** was synthesized in the same way as described for isomer **2a** starting from **ent. 13c** (172 mg, 0.45 mmol). Recrystallisation from ethyl acetate/diisopropyl ether afforded **ent. 2c** as colourless crystals in 46% yield. mp: 177.5-178.5°C.$[\alpha]^{21}_D$=-286.5° (c=0.21, $CHCl_3$). purity 98.9%, e.e.>99% (determined by HPLC). ^{1}H NMR, ^{13}C NMR and mass data were identical to those of its enantiomer **2c**. Anal. calcd. for $C_{18}H_{20}O_5$: C, 68.34; H, 6.37 found: C, 68.26; H, 6.34. CD (CH_3CN, c=33.2mM): λ_{max}=225nm, Δε=-23 L mol^{-1} cm^{-1}.

8*(R)*-Methyl-3-[4'-methyl-5'-oxo-2',5'-dihydrofuran-2'*(S)*-yloxymethylene]-3,3a*(R)*,4,5,6,7,8,8b *(S)*-octahydro indeno[1,2-b]-furan-2-one (2d). Sorgolactone stereoisomer **2d** was prepared in the same manner as described for isomer **2a** starting from adduct **13d** (229 mg, 0.6 mmol). **2d** was obtained as colourless crystals in 58% yield. An analytically pure sample was obtained by recrystallisation from ethyl acetate/diisopropyl ether. mp: 136-138.°C.$[\alpha]^{21}_D$=+170.8° (c=0.16, $CHCl_3$). purity 96.7%, e.e.>99% (determined by HPLC). ^{1}H NMR, ^{13}C NMR and mass data were identical to those of its enantiomer **ent 2d**. Anal. calcd. for $C_{18}H_{20}O_5$: C, 68.34; H, 6.37 found: C, 68.52; H, 6.31. CD (CH_3CN, c=33.2mM): λ_{max}=243nm, Δε=+3 L mol^{-1} cm^{-1}.

8*(S)*-Methyl-3-[4'-methyl-5'-oxo-2',5'-dihydrofuran-2'*(R)*-yloxymethylene]-3,3a*(S)*,4,5,6,7,8,8b *(R)*-octahydro-indeno[1,2-b]-furan-2-one (ent. 2d). Sorgolactone stereoisomer **ent. 2d** was synthesized in the same manner as reported for isomer **2a** starting from **ent. 13d** (191 mg, 0.5 mmol). **ent. 2d** was obtained as colourless crystals in 67% yield. An analytically pure sample was obtained by recrystallisation from ethyl acetate/diisopropyl ether. mp: 135-137°C. $[\alpha]^{21}_D$=-167.2° (c=0.25, $CHCl_3$). purity 97.4%, e.e.>99% (determined by HPLC). 400MHz ^{1}H NMR ($CDCl_3$): δ

1.12 (d, 3H, J=7.0Hz, 3xH9); 1.36 (m, 1H, H7); 1.54 (m, 1H, H6); 1.72 (m, 2H, H6+H7); 1.95 (m, 2H. 2xH5); 2.03 (s, 3H, 3xH7'); 2.31 (m, 1H, H8); 2.33 (bd, 1H, J=16.9Hz, H4); 2.69 (ddd, 1H, J=2.9Hz, 12.1Hz, 16.9Hz, H4); 3.61 (m, 1H, H3a); 5.35 (d, 1H, J=7.7Hz, H8b); 6.13 (s, 1H, H2'); 6.93 (s, 1H, H3'); 7.42 (d, 1H, J=2.5Hz, H6'). ^{13}C NMR ($CDCl_3$): δ 10.8; 20.0; 20.3; 26.2; 30.2; 31.7; 36.8; 41.5; 90.9; 100.6; 114.6; 135.9; 136.5; 141.0; 143.0; 149.9; 170.3; 171.9. MS [EI *m/z*, rel. intensity (%)]: 316 ([M]$^+$, 7.9); 219 ([$C_{13}H_{15}O_3$]$^+$, 32.1); 201 ([$C_{13}H_{13}O_2$]$^+$, 47.7); 173 ([$C_{12}H_{13}O$]$^+$, 20.2); 97 ([$C_5H_5O_2$]$^+$, 100); 91 ([C_7H_7]$^+$, 15.5). Anal. calcd. for $C_{18}H_{20}O_5$: C, 68.34; H, 6.37 found: C, 68.63; H, 6.36. CD (CH_3CN, c=31.0mM): λ_{max}=240nm, Δε=-2.3 L mol^{-1} cm^{-1}.

2-Methoxycarbonylmethyl-4-methyl-3-oxo-2,3,4,5,6,7-hexahydro-1H-indene-2-carboxylic acid methyl ester (8). Diester **8** was prepared starting from **7** (12.0 g, 80 mmol) analogous to the procedure described by Mangnus *et al.*[21] Yield: 89%. An analytically pure sample was obtained by recrystallisation from hexane/ethyl acetate. mp: 53.5-54.5°C. bp: 121.5°C (0.5 mm Hg). 400MHz ^{1}H NMR ($CDCl_3$): δ 1,09 (d, 3H, J=7.0Hz, CH_3); 1.43 (m, 1H, CH_3CHCH_2 A-ring); 1.67-1.80 (m, 3H, $CH_3CHCH_2CH_2$ A-ring); 2.23-2.55 (m, 3H, =CCH_2 A-ring, =CCH A-ring); 2.48 and 3.25 (AB, 2H, ^{2}J=17.1Hz; CH_2CO_2Me); 2.46 and 3.29 (AB, 2H, ^{2}J=17.3Hz; CH_2 B-ring); 3.67 and 3.69 (2xs, 2x3H, 2xOCH_3). ^{13}C NMR ($CDCl_3$): δ 18.4; 19.0; 26.1; 28.7; 30.1; 38.6; 41.6; 51.9; 52.9; 56.2; 139.8; 170.5; 171.6; 173.7; 202.0. MS [EI *m/z*, rel. intensity (%)]: 280 ([M]$^+$, 40.7); 220 ([$C_{13}H_{16}O_3$]$^+$, 100); 192 ([$C_{12}H_{16}O_2$]$^+$, 47.7); 133 ([$C_{10}H_{13}$]$^+$, 73.6); 105 ([C_8H_9]$^+$, 19.2); 91 ([C_7H_7]$^+$, 35.6). Anal. calcd for $C_{15}H_{20}O_5$: C, 64.27; H, 7.19 found: C, 64.48; H, 7.11.

4-Methyl-3-oxo-2,3,4,5,6,7-hexahydro-1H-inden-2-yl-acetic acid (9). Carboxylic acid **9** was synthesized from **8** (11.2 g, 40 mmol) in 97% yield according to the procedure reported by Mangnus *et al.*[21] and obtained as 1:1 mixture of *cis*- and *trans*-carboxylic acids **9**. Racemic *trans*-acid **9a** precipitated when mixture **9** was dissolved in diisopropyl ether. Recrystallization from diisopropyl ether gave acid **9a** in 87% d.e.

9a: mp: 111-113°C. (d.e. 87% (determined by NMR). 400MHz ^{1}H NMR ($CDCl_3$): δ 1,11 (d, 3H, J=7.0Hz, CH_3); 1.42 (m, 1H, CH_3CHCH_2 A-ring); 1.64-1.81 (m, 3H, $CH_3CHCH_2CH_2$ A-ring); 2.21-2.30 (m, 3H); 2.41 (dd, 1H, J=7.8Hz, 16.3Hz); 2.52 (m, 1H); 2.74-2.88 (m, 3H) (=CCH_2 A-ring, CH A-ring, CH B-ring, CH_2 B-ring, CH_2COOH). ^{13}C NMR ($CDCl_3$): δ 18.5; 19.1; 25.9; 28.8; 30.2; 35.5; 37.2; 41.5; 141.7; 173.0; 176.0; 209.3. MS [EI *m/z*, rel. intensity (%)]: 208 ([M]$^+$, 38.1); 190 ([$C_{12}H_{14}O_2$]$^+$, 36.7); 163 ([$C_{11}H_{15}O$]$^+$, 32.9); 147 ([$C_{11}H_{15}$]$^+$, 22.3); 105 ([C_8H_9]$^+$, 14.1); 91 ([C_7H_7]$^+$, 23.3); 28 ([CO]$^+$, 100). Anal. calcd for $C_{12}H_{16}O_3$: C, 69.21; H, 7.74 found: C, 69.24; H, 7.61. The *cis*-diastereomer of **9** could not be obtained in diastereomerically pure form.

8*(SR)*-Methyl-3,3a*(RS)*,4,5,6,7,8,8b*(SR)*-octahydro-indeno[1,2-b]furan-2-one (rac. 10a). To a solution of *trans*-carboxylic acid **9a** (2.7 g, 13 mmol) in methanol (100 mL) were added 2 equiv. of $CeCl_3.7H_2O$ (9.7 g, 26 mmol) dissolved in methanol (100 mL) and 4 equiv. of $NaBH_4$ (2.0 g, 52 mmol). The reaction mixture was stirred at room temperature for 3 hours and conversion was

followed by GC. When no further progress could be detected, another equiv. of $CeCl_3.7H_2O$ (4.8 g, 13 mmol) dissolved in methanol (50 mL) and 2 equiv. of $NaBH_4$ (0.98 g, 26 mmol) were added and stirring was continued overnight. Thereafter 1.5 equiv. of $NaBH_4$ was added and the reaction was allowed to stir until all starting material had disappeared. Methanol was evaporated *in vacuo* and the residue was dissolved in a mixture of diethyl ether and a 20% aqueous solution of H_2SO_4. The aqueous phase was extracted with diethyl ether (2 times) and the combined organic layers were dried ($MgSO_4$) and concentrated *in vacuo*. Silicagel column chromatography (hexane/ethyl acetate 6/1) yielded racemic *trans*-tricyclic lactone **rac. 10a** as a colourless oil. (2.0 g, 80%). d.e. 87%[30] (determined by NMR). 400MHz ^{1}H NMR ($CDCl_3$): δ 1,05 (d, 3H, J=7.0Hz, CH_3); 1.25 (m, 1H, H7); 1.60 (m, 1H, H6); 1.76 (m, 2H, H6+H7); 1.97 (m, 2H, 2xH5); 2.14 (dd, 1H, J=1.9Hz, 16.5Hz, H4); 2.29 (dd, 1H, J= 5.7Hz, 18.4Hz, H3); 2.36 (m, 1H, H8); 2.71 (dd, 1H, J=8.2Hz, 16.6Hz, H4); 2.83 (dd, 1H, J=10.7Hz, 18.3Hz, H3); 3.05 (m, 1H, H3a); 5.47 (d, 1H, J=7.5Hz, H8b). 400MHz 2D-NOESY NMR ($CDCl_3$): δ 5.31 (H8b) cross peak with δ 3.04 (H3a), δ 1.11 (CH_3) not with δ 2.32 (H8). ^{13}C NMR ($CDCl_3$): δ 18.6; 20.7; 26.1; 27.9; 31.2; 34.0; 36.6; 42.6; 89.9; 137.4; 140.9; 177.7. MS [EI *m/z*, rel. intensity (%)]: 192 ($[M]^+$, 72.8); 148 ($[C_{11}H_{16}]^+$, 49.0); 133 ($[C_{10}H_{13}]^+$, 100); 108 ($[C_8H_{12}]^+$, 89.6); 105 ($[C_8H_9]^+$, 64.3); 91 ($[C_7H_7]^+$, 76.4).

8*(RS)*-Methyl-3,3a*(RS)*,4,5,6,7,8,8b*(SR)*-octahydro-indeno[1,2-b]furan-2-one (rac. 10b). The reaction was performed as described for compound **rac. 10a.** The product was obtained, starting from the 2:1 mixture of *cis*- and *trans*-acids **9b** (5.4g, 26 mmol), as an oily mixture of *cis*- and *trans*-lactones **10** (4.6 g, 92%) from which *cis*-tricyclic lactone **rac. 10b** crystallized. Recrystallization from n-heptane provided **rac. 10b** in 87% d.e. mp: 54-57°C. d.e. 87%[30] (determined by NMR). 400MHz ^{1}H NMR ($CDCl_3$): δ 1,11 (d, 3H, J=7.1Hz, CH_3); 1.35 (m, 1H, H7); 1.55 (m, 1H, H6); 1.73 (m, 2H, H6+H7); 1.98 (m, 2H, 2xH5); 2.19 (bd, 1H, J=16.5Hz, H4); 2.32 (m, 1H, H8); 2.35 (dd, 1H, J=4.4Hz, 18.2Hz, H3); 2.61 (ddd, 1H, J=3.0Hz, 8.8Hz, 16.8Hz, H4); 2.81 (dd, 1H, J=10.4Hz, 18.0Hz, H3); 3.04 (m, 1H, H3a); 5.31 (d, 1H, J=7.3Hz, H8b). 400MHz 2D-NOESY NMR ($CDCl_3$): δ 5.31 (H8b) cross peak with d 3.04 (H3a), d 1.11 (CH_3) and d 2.32 (H8). ^{13}C NMR ($CDCl_3$): δ 19.9; 20.4; 26.2; 30.1; 31.6; 34.7; 36.2; 42.4; 92.6; 136.9; 142.8; 177.8. MS [EI *m/z*, rel. intensity (%)]: 193 ($[M+1]^+$, 100); 148 ($[C_{11}H_{16}]^+$, 13.0); 133 ($[C_{10}H_{13}]^+$, 79.3); 105 ($[C_8H_9]^+$, 37.0); 91 ($[C_7H_7]^+$, 15.0). HRMS/EI, *m/z* calcd for $C_{12}H_{16}O_2$: 192.11503, found: 192.11511 ± 0.00054.

8*(S)*-Methyl-3-[6'*(S)*-methyl-5'-oxo-4'-oxa-tricyclo[5.2.1.0$^{2',6'}$]dec-8'-en-3'*(R)*-yloxymethylene]-3,3a*(R)*,4,5,6,7,8,8b*(S)*-octahydro-indeno[1,2-b]furan-2-one (13a) and its 3a*(S)*,8*(R)*,8b*(R)* diastereomer **(ent. 13b).** To a cooled (0°C) and stirred solution of racemic *trans*-tricyclic lactone **rac. 10a** (576 mg, 3.0 mmol) in diethyl ether (15 mL) were added, under a continuous stream of nitrogen, 3 equiv. of ethyl formate (666 mg, 9 mmol) and 1.1 equiv. of potassium *tert*-butoxide (370 mg, 3.3 mmol). The mixture was stirred overnight at room temperature. Ether was removed by a nitrogen flow and the potassium salt of formylated **rac. 10a** was dissolved in DMF (20 mL)

and cooled to -50°C. A solution of chlorolactone **12** (596 mg, 3.0 mmol) in DMF (5 mL) was gradually added at -50°C under nitrogen. After stirring overnight the mixture was quenched with acetic acid (0.5 mL) and the solvent was removed under reduced pressure. The residue was dissolved in a mixture of water and ethyl acetate. The aqueous phase was extracted with ethyl acetate (2 times) and the combined organic layers were washed with brine (2 times), dried ($MgSO_4$) and concentrated *in vacuo*. The crude product was purified using flash chromatography (SiO2, hexane/ethyl acetate 4/1) to give fast moving diastereomer **13a** (444 mg, 39%) and slow moving diastereomer **ent. 13b** (558 mg, 49%) as colourless crystals, that were recrystallized from ethyl acetate.

13a: mp: 150-152°C. d.e. 93% (determined by NMR). $[\alpha]^{21}_D$=+174.8° (c=0.5, CH_2Cl_2). 400MHz ^{1}H NMR ($CDCl_3$): δ 1.06 (d, 3H, J=7.0Hz, 3xH9); 1.26 (m, 1H, H7); 1.56 (m, 4H, 3xH12'+H6); 1.74 (m, 4H, H6+H7+2xH10'); 1.94 (m, 2H 2xH5); 2.35 (m, 2H, H4+H8); 2.69 (d, 1H, J=4.1Hz, H2'); 2.76 (dd, 1H, J=8.8Hz, 15.6Hz, H4); 2.90 (m, 1H, H7'); 3.22 (m, 1H, H1'); 3.60 (m, 1H, H3a); 5.20 (d, 1H, J<1Hz, H3'); 5.47 (d, 1H, J=7.2Hz, H8b); 6.25 (2m, 2H, H8'+H9'); 7.34 (d, 1H, J=2.5Hz, H11'). ^{13}C NMR ($CDCl_3$): δ 18.6; 20.7; 22.6; 26.1; 27.8; 31.1; 36.5; 41.4; 45.2; 49.8; 52.0; 53.4; 54.5; 87.8; 103.8; 113.7; 133.5; 137.5; 137.8; 141.1; 151.0; 171.9; 178.7. MS [EI *m/z*, rel. intensity (%)]: 382 ($[M]^+$, 2.8); 316 ($[C_{18}H_{20}O_5]^+$, 1.6); 202 ($[C_{13}H_{14}O_2]^+$, 34.3); 163 ($[C_{10}H_{11}O_2]^+$, 89.2); 97 ($[C_5H_5O_2]^+$, 100); 91 ($[C_7H_7]^+$, 15.0). Anal. calcd. for $C_{23}H_{26}O_5$: C, 72.23; H, 6.85 found: C, 72.14; H, 6.81.

ent 13b: mp: 170-171°C. d.e. 87% (determined by NMR). $[\alpha]^{21}_D$=-234.0° (c=0.5, CH_2Cl_2). 400MHz ^{1}H NMR ($CDCl_3$): δ 1.05 (d, 3H, J=7.0Hz, 3xH9); 1.22 (m, 1H, H7); 1.55 (m, 1H, H6); 1.57 (s, 3H, 3xH12'); 1.68 (m, 1H, H6); 1.71 (m, 2H, 2xH10'); 1.75 (m, 1H, H7); 1.93 (m, 2H 2xH5); 2.29 (bd, 1H, J=16.5Hz, H4); 2.36 (m, 1H, H8); 2.70 (dd, 1H, J=8.2Hz, 16.5Hz, H4); 2.72 (d, 1H, J=4.2Hz, H2'); 2.88 (m, 1H, H7'); 3.21 (m, 1H, H1'); 3.58 (m, 1H, H3a); 5.19 (d, 1H, J<1Hz, H3'); 5.47 (d, 1H, J=7.2Hz, H8b); 6.25 (2m, 2H, H8'+H9'); 7.32 (d, 1H, J=2.6Hz, H11'). ^{13}C NMR ($CDCl_3$): δ 18.6; 20.7; 22.5; 26.0; 27.8; 31.2; 36.5; 41.1; 45.2; 49.8; 52.0; 53.4; 54.3; 87.8; 103.7; 113.8; 133.5; 137.3; 137.8; 141.3; 151.1; 171.8; 178.8. MS [EI *m/z*, rel. intensity (%)]: 382 ($[M]^+$, 1.0); 316 ($[C_{18}H_{20}O_5]^+$, 1.1); 202 ($[C_{13}H_{14}O_2]^+$, 29.5); 163 ($[C_{10}H_{11}O_2]^+$, 78.2); 97 ($[C_5H_5O_2]^+$, 100); 91 ($[C_7H_7]^+$, 13.2). Anal. calcd. for $C_{23}H_{26}O_5$: C, 72.23; H, 6.85 found: C, 72.16; H, 6.77.

8*(R)*-Methyl-3-[6'*(R)*-methyl-5'-oxo-4'-oxa-tricyclo[5.2.1.0$^{2',6'}$]dec-8'-en-3'*(S)*-yloxymethylene]-3,3a*(S)*,4,5,6,7,8,8b*(R)*-octahydro-indeno[1,2-b]furan-2-one (ent. 13a) and its 3a*(R)*,8*(S)*,8b*(S)* diastereomer **(13b).** These compounds were prepared in the same way as described for **13a** and **ent. 13b**, starting from tricyclic lactone **rac. 10a** (442 mg, 2.3 mmol) and chlorolactone **ent. 12** (457 mg, 2.3 mmol). Yields were 33% of fast moving diastereomer **ent. 13a** and 39% of slow moving diastereomer **13b,** which were both recrystallized from ethyl acetate.

ent 13 a: mp: 111-114°C.[31] d.e. 97% (determined by NMR). $[\alpha]^{21}_D$=-169.6° (c=0.5, CH_2Cl_2). ^{1}H NMR, ^{13}C NMR and mass data were identical to those of its enantiomer **13a**. Anal. calcd. for $C_{23}H_{26}O_5$: C, 72.23; H, 6.85 found: C, 72.40; H, 6.89

13b: mp: 172-173°C. d.e. 95% (determined by NMR). $[\alpha]^{21}_D$=+232.8° (c=0.5, CH_2Cl_2). ^1H NMR, ^{13}C NMR and mass data were identical to those of its enantiomer **ent. 13b**. Anal. calcd. for $C_{23}H_{26}O_5$: C, 72.23; H, 6.85 found: C, 72.09; H, 6.80

8*(R)*-Methyl-3-[6'*(S)*-methyl-5'-oxo-4'-oxa-tricyclo[5.2.1.0$^{2',6'}$]dec-8'-en-3'*(R)*-yloxymethylene]-3,3a*(R)*,4,5,6,7,8,8b*(S)*-octahydro-indeno[1,2-b]furan-2-one (13c) and its 3a*(S)*,8*(S)*,8b*(R)* diastereomer **(ent 13d).** These compounds were prepared in the same way as described for **13a** and **ent. 13b**, starting from tricyclic lactone **rac. 10b** (442 mg, 2.3 mmol) and chlorolactone **12** (457 mg, 2.3 mmol). Yields were 28% of fast moving diastereomer **13c** and 35% of slow moving diastereomer **ent. 13d**, which were both recrystallized from ethyl acetate.

13c: mp: 240-242°C. d.e. >99% (determined by NMR). $[\alpha]^{21}_D$=+182.4° (c=0.5, CH_2Cl_2). 400MHz ^1H NMR ($CDCl_3$): δ 1.12 (d, 3H, J=7.0Hz, 3xH9); 1.34 (m, 1H, H7); 1.54 (m, 1H, H6); 1.58 (s, 3H, 3xH12'); 1.72 (m, 4H, H6+H7+2xH10'); 1.94 (m, 2H 2xH5); 2.33 (m, 2H, H4+H8); 2.68 (d, 1H, J=4.1Hz, H2'); 2.71 (ddd, 1H, J=3.1Hz, 9.3Hz, 16.8Hz, H4); 2.89 (m, 1H, H7'); 3.22 (m, 1H, H1'); 3.60 (m, 1H, H3a); 5.20 (d, 1H, J<1Hz, H3'); 5.33 (d, 1H, J=7.7Hz, H8b); 6.25 (2m, 2H, H8'+H9'); 7.33 (d, 1H, J=2.4Hz, H11'). ^{13}C NMR ($CDCl_3$): δ 19.8; 20.3; 22.6; 26.2; 30.1; 31.6; 36.9; 41.5; 45.2; 49.8; 52.0; 53.4; 54.5; 90.7; 103.8; 113.7; 133.5; 136.8; 137.8; 142.6; 150.7; 171.9; 178.8. MS [EI *m/z*, rel. intensity (%)]: 382 ([M]$^+$, 0.8); 316 ([$C_{18}H_{20}O_5$]$^+$, 0.5); 202 ([$C_{13}H_{14}O_2$]$^+$, 28.3); 163 ([$C_{10}H_{11}O_2$]$^+$, 80.2); 97 ([$C_5H_5O_2$]$^+$, 100); 91 ([C_7H_7]$^+$, 8.2). Anal. calcd. for $C_{23}H_{26}O_5$: C, 72.23; H, 6.85 found: C, 72.09; H, 6.80.

ent. 13d: mp: 217-218.5°C. d.e. >99% (determined by NMR). $[\alpha]^{21}_D$=-242.8° (c=0.5, CH_2Cl_2). 400MHz ^1H NMR ($CDCl_3$): δ 1.12 (d, 3H, J=7.0Hz, 3xH9); 1.37 (m, 1H, H7); 1.54 (m, 1H, H6); 1.58 (s, 3H, 3xH12'); 1.71 (m, 2H, H6+H7); 1.73 (m, 2H, 2xH10'); 1.94 (m, 2H 2xH5); 2.31 (m, 2H, H4+H8); 2.66 (ddd, 1H, J=3.2Hz, 9.0Hz, 16.8Hz, H4); 2.73 (d, 1H, J=4.2Hz, H2'); 2.90 (m, 1H, H7'); 3.22 (m, 1H, H1'); 3.60 (m, 1H, H3a); 5.20 (d, 1H, J<1Hz, H2'); 5.35 (d, 1H, J=7.8Hz, H8b); 6.25 (2m, 2H, H8'+H9'); 7.33 (d, 1H, J=2.5Hz, H11'). ^{13}C NMR ($CDCl_3$): δ 20.0; 20.2; 22.5; 26.2; 30.1; 31.6; 36.7; 41.1; 45.1; 49.8; 51.9; 53.4; 54.3; 90.7; 103.6; 113.7; 133.5; 136.5; 137.8; 142.7; 150.8; 171.8; 178.8. MS [EI *m/z*, rel. intensity (%)]: 382 ([M]$^+$, 0.9); 316 ([$C_{18}H_{20}O_5$]$^+$, 0.5); 202 ([$C_{13}H_{14}O_2$]$^+$, 20.4); 163 ([$C_{10}H_{11}O_2$]$^+$, 64.1); 97 ([$C_5H_5O_2$]$^+$, 100); 91 ([C_7H_7]$^+$, 4.1). Anal. calcd. for $C_{23}H_{26}O_5$: C, 72.23; H, 6.85 found: C, 72.21; H, 6.76.

8*(S)*-Methyl-3-[6'*(R)*-methyl-5'-oxo-4'-oxa-tricyclo[5.2.1.0$^{2',6'}$]dec-8'-en-3'*(S)*-yloxymethylene]-3,3a*(S)*,4,5,6,7,8,8b*(R)*-octahydro-indeno[1,2-b]furan-2-one (ent. 13c) and its 3a*(R)*,8*(R)*,8b*(S)* stereoisomer **(13d).** These compounds were prepared in the same way as described for **13a** and **ent. 13b**, starting from tricyclic lactone **rac. 10b** (442 mg, 2.3 mmol) and chlorolactone **ent. 12** (457 mg, 2.3 mmol). Yields were 46% of fast moving diastereomer **ent. 13c** and 40% of slow moving diastereomer **13d**, which were both recrystallized from ethyl acetate.

ent. 13c: mp: 238-240°C. d.e. 97% (determined by NMR). $[\alpha]^{21}_D$=-187.0° (c=0.5, CH_2Cl_2). ^1H NMR, ^{13}C NMR and mass data were identical to those of its enantiomer **13c.** Anal. calcd. for $C_{23}H_{26}O_5$: C, 72.23; H, 6.85 found: C, 71.69; H, 6.79

13d: mp: 217-219°C. d.e. 94% (determined by NMR). $[\alpha]^{21}_D$=+250.2° (c=0.5, CH_2Cl_2). ^1H NMR, ^{13}C NMR and mass data were identical to those of its enantiomer **ent. 13d**. Anal. calcd. for $C_{23}H_{26}O_5$: C, 72.23; H, 6.85 found: C, 72.52; H, 6.84

Biological Activity.

Plant material. Seeds of *Striga hermonthica* (Del.) Benth. (from *Sorghum bicolor* (L.) Moench) and *Orobanche crenata* Forsk. (from *Vicia faba* L.) were harvested in Burkina Faso in 1994 and Egypt in 1991 respectively, and were stored in the dark at room temperature until use in germination tests. Bioassays were carried out essentially following the procedure of Mangnus *et al.*[32] with minor modifications.

Preparation of test Solutions. A compound to be tested was weighed out accurately to the amount of 1 mg, dissolved in 1 mL acetone p.a. and diluted with demineralized water to 100 mL. Aliquots of this stock solution were diluted further with water to obtain test solutions containing 0.1, 0.01, 0.001 and 0.0001 mg/L test compound and 0.01, 0.001, 0.0001 and 0.00001% (v/v) acetone respectively.

Bioassays. The sterilization, conditioning and stimulation of the seeds were performed as described by Mangnus. *et al.*[30] for *Orobanche crenata* and as described by Kuiper[33] for *Striga hermonthica.* In each test series aqueous solutions with 0.1% (v/v) acetone were used as the control. Test solutions of the stimulant GR 24 (concentrations of 0.1, 0.01, 0.001 and 0.0001 mg/L) were used as references, which enables comparison among results obtained in different test series. These positive controls are important, since the response of seeds of parasitic weeds varies considerably from test to test. All tests were performed at least in duplicate and in each test the germination percentages were determined on 6 disks per treatment.

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33 Kuiper, E. Comparative studies on the parasitism of *Striga aspera* and *Striga hermonthica* on tropical grasses. Ph.D. Thesis, Free University Amsterdam, The Netherlands, ISBN 90-5651-035-5, **1997**.

4

Dose-Response of Seeds of the Parasitic Weeds *Striga* and *Orobanche* towards the Synthetic Germination Stimulants GR 24 and Nijmegen-1

Abstract *Striga* and *Orobanche* seeds germinate in response to a host derived germination stimulant. Dose-response curves of the synthetic strigolactone analogues GR 24 and Nijmegen-1 were determined and their activities were compared to that of the naturally occurring stimulant sorgolactone. Typical sigmoidal curves were obtained. ED_{50} values for GR 24 were in the order of 10^{-9} to 10^{-8} mol/L; for Nijmegen-1 these values were three orders of magnitude higher. Both synthetic stimulants are appreciably active at low concentrations and merit investigation as agents for the suicidal germination approach (*i.e.* treatment of the soil with stimulant in the absence of a host).

Introduction

The root parasitic weeds *Striga* (witchweed) and *Orobanche* (broomrape) are serious pests in agriculture.[1,2] *Orobanche* parasitizes dicotyledonous crops, such as legumes, tomato and sunflower and predominantly occurs in the Mediterranean region. *Striga* mainly infects cereals such as sorghum, maize and millet in tropical and subtropical areas. The lives of millions of people in Africa, India and the Middle East are directly affected by the severe harvest reductions, due to heavy infestations of susceptible crops with these parasites.[3] *S. hermonthica* and *S. asiatica* are the species which cause the most economically significant damage to cereals.[4]

The lifecycle of the parasitic weeds is closely adapted to that of their host plants. The seeds of the parasites only germinate if they are exposed to stimulant molecules, which are present in the root exudate of a suitable host plant.[1,2]

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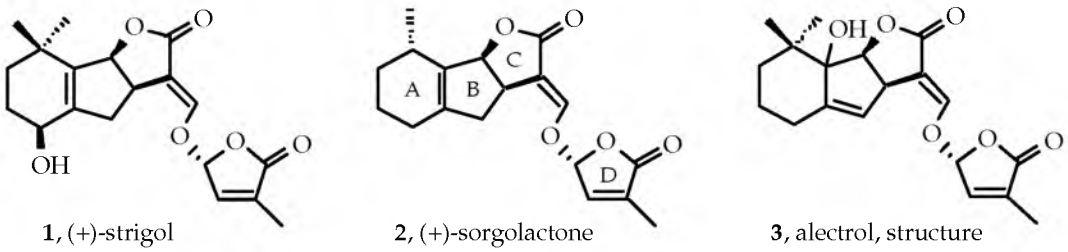
Figure 1



1, (+)-strigol

2, (+)-sorgolactone

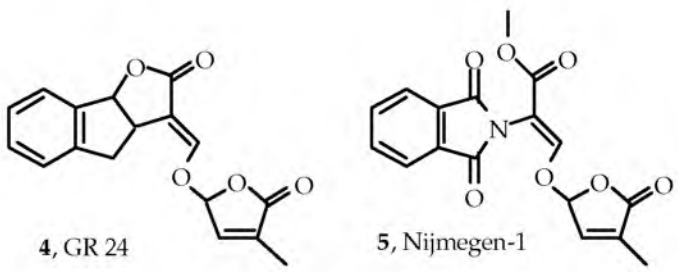
3, alectrol, structure proposed by Müller *et al.*

Thus far, three naturally occurring germination stimulants, belonging to the class of the strigolactones[4], *viz.* strigol[5] (**1**), sorgolactone[6] (**2**) and alectrol[7,8] (**3**) have been isolated from *Striga* hosts (Figure 1). Recently, another strigolactone germination stimulant, orobanchol, was isolated from the *Orobanche* host red clover[9] (see chapter 1).

To control parasitic weeds, various methods have been developed over the years, among others, hand pulling, fallow, crop rotation, resistant crops and the use of herbicides. Hand weeding is not very practical, as when the parasite plant emerges from the soil, most of the damage to the host crop has already been done. Crop rotation with non-host crops which do stimulate *Striga* seed germination, but which are not parasitized themselves (*e.g.* cotton) in addition to periods of fallow have been used to keep *Striga* infestations at tolerable levels.[10] However, growth of the human population and the increasing demand for production of food lead to intensified use of land, with more monocropping and little or no fallow, thus enlarging the numbers of parasite seeds. Although the development of resistant crops is quite difficult, the approach is promising and recently some success has been achieved.[11] The use of herbicides is, in general, not attractive from an environmental point of view and the cost of sophisticated chemicals limits their use in the developing countries. So far, none of the above mentioned methods is very effective in eradicating *Striga* and *Orobanche*.[2,12,13]

In principle, an alternative approach to control *Striga* infestations is the reduction of the amount of parasite seeds in the soil by suicidal germination, *i.e.* the application of a synthetic germination stimulant to the soil, before planting the desired crop.[14,15] The *Striga* seeds will germinate, but, in the absence of a suitable host plant, the seedlings will die. This method should generally be applicable for all *Striga* and *Orobanche* infestations, provided a suitable germinating agent is available.

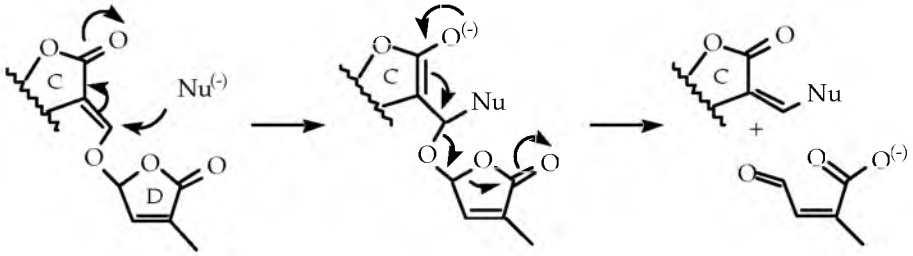
Figure 2



4, GR 24

5, Nijmegen-1

Scheme 1



A germination stimulant with a simplified strigolactone structure, which can readily be prepared in large quantities would be an attractive candidate in this suicidal germination approach. In this context, the synthesis of strigol[16] (**1**), sorgolactone[17] (**2**) and their analogues *e.g.* GR 24 (**4**) (Figure 2) has received much attention.[18,19,20,21] A standardized bioassay to determine the germination inducing activity of a compound was developed and structure activity studies were conducted.[22] These studies revealed that the bioactiphore resides in the CD-part of the strigolactone molecule and a tentative molecular mechanism for the induction of germination was proposed.[23]

This mechanism involves addition of a nucleophilic species, present at the receptor site, in a Michael fashion, followed by elimination of the D-ring. The ultimate result is that the ABC-part of the stimulant is covalently bound to the receptor, a chemical change that may be responsible for triggering germination (Scheme 1).

GR 24 (**4**) is a very potent synthetic germination stimulant[18,24], which is used world wide in parasitic weed research to stimulate germination and as a standard for comparison of new germinating agents (Figure 2). Despite its frequent use, dose-response curves of GR 24 have never been reported, except in one paper on *O. crenata* seeds.[25] Multigram scale production of GR 24 is not (yet) economically feasible, which prevents its use as a suicidal germination agent. Nijmegen-1 (**5**), which has a much more simplified structure[26], was designed to contain the essential structural features required for bioactivity, according to the mechanism depicted in scheme 1. Nijmegen-1 can easily be prepared in multigram quantities and is an attractive candidate for *Striga* control by the suicidal germination approach (Figure 2).

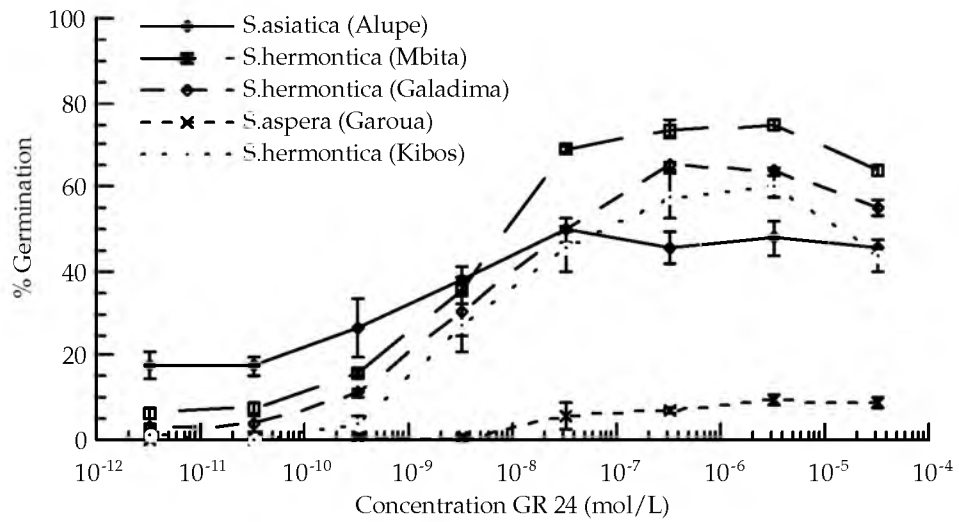
The aim of the research presented in this chapter, was to determine the dose-response curves of the synthetic germination stimulants GR 24 (**4**) and Nijmegen-1 (**5**) for several *Striga* species and *Orobanche crenata*. This information is highly relevant to the possible use of these stimulants in the suicidal germination approach in the field. The activities of **4** and **5** were compared with that of the natural stimulant sorgolactone (**2**). In addition, a modified procedure for the preparation of GR 24 is presented.

Results and Discussion

The strigolactone analogue GR 24 is used world wide in parasitic weed research, often as a reference compound in bioassays in which the germination inducing activity of a certain compound is investigated. In these assays, usually only two or three concentrations of the compounds are tested, frequently 1, 0.1 and/or 0.01 mg/L. It is important to note here, that the unit mol/L is a better way to express concentrations, because the molecular masses of the compounds tested are usually not the same. The percentages of germinated seeds of five different *Striga* populations were determined, induced by a racemic mixture of GR 24 (the form in which it is usually applied) at eight concentrations, ranging from 10^{-12} to 10^{-5} mol/L. Details of the populations used are given in the materials and methods section and the resulting dose-response curves are depicted in figure 3.

Figure 3 reveals that not all *Striga* populations responded equally well to stimulation by GR 24. *S. aspera* was hardly sensitive to GR 24, although it did give much higher percentages of germinated seeds (up to 40%) when stimulated with maize root exudate.[35] This is an example of a seed population that is obviously very sensitive to the structure of its original stimulant, present in maize root exudate. This phenomenon was observed for a variety of *S. aspera* populations.[35] The majority of the populations investigated here, however, responded well to GR 24. Germination generally began at a GR 24 concentration of 10^{-10} mol/L and reached a maximum value when [GR 24] was 10^{-7} mol/L. When the stimulant concentration became higher than 10^{-5} mol/L a decrease in the germinating activity was observed. The concentration that induces half-maximal response, the ED_{50} value, is for GR 24 in all cases approximately 5 x 10^{-9} mol/L. The maximum percentages of germinated seeds are quite different and are apparently species dependent.

Figure 3. Percentage germination of several populations of Striga seeds stimulated by aqueous solutions of the synthetic germination stimulant GR 24. Data presented are the mean ± S.E. of one representative experiment.



In addition there is also a certain host specificity, which might explain a different response of various *Striga* populations, belonging to the same species, to the same synthetic stimulant. If GR 24 is considered as a reference compound in experimental set-ups, a concentration should be used in the range where maximum germination is induced (1 mg/L corresponds to 3.35×10^{-6} mol/L and is a good choice), as well as a concentration around the ED_{50} value, *i.e.* 5×10^{-9} mol/L.

The dose-effect curves depicted in figure 3 were determined in 1994. Recently the synthesis of all 4 diastereomers of GR 24 was described.[27] Biological testing revealed that only the GR 24 diastereomer possessing the same stereochemical configuration (at all its stereocenters) as the naturally occurring strigolactones, expressed significant biological activity at the sensitive concentration of 0.001 mg/L (= 3.35×10^{-9} mol/L). The same concentration of racemic GR 24 (which only contains 25% of the "natural configuration" isomer) induced equally high numbers of seeds to germinate. Because the racemic form is always used in parasitic weed research and its activity is comparable to that of the "natural" GR 24 diastereomer, only racemic GR 24 was included in the experiments discussed below.

In 1997, the synthesis of Nijmegen-1 (**5**) was reported by Nefkens *et al.*[26] This strigolactone analogue contains only one stereogenic center, and therefore its synthesis is not complicated by the formation of diastereomers, which would require chromatographic separation. Nijmegen-1 can be synthesized in only 2 steps, provided the D-ring synthon is available. Because of its biological activity in germinating parasitic weed seeds, and its easy preparation, Nijmegen-1 may be a potential candidate for suicidal germination in the field.

In 1998, all diastereomers of the naturally occurring stimulant sorgolactone were prepared as single stereoisomers in the Nijmegen laboratory.[17] This enabled us to compare the activity of GR 24 (**4**) and Nijmegen-1 (**5**) with the biological activity of the naturally occurring diastereomer of sorgolactone (**2**). To this end two *S. hermonthica* populations were selected (a population from maize in Kana, Benin and a population from sorghum in Shire, Ethiopia), as well as a population of *S. asiatica* because these species are the most economically significant ones. A population of *O. crenata* was also included. First, concentrations of 10^{-15}, 10^{-14}, 10^{-13} etc. up to 10^{-4} mol compound/L were assayed (data not shown). This rough test clearly indicated that in almost every case, germination started when the stimulant concentration was at least 10^{-10} mol/L and that maximum percentages of germination were generally obtained at stimulant concentrations $\geq 10^{-6}$ mol/L. Therefore, a more refined selection of test solutions was prepared with which more data points were collected in the sensitive regions of the germination curves. The resulting curves are depicted in Figures 4, 5, 6 and 7 for *S. hermonthica* (maize), *S. hermonthica* (sorghum), *S. asiatica* and *O. crenata* respectively. In all cases the percentage germination induced by the aqueous control solution was practically zero.

Figure 4. Percentage germination of Striga hermonthica seeds (collected from maize) stimulated by aqueous solutions of GR 24, Nijmegen-1 and sorgolactone. Data presented are the mean ± S.E. of one representative experiment.

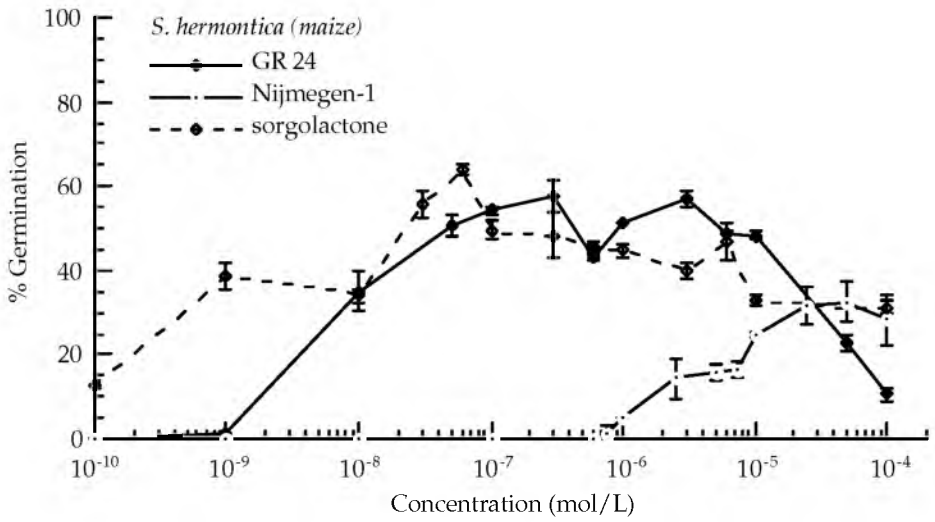


Figure 5. Percentage germination of Striga hermonthica seeds (collected from sorghum) stimulated by aqueous solutions of GR 24, Nijmegen-1 and sorgolactone. Data presented are the mean ± S.E. of one representative experiment.

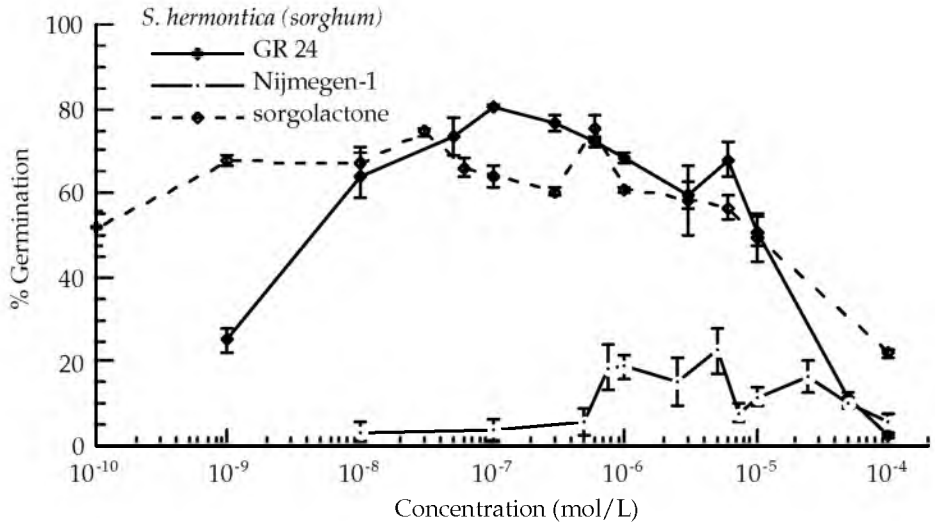


Figure 6. Percentage germination of Striga asiatica seeds (collected from maize) stimulated by aqueous solutions of GR 24, Nijmegen-1 and sorgolactone. Data presented are the mean ± S.E. of one representative experiment.

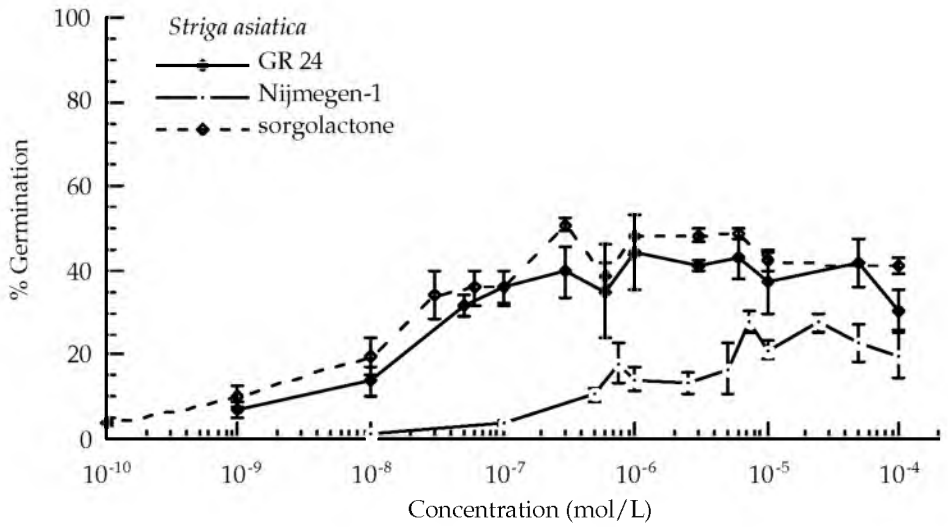
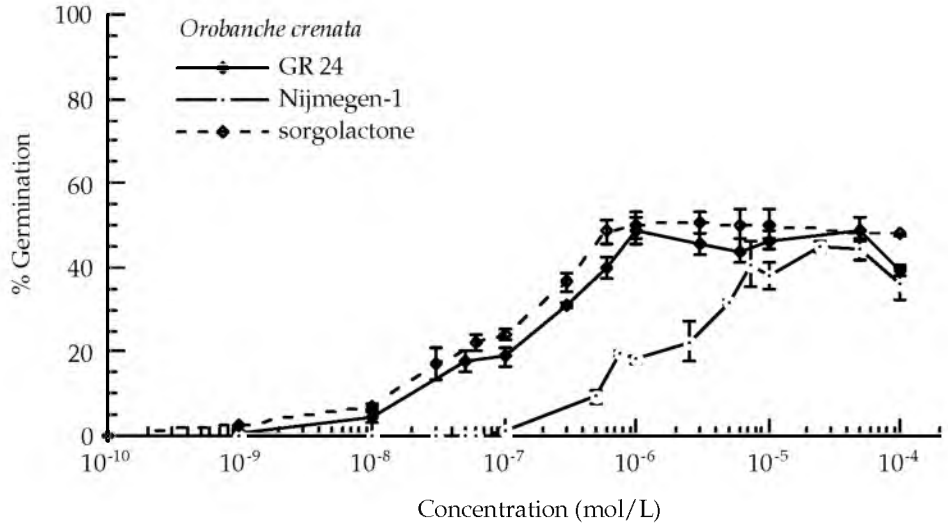


Figure 7. Percentage germination of Orobanche crenata seeds (collected from faba bean) stimulated by aqueous solutions of GR 24, Nijmegen-1 and sorgolactone. Data presented are the mean ± S.E. of one representative experiment.



The most striking result, which is evident from all four figures, is the extremely high activity of GR 24. For every seed population that was used in this experiment, racemic GR 24 is as active as the single isomer of sorgolactone. This is in agreement with the results of Thuring *et al.*[27] ED_{50} values and maximum percentages of germinated seeds induced by GR 24, Nijmegen-1 and sorgolactone are summarized in table 1.

Table 1. ED_{50} values and maximum percentages of germinated seeds obtained when four populations of parasitic weed seeds were stimulated with solutions of GR 24, Nijmegen-1 and sorgolactone. n.d. = not determined.

population	GR 24		Nijmegen-1		sorgolactone	
	ED_{50} (mol/L)	max. % germination	ED_{50} (mol/L)	max. % germination	ED_{50} (mol/L)	max. % germination
S. hermonthica (maize)	6×10^{-9}	57.5	5×10^{-6}	32.5	5×10^{-10}	64.0
S. hermonthica (sorghum)	2×10^{-9}	80.6	n.d.	22.5	$<10^{-10}$	74.4
S. asiatica	2×10^{-8}	44.4	6×10^{-7}	28.0	2×10^{-8}	50.9
O. crenata	2×10^{-7}	49.0	2×10^{-6}	44.9	10^{-7}	50.8

In complete agreement with the results obtained in 1994 (Figure 3), the ED_{50} values of GR 24 are between 10^{-9} and 10^{-8} mol/L. In the case of *O. crenata* half-maximum percentages of germination are obtained at stimulant concentrations at least two orders of magnitude higher than in the case of *Striga*. This trend was also observed in the bioassay of all diastereomers of sorgolactone.[17] Sorgolactone is the major germination stimulant exuded by sorghum roots, whereas in the case of maize this is strigol.[28] The lower ED_{50} value of sorgolactone in stimulating *S. hermonthica* from sorghum as compared to that for *S. hermonthica* from maize may be an example of a host specific response of parasitic weed seeds.[29] From the results summarized in table 1, it can also be concluded that *O. crenata* is less sensitive to structural modifications of the applied germination stimulant than *Striga:* in the case of Nijmegen-1, sorgolactone and GR 24 approximately the same maximum percentage of germinated seeds was obtained and there is also not much difference in their respective ED_{50} values. As for *S. asiatica,* its response is somewhat intermediate between those of *S. hermonthica* and *O. crenata.* The ED_{50} values of the three compounds are not as different as the maximum percentages of germination induced by each stimulant.

Nijmegen-1 is considerably less active in stimulating the germination of *Striga* seeds: its ED_{50} values are up to 3 orders of magnitude higher than those of GR 24 and sorgolactone and also the maximum percentages of germination that were observed are significantly lower than the corresponding values determined for GR 24 and sorgolactone. This drawback could be overcome by repeated applications of the compound to soils infested with parasitic

weed seeds. Its activity and the fact that Nijmegen-1 can easily be prepared in large quantities, from cheap starting materials does merit further investigations of this compound in the field.

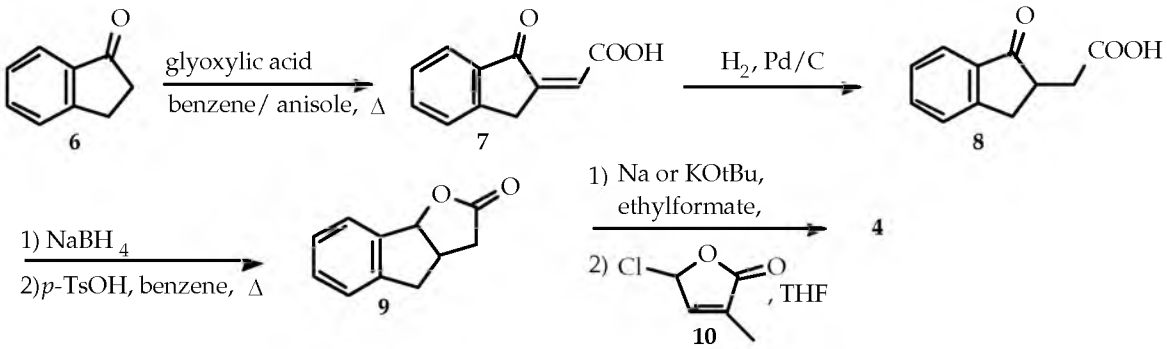
The results presented above clearly show that the dose-response curves of sorgolactone, Nijmegen-1 and GR 24 have a sigmoidal shape. In the case of GR 24 germination generally starts at a concentration of 10^{-9} mol/L and reaches a maximum value at concentrations of 10^{-7} mol/L. At concentrations below 10^{-10} mol/L any germination inducing activity was never observed. We therefore have doubts about the validity of the results published by Rugutt and Rugutt.[30] They reported a non-sigmoidal dose-response curve of GR 24, on *S. hermonthica* seeds, with germination values varying from 60-80% at concentrations between 10^{-21} and 10^{-2} mol/L. Even at the lowest concentration they observed 70% germination.

The main conclusion from the results presented above is that GR 24 is clearly the superior candidate for application as a suicidal germination agent in *Striga* control. However, the synthesis of GR 24 does not yet allow large scale production of the compound in an economically feasible manner. Recently, a few improvements were made in the synthesis of the ABC-part. Following the route presented in scheme 2, the entire ABC-part can be constructed without the necessity of any distillation or chromatography procedure, which is highly desirable from an industrial point of view.

Regarding the stability and effectiveness of GR 24 and its analogue GR 7 in soil, two accounts of preliminary research have been published.[31,32] The conclusions were that soil moisture and pH have a strong influence on stimulant activity. The compounds were stable at neutral and acidic pH, whereas in alkaline soil the activity decreased rapidly. Most likely the instability of strigolactones under basic conditions can be explained by the reaction of hydroxide ion as the nucleophile in a Michael fashion, followed by hydrolysis of the lactone rings (see scheme 1). Also, microbial degradation may be a cause for the breakdown of strigolactones in soil. These instability problems can be overcome by creating an appropriate controlled release formulation.

Our laboratory experiments show that GR 24 as well as Nijmegen-1 are active germination stimulants for many species of the parasitic weeds *Striga* and *Orobanche* in the concentration range of 10^{-9}-10^{-5} mol/L. However, to evaluate the usefulness of these compounds in parasitic weed control, field studies should be undertaken to investigate the behaviour of GR 24 and Nijmegen-1 in soil. Large scale preparation of Nijmegen-1 is easy and this counterbalances its diminished activity as compared to that of GR 24. Furthermore, with the simplified synthesis of GR 24 presented above, multigram preparation of this compound is now feasible, which allows large scale field studies at relatively low costs. Both synthetic stimulants have great potential in the control of parasitic weed pests.

Scheme 2. Modified synthesis of GR 24.



Experimental

Synthesis. General methods and instrumentation are the same as described in chapter 5.

Nomenclature. Systematic names were generated using the ACD/Name programme, provided by Advanced Chemistry Development Inc. (Toronto, Canada).

8*(S)*-Methyl-3-[4'-methyl-5'-oxo-2',5'-dihydro-furan-2'*(R)*-yloxymethylene]-3,3a(R),4,5,6,7,8,8b *(S)*-octahydro-indeno[1,2-b]-furan-2-one (2). The naturally occurring diastereomer of sorgolactone was synthesized as reported by Sugimoto *et al.*[17]

3-[(2,5-dihydro-3-methyl-2-oxo-5-furanyl)oxymethylene]-3,3a,4,8b-tetrahydroindeno[1,2-b]-furan-2-one (4). Racemic GR 24 (an equimolar mixture of four diastereomers) was essentially prepared as described by Mangnus *et al.* [24] with modifications in the synthesis of the ABC-part *(vide infra).*

Methyl 2-(1,3-dioxo-1,3-dihydroisoindol-2-yl)-3-[4-methyl-5-oxo-2,5-dihydrofuran-2-yloxy]-acrylate (5). Racemic Nijmegen-1 (an equimolar mixture of two enantiomers) was prepared as described previously.[26]

2-(1-oxo-2,3-dihydro-1H-2-indanyliden)acetic acid (7). 65.1 g (0.5 mol) of indanone (**6**) was dissolved in 150 mL anisole. To this solution was added 50 g (0.54 mol) of glyoxylic acid monohydrate in 100 mL benzene. The mixture was heated under reflux overnight using Dean-Stark conditions. When no more starting material was present, the reaction mixture was cooled to 0 °C whereupon the product precipitated. The white precipitate was filtered off, washed with plenty of diisopropyl ether and air dried. Yield: 79%; Analytical data were in complete accordance with those reported in the literature.[33]

2-(1-oxo-2,3-dihydro-1H-2-indenyl)acetic acid (8). 18.8 g (0.1 mol) of acid **7** was dissolved in a mixture of methanol (35 mL) and water (35 mL) in which 6 g (0.1 mol) of potassium hydroxide was dissolved, to enhance solubilization of the starting material. A catalytic amount of palladium on activated charcoal was added and the mixture was hydrogenated

employing a Parr apparatus until hydrogen uptake ceased. Then the solution was filtered over hyflo and methanol was evaporated under reduced pressure. 150 mL of water was added and the product was precipitated by slow addition of 6N HCl. The resulting white solid was washed with water (1x), diisopropyl ether (1x) and dried *in vacuo*. Yield: 14.6 g (77%); Analytical data were in complete accordance with those reported previously.[24,34]

The following steps in the reaction sequence depicted in scheme 2 were performed as described by Mangnus *et al.* [24]

Biological Activity

Plant material. Five populations of *Striga hermonthica* (Del.) Benth. seeds were used. They were collected from the following host plants: Pearl millet *(Pennisetum americanum* (L.) K. Schum.) in Galadima, Nigeria in 1988; Sorghum *(Sorghum bicolor* (L.) Moench) on Mbita-point Road near Homa Bay, Kenya in 1993; Sorghum *(Sorghum bicolor* (L.) Moench) in Shire, Ethiopia in 1996; Wild sorghum *(Sorghum arundinaceum* (Desv.) Stapf.) in Kibos, Kenya in 1993; Maize *(Zea mays* L.) in Kana, Benin in 1993. *S. asiatica* (L.) Kuntze seeds were harvested in Alupe, Kenya from finger millet *(Eleusine coracana* (L.) Gaertn) and maize *(Zea mays* L.) in 1993 and 1995 respectively. *S. aspera* (Willd.) Benth. seeds from maize *(Zea mays* L.) were obtained in Garoua, Nigeria in 1989. *Orobanche crenata* Forsk. seeds were harvested from faba bean *(Vicia faba* L.) in Beheira, Egypt in 1993. All seeds were stored in glass vials in the dark at room temperature until use in germination tests.

Preparation of the test solutions. For the bioassay performed in 1994: Exactly 5 mg of GR 24 was dissolved in 1 mL of acetone p.a. and diluted with demineralized water to 500 mL. Thus a GR 24 solution of 10 mg/L (3.35 x 10^{-5} mol/L) was obtained. A stock solution of 1 mg GR 24/L (3.35 x 10^{-6} mol/L) was prepared as follows: 5 mg GR 24 was dissolved in 10 mL acetone p.a. and 1 mL of this solution was diluted with demineralized water to 500 mL. From this stock, GR 24 test solutions of 3.35 x 10^{-7} to 3.35 x 10^{-12} mol/L were prepared. For the bioassay performed in 1998: A compound to be tested was weighed out very accurately to the amount of 1.5 mg, dissolved in 1 mL acetone p.a. and diluted with demineralized water to 5 mL. These stock solutions of approximately 10^{-3} mol/L (the exact concentration depending on the molecular mass of the compound used) were further diluted with demineralized water to obtain test solutions with concentrations ranging from 10^{-4} to 10^{-15} mol/L. All solutions were prepared one day prior to use.

Bioassays. All bioassays were performed at the Department of Ecology and Ecotoxicology of Plants of the Vrije Universiteit in Amsterdam, The Netherlands in 1994 and 1998. For surface sterilization all seeds were exposed to 70% (v/v) aqueous ethanol for 5 minutes and then for 2 minutes to a 50% (v/v) aqueous solution of commercial bleach (2% hypochlorite). Subsequently, the seeds were thoroughly rinsed with demineralized water and air-dried. For conditioning the seeds were spread on glass fiber filter paper disks (8 mm diameter, approximately 60-100 seeds per disk) in Petri dishes, wetted with demineralized water and

incubated in the dark at 20 °C for *Orobanche* and at 30 °C for *Striga*. Thereafter the conditioning water was removed and the seeds were placed in new Petri dishes and exposed to the test solutions (100µL per disk). After incubation for 24 hours*(Striga)* and 5 days *(Orobanche)* in the dark at the indicated temperatures the percentage of germinated seeds was determined under a microscope. Seeds were considered germinated when the radicle protruded through the seed coat. In each test series an aqueous solution of 0.1% (v/v) acetone was included as a negative control. For full details of the bioassay see Mangnus *et al.* and Kuiper.[22,35] All tests were performed twice with three replicates per test solution. Each replicate contained 2 disks with parasite seeds. Data handling was performed using Microsoft Excel. For each replicate the mean percentage of germinated seeds was calculated. Consequently, the mean percentage of germinated seeds of all three replicates (for each test solution) was determined as well as the S.E. value of the mean. Numerical values of the dose-response curves depicted in figures 3-7 are collected in tables 2-5.

Table 2. Germination percentages ± S.E. for seeds of several Striga populations after exposure to various concentrations of GR 24. (Data corresponding with figure 3).

[GR 24] (mol/L)	*S. hermonthica* (Mbita)	*S. hermonthica* (Galadima)	*S. hermonthica* (Kibos)	*S. asiatica* (Alupe)	*S. aspera* (Garoua)
3.35×10^{-5}	63.8 ± 1.4	55.2 ± 1.8	43.6 ± 3.6	45.7 ± 1.6	9.0 ± 1.2
3.35×10^{-6}	74.8 ± 1.3	63.8 ± 0.9	60.1 ± 2.8	48.0 ± 4.1	9.5 ± 1.4
3.35×10^{-7}	73.5 ± 2.2	64.9 ± 0.9	57.8 ± 5.5	45.6 ± 4.0	7.0 ± 0.8
3.35×10^{-8}	69.0 ± 0.7	49.9 ± 2.9	45.3 ± 5.6	50.1 ± 0.5	5.7 ± 3.4
3.35×10^{-9}	35.5 ± 2.9	30.1 ± 5.4	27.5 ± 6.5	38.0 ± 2.9	0.6 ± 0.4
3.35×10^{-10}	15.6 ± 0.6	11.1 ± 1.0	4.0 ± 1.9	26.5 ± 6.8	0.5 ± 0.5
3.35×10^{-11}	7.6 ± 1.4	3.9 ± 2.0	0.3 ± 0.3	17.5 ± 2.1	n.d.
3.35×10^{-12}	6.5 ± 1.1	3.0 ± 0.6	1.3 ± 0.6	17.8 ± 3.2	n.d.

n.d. = not determined.

Table 3. Germination percentages ± S.E. for seeds of several Striga populations and Orobanche crenata, after exposure to various concentrations of GR 24. (Data corresponding with GR 24 curves in figure 4-7).

[GR 24] (mol/L)	*S. hermonthica* (maize)	*S. hermonthica* (sorghum)	*S. asiatica* (maize)	*O. crenata* (faba bean)
1×10^{-4}	10.5 ± 1.6	2.5 ± 0.4	30.6 ± 4.9	39.4 ± 1.4
5×10^{-5}	23.0 ± 2.0	11.2 ± 1.4	41.9 ± 5.8	49.0 ± 2.9
1×10^{-5}	48.4 ± 0.9	50.8 ± 3.4	37.1 ± 7.3	46.5 ± 2.4
6×10^{-6}	49.0 ± 0.4	67.9 ± 4.1	42.9 ± 4.8	43.9 ± 2.9
3×10^{-6}	56.8 ± 1.8	59.6 ± 3.0	41.0 ± 1.4	45.6 ± 2.6
1×10^{-6}	51.2 ± 0.2	68.5 ± 1.1	44.4 ± 9.0	48.7 ± 3.4
6×10^{-7}	43.3 ± 0.6	72.1 ± 1.4	35.0 ± 11.2	39.8 ± 2.4
3×10^{-7}	57.5 ± 3.8	76.6 ± 1.7	39.7 ± 5.9	31.1 ± 0.7
1×10^{-7}	54.2 ± 1.2	80.6 ± 0.2	35.9 ± 3.8	18.7 ± 2.1
5×10^{-8}	50.7 ± 2.8	73.3 ± 4.4	31.6 ± 2.7	17.8 ± 2.4
1×10^{-8}	34.9 ± 4.8	64.2 ± 5.3	13.7 ± 3.3	4.4 ± 1.0
1×10^{-9}	1.0 ± 0.2	25.1 ± 2.7	6.8 ± 2.0	0.7 ± 0.4

Table 4. Germination percentages ± S.E. for seeds of several Striga populations and Orobanche crenata, after exposure to various concentrations of Nijmegen-1. (Data corresponding with Nijmegen-1 curves in figure 4-7).

[Nijmegen-1] (mol/L)	*S. hermonthica* (maize)	*S. hermonthica* (sorghum)	*S. asiatica* (maize)	*O. crenata* (faba bean)
1×10^{-5}	28.2 ± 6.3	5.7 ± 2.2	19.8 ± 5.3	36.0 ± 3.8
5×10^{-5}	32.5 ± 4.6	10.2 ± 1.5	22.8 ± 4.5	44.2 ± 2.7
2.5×10^{-5}	31.6 ± 4.6	16.6 ± 3.7	27.7 ± 2.2	44.9 ± 1.3
1×10^{-5}	24.8 ± 0.7	11.7 ± 2.2	21.2 ± 2.0	38.0 ± 3.4
7.5×10^{-6}	16.6 ± 2.0	7.7 ± 2.2	28.0 ± 2.7	40.7 ± 5.4
5×10^{-6}	15.7 ± 2.0	22.5 ± 5.6	16.6 ± 6.0	31.4 ± 0.6
2.5×10^{-6}	14.4 ± 4.9	15.2 ± 5.4	13.4 ± 2.5	22.4 ± 4.8
1×10^{-6}	5.1 ± 0.3	18.7 ± 2.7	14.0 ± 2.9	18.1 ± 1.1
7.5×10^{-7}	1.5 ± 1.5	18.6 ± 5.3	18.0 ± 4.9	19.5 ± 0.7
5×10^{-7}	n.d.	5.6 ± 3.1	10.5 ± 1.4	9.4 ± 1.5
1×10^{-7}	n.d.	3.9 ± 2.7	3.8 ± 0.9	1.5 ± 0.5
1×10^{-8}	n.d.	3.3 ± 2.3	1.1 ± 0.3	n.d.

n.d. = not determined.

Table 5. Germination percentages ± S.E. for seeds of several Striga populations and Orobanche crenata, after exposure to various concentrations of sorgolactone. (Data corresponding with sorgolactone curves in figure 4-7).

[sorgolactone] (mol/L)	*S. hermonthica* (maize)	*S. hermonthica* (sorghum)	*S. asiatica* (maize)	*O. crenata* (faba bean)
1×10^{-4}	31.3 ± 1.6	22.0 ± 0.8	41.0 ± 1.8	47.9 ± 0.2
1×10^{-5}	32.9 ± 1.4	49.4 ± 5.5	42.3 ± 2.4	50.2 ± 3.5
6×10^{-6}	47.0 ± 4.3	56.5 ± 2.6	48.8 ± 1.5	50.1 ± 3.4
3×10^{-6}	39.9 ± 2.2	58.4 ± 8.2	48.3 ± 1.6	50.8 ± 2.7
1×10^{-6}	44.9 ± 1.6	60.9 ± 0.5	48.3 ± 4.9	49.8 ± 3.1
6×10^{-7}	45.8 ± 1.2	75.6 ± 2.6	38.5 ± 3.5	48.7 ± 3.0
3×10^{-7}	48.4 ± 5.2	60.4 ± 1.1	50.9 ± 1.4	36.3 ± 2.3
1×10^{-7}	49.6 ± 2.1	64.0 ± 2.4	35.8 ± 3.9	24.3 ± 1.2
6×10^{-8}	64.0 ± 1.4	66.0 ± 2.2	35.8 ± 4.1	22.3 ± 2.0
3×10^{-8}	55.8 ± 3.2	74.4 ± 0.8	34.4 ± 5.7	17.3 ± 3.9
1×10^{-8}	33.9 ± 1.7	67.4 ± 3.6	19.5 ± 4.4	6.9 ± 0.7
1×10^{-9}	38.6 ± 3.2	67.7 ± 1.5	10.1 ± 2.8	2.4 ± 0.1
1×10^{-10}	12.4 ± 0.3	51.7 ± 4.3	4.0 ± 0.7	0.2 ± 0.2

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5

An Expeditious Preparation of All Enantiopure Diastereoisomers of Aromatic A-Ring Analogues of Strigolactones, Germination Stimulants for Seeds of the Parasitic Weeds *Striga* and *Orobanche*

Abstract An expeditious manner to prepare all enantiopure diastereoisomers of aromatic A-ring strigolactone analogues is described. The racemic diastereoisomers of 8-methyl-GR 24 and of its regio isomer 6-methyl-GR 24 were prepared and separated, and subsequently chromatographed to give the pure enantiomers, using a Chiralcel OD HPLC column. The biological activity of all enantiopure strigolactone analogues towards seeds of *Striga hermonthica* and *Orobanche crenata* was determined. The presence of a methyl group on position 8 of GR 24 did not result in increased biological activity, whereas a 6-methyl substituent on GR 24 resulted in higher percentages of germinated *O. crenata* seeds, when compared with GR 24.

Introduction

The angiosperms *Striga, Alectra* (Scrophulariaceae) and *Orobanche* (Orobanchaceae) are root parasitic plants, which can only survive when attached to the roots of an appropriate host plant. Host plants for *Striga* include cereals such as maize and sorghum, whereas *Orobanche* mainly parasitizes legumes *e.g.* tomato and eggplant. Parasitic weeds deprive their host plants from nutrients, minerals and water and have an extremely devastating effect on the host crop yield.[1,2]

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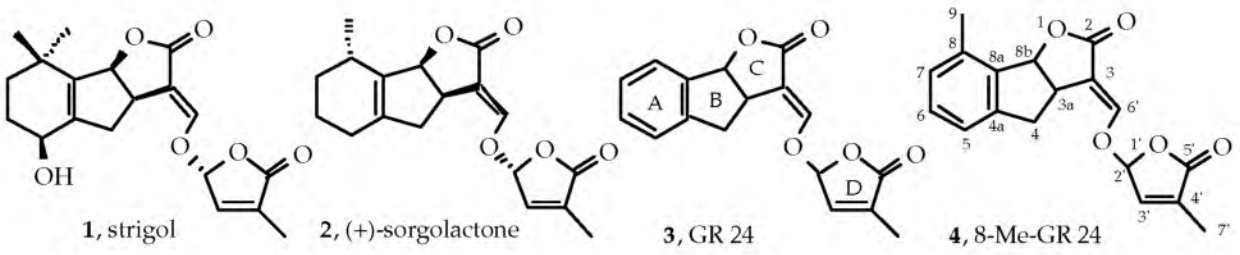
Figure 1



1, strigol **2**, (+)-sorgolactone **3**, GR 24 **4**, 8-Me-GR 24

The lifecycle of *Striga, Alectra* and *Orobanche* is closely adapted to that of their host plants. The seeds only germinate after exposure to a specific germination stimulant, which is exuded by the roots of suitable host plants and also by some non-hosts. Thus far, four naturally occurring germination stimulants, all belonging to the class of strigolactones[3], have been identified, *viz.* strigol (**1**)[4], sorgolactone (**2**)[5], alectrol[6], and orobanchol[7] (Figure 1).

Several synthetic strigol analogues, *e.g.* GR 24 (**3**) have been prepared in order to gain more insight in the germination process at the molecular level.[8,9] Considerable attention has been given to the influence of the stereochemistry of the stimulants on the biological activity.[10,11,12,13] In the case of GR 24, only the stereoisomer possessing the same stereochemical configuration as the naturally occurring stimulants, exhibited appreciable activity at low concentrations, whereas the other three stereoisomers were virtually inactive at sensitive concentrations.[11] The synthesis and biological testing of all eight diastereoisomers of sorgolactone, **2**, showed the same trend.[13] In the latter case, the methyl substituent on the sorgolactone A-ring also clearly influences the biological activity. It would therefore be interesting to study the biological activity of all diastereoisomers of 8-methyl-GR 24 (**4**),which can be considered as the most genuine aromatic A-ring analogue of sorgolactone.

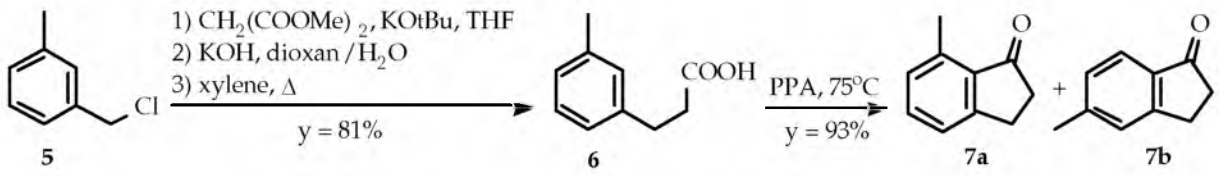
Enantiopure samples of strigol, and several of its analogues have been obtained both by resolution using a chiral auxiliary, and by asymmetric synthesis.[14,15,16,17] Usually, these methods are relatively time consuming. To evaluate the influence of the stereochemistry of the stimulants on the germination of *Striga* and *Orobanche* seeds, a more expeditious route to obtain enantiopure strigolactone analogues would be desirable. In this chapter, a facile route to the enantiopure aromatic strigolactone analogues 8-methyl GR 24 (**4**), and its regio isomer 6-methyl GR 24, (**12**) is presented. The biological activity of all compounds towards *S. hermonthica* and *O. crenata* was determined.

Results and Discussion

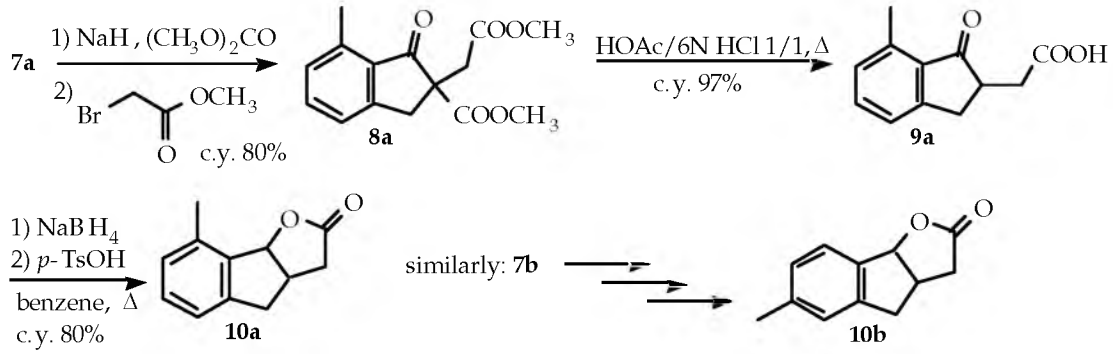
Synthesis

The strigolactones 8-methyl-GR 24 (**4**) and 6-methyl-GR 24 (**12**) were prepared following the same strategy as used for the synthesis of GR 24, namely first construction of the ABC-part, followed by coupling with the D-ring. The ABC-part of the target compounds was synthesized starting from 1-(chloromethyl)-3-methylbenzene (**5**), as outlined in scheme 1.

Scheme 1



Scheme 2

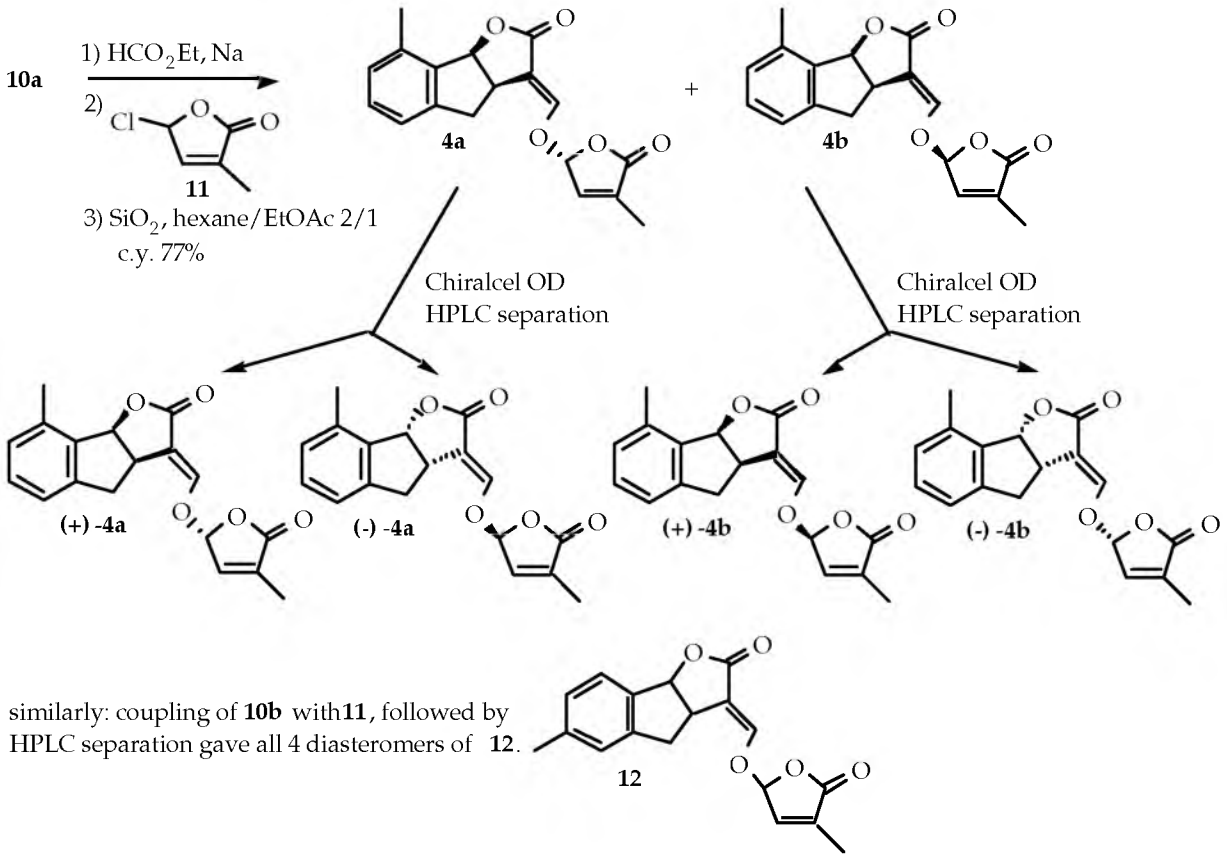


Treatment with dimethyl malonate and subsequent decarboxylation in the usual manner, followed by ring closure using polyphosphoric acid[18] gave a 1:1 mixture of **7a** and **7b** in good yields. The annelation of ring C was performed as described previously[9] (see scheme 2) to give the ABC-lactones **10a** and **10b**, respectively.

Before the coupling with the D-ring, **10a** and **10b** were formylated using ethyl formate and metallic sodium in the rather short reaction time of 1.5 hours. It should be noted that usually the formylation of strigolactone ABC-parts is performed in THF or diethyl ether with 10 equivalents of ethyl formate employing KOtBu or NaH as the base in an overnight reaction. The actual coupling with chlorobutenolide **11** was most conveniently conducted in THF, instead of the usual solvent DMF. In the present case, the initially obtained sodium enolate is a suspension in THF, which smoothly reacts with butenolide **11**. The thus obtained 1:1 mixture of diastereoisomers **4a** and **4b** was separated by flash chromatography over silicagel. Similarly **12a** and **12b** were acquired starting from **10b** (Scheme 3).

The racemic diastereoisomers **4a** and **4b** were both subjected to chromatographic separation using a semi-preparative cellulose carbamate Chiralcel OD HPLC column, to give enantiomerically pure compounds **(+)-4a** and **(-)-4a**, and **(+)-4b** and **(-)-4b**, respectively. Similarly, all four diastereoisomers of **12** were obtained as single enantiomers. A semi-preparative chiral HPLC system was preferred over a preparative Chiralcel OD column, because of the very high costs of large scale chiral HPLC material. In a single run the semi-preparative method allowed the separation of 1 mg of racemate in 30-90 minutes runtime. Repetitive chromatography readily afforded sufficient material for analysis and biological testing (5 mg).

Scheme 3



Also with racemic mixtures of sorgolactone, deshydroxy strigol and several simpler strigol analogues good separation was achieved on this type of cellulose carbamate column, demonstrating the scope of the method.

This method, involving the separation of enantiomers on a chiral semi-preparative HPLC column, is very useful when only small amounts of material are required for biological evaluation. Thus far, only (+)-strigol and its enantiomer have been separated on a column of cellulose triacetate.[19] Analogously, the ABC-parts of strigol[20] and GR 24[11] were separated on the aforementioned type of column.

The absolute configuration of the enantiopure compounds **4** and **12** was determined by comparing their optical rotation with the $[\alpha]^{20}_{D}$ values reported for all diastereoisomers of GR 24.[11] Clearly, the influence of the methyl group at the A-ring does not affect the optical rotation. For chiral strigol analogues containing the same chromophores as strigol and sorgolactone (*i.e.* the α,β unsaturated system of the CD-moiety) and no other chromophoric systems, circular dichroism can be used to determine their absolute stereochemistry. If no reference data are available, X-ray crystallography must be applied.

Biological activity

All enantiopure stereoisomers of **4** and **12** respectively, as well as their racemic mixtures, were assayed for their capacity to induce germination of *Striga hermonthica* and *Orobanche crenata* seeds. In all germination assays an aqueous solution of acetone (0.1% v/v) was included as a negative control and a diastereomeric 1:1 mixture of GR 24 as a positive control. This procedure enables comparison between results obtained in different test series.

In previous research, it was established that the concentration that induces half-maximal response (the ED_{50} value) of *S. hermonthica* seeds toward GR 24 is 5×10^{-9} M. Maximum germination is induced at concentrations $\geq 5 \times 10^{-6}$ M. For *O. crenata*, the ED_{50} value was established to be 2×10^{-7} M in the case of GR 24, whereas concentrations $\geq 5 \times 10^{-5}$ M cause maximum germination.[21] Because compounds **4** and **12** are very similar to GR 24, these concentrations were also used in the present bioassay.

The results of the bioassays with *S. hermonthica* are collected in tables 1 and 2, and a bar representation of this data is given in figure 2. The percentages of germinated *O. crenata* seeds are presented in tables 3 and 4 and in figure 3. Biological testing of all four diastereoisomers of GR 24 revealed a very marked difference in germinating activity, between the diastereomer possessing the 'natural' absolute stereochemistry and its optical antipode. The 'natural' diastereomer was the most active one, whereas its mirror image was virtually inactive, both at the high and at the low concentrations that were tested (concentrations that are similar to the ones used in the present assay).[11]

Figure 2. Bar representation of the biological activities of methyl-GR 24 stereoisomers ***4*** *and* ***12****, their racemates and the positive control GR 24 toward seeds of Striga hermonthica.*

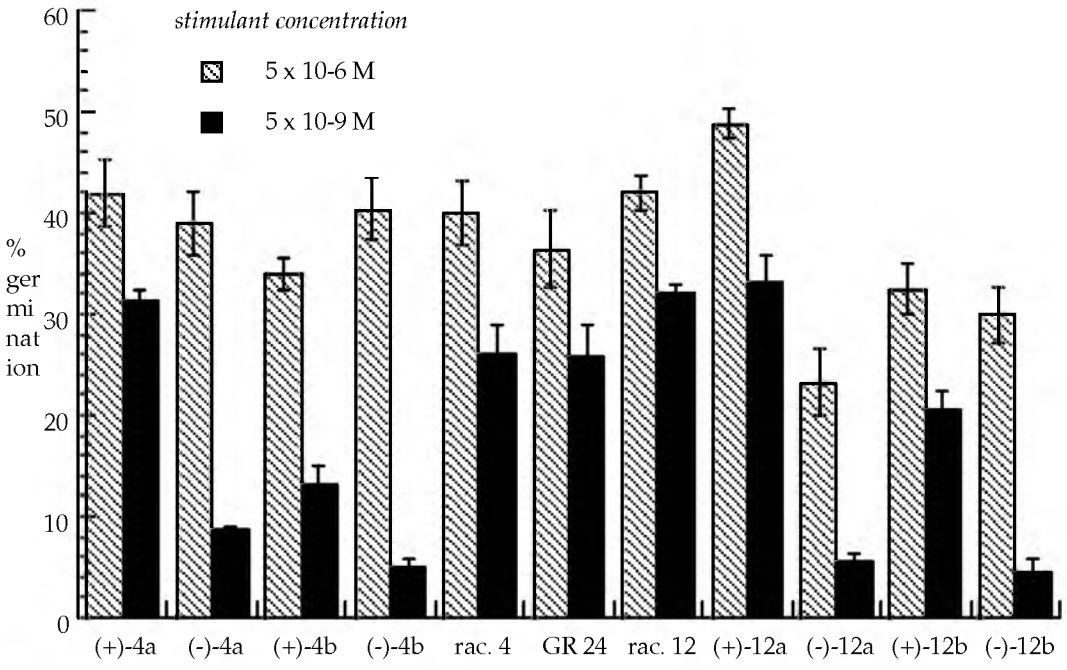


Table 1. Percentages of germinated seeds of S. hermonthica after exposure to solutions (5 x 10^{-6} and 5 x 10^{-9} mol/L) of 8-methyl-GR 24 diastereoisomers **4** *and the control GR 24.*[a]

		% germination ± SE at a concentration of	
entry	compound	5 x 10^{-6} mol/L	5 x 10^{-9} mol/L
1	**(+)-4a**	41.9 ± 3.3	31.3 ± 1.1
2	**(-)-4a**	38.9 ± 3.1	8.8 ± 0.3
3	**(+)-4b**	34.0 ± 1.5	13.1 ± 1.9
4	**(-)-4b**	40.4 ± 3.0	5.1 ± 0.7
5	**racemic 4**[c]	40.1 ± 3.2	26.1 ± 3.0
6	**GR 24**[c]	36.4 ± 3.8	25.8 ± 3.1

[a]Data presented are the mean ± SE of one representative experiment. [b]Not significantly different from aqueous control (without stimulant). [c]Equimolar mixture of two racemic diastereomers.

Table 2. Percentages of germinated seeds of S. hermonthica after exposure to solutions (5 x 10^{-6} and 5 x 10^{-9} mol/L) of 6-methyl-GR 24 diastereoisomers **12** *and the control GR 24.*[a]

		% germination ± SE at a concentration of	
entry	compound	5 x 10^{-6} mol/L	5 x 10^{-9} mol/L
1	**(+)-12a**	48.8 ± 1.5	33.3 ± 2.5
2	**(-)-12a**	23.3 ± 3.3	5.6 ± 0.8[b]
3	**(+)-12b**	32.5 ± 2.5	20.6 ± 1.7
4	**(-)-12b**	29.9 ± 2.7	4.6± 1.1[b]
5	**racemic 12**[c]	42.0 ± 1.7	32.0 ± 1.0
6	**GR 24**[c]	36.4 ± 3.8	25.8 ± 3.1

[a]Data presented are the mean ± SE of one representative experiment. [b]Not significantly different from aqueous control (without stimulant). [c]Equimolar mixture of two racemic diastereomers.

In contrast, in the case of sorgolactone, only at sensitive concentrations clear differences in activity between the eight diastereoisomers were observed. At higher concentrations, all sorgolactone diastereoisomers exhibited rather high activity.[13] The results for compound **4** (Tables 1 and 3) are, in this respect, similar to those of sorgolactone: only at low stimulant concentrations, there were significant differences in activity between the diastereoisomers; the 'natural' diastereomer, **(+)-4a**, was the most active one. For 6-methyl-GR 24 (**12**), the results are more similar to those of GR 24 itself: even at the higher stimulant concentration, there are significant differences in activity between the 'natural' analogue **(+)-12a** and its antipode **(-)-12a**, especially so in the case of *Orobanche*.

Table 3. Percentages of germinated seeds of O. crenata after exposure to solutions (5 x 10^{-5} and 2 x 10^{-7} mol/L) of 6-methyl-GR 24 diastereoisomers **4** *and the control GR 24.*[a]

		% germination ± SE at a concentration of	
entry	compound	5 x 10^{-5} mol/L	2 x 10^{-7} mol/L
1	**(+)-4a**	47.3 ± 3.4	20.2 ± 2.7
2	**(-)-4a**	32.5 ± 1.6	3.1 ± 0.9
3	**(+)-4b**	42.3 ± 3.9	12.8 ± 1.4
4	**(-)-4b**	41.1 ± 2.5	15.2 ± 2.6
5	**racemic 4**[c]	42.2 ± 2.9	17.9 ± 1.9
6	**GR 24**[c]	44.0 ± 1.3	15.7 ± 0.9

[a]Data presented are the mean ± SE of one representative experiment. [b]Not significantly different from aqueous control (without stimulant). [c]Equimolar mixture of two racemic diastereomers.

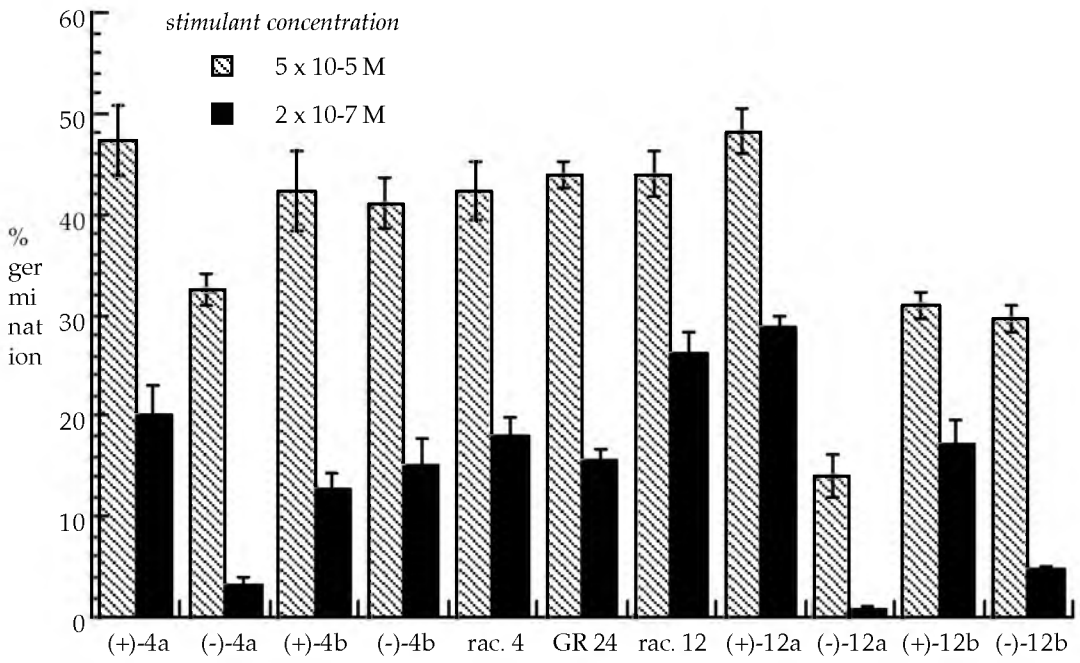
Table 4. Percentages of germinated seeds of O. crenata after exposure to solutions (5 x 10^{-5} and 2 x 10^{-7} mol/L) of 6-methyl-GR 24 diastereoisomers **12** *and the control GR 24.*[a]

		% germination ± SE at a concentration of	
entry	compound	5 x 10^{-5} mol/L	2 x 10^{-7} mol/L
1	**(+)-12a**	48.2 ± 2.2	28.9 ± 0.9
2	**(-)-12a**	14.1 ± 2.1	0.7 ± 0.3[b]
3	**(+)-12b**	30.9 ± 1.4	17.1 ± 2.4
4	**(-)-12b**	29.6 ± 1.3	4.8 ± 0.3
5	**racemic 12**[c]	44.0 ± 2.3	26.1 ± 2.2
6	**GR 24**[c]	44.0 ± 1.3	15.7 ± 0.9

[a]Data presented are the mean ± SE of one representative experiment. [b]Not significantly different from aqueous control (without stimulant). [c]Equimolar mixture of two racemic diastereomers.

Racemic GR 24 induced equally high percentages of germinated seeds as the same concentration of the enantiopure 'natural' GR 24 isomer.[11] The same feature was observed for 8-methyl-GR 24 and 6-methyl-GR 24. The bioassays reveal that, 8-methyl-GR 24 (**4**) and 6-methyl-GR 24 (**12**) are equally good synthetic germination stimulants as GR 24 itself. Only in the case of *O. crenata*, the extra methyl group on the 6-position, in comparison to GR 24, lead to increased activity at the lower concentration. Therefore, it may be concluded that a small hydrophobic moiety on the 6-position of the A-ring leads to a somewhat more favourable interaction with the strigolactone receptor on *O. crenata* seeds, resulting in a higher germination inducing activity.

Figure 3. Bar representation of the biological activities of methyl-GR 24 stereoisomers **4** *and* **12**, *their racemates and the positive control GR 24 toward seeds of Orobanche crenata.*



In conclusion, the activity of all diastereoisomers of 8-methyl-GR 24 and 6-methyl-GR 24 followed the expected trend: the 'natural' diastereomer is the most active one, whereas its optical antipode is the least active. A methyl group at C8 did not significantly increase the activity of the molecule toward *S. hermonthica* or *O. crenata*, compared with GR 24 and the results of the germination assay are similar to those of the bioassay for all sorgolactone stereoisomers. When an extra methyl group is located on the 6-position, the results of the bioassay are similar to these of all diastereoisomers of GR 24, with the exception of the more active diastereomer **(+)-12b**. Especially for *O. crenata* seeds, racemic **12** is a very active germination stimulant.

Experimental

Synthesis

General. ^{1}H NMR (300 MHz) and ^{13}C NMR spectra were recorded on a Bruker AC 300 spectrometer, using Me_4Si as internal standard. All coupling constants are given as ^{3}J in Hz , unless indicated otherwise. Melting points were measured with a Reichert thermopan microscope and are uncorrected. IR spectra were recorded on a Bio-Rad FTS-25 instrument. For mass spectra a double focusing VG7070E mass spectrometer was used. GC-MS spectra 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 x 0.25 mm), helium was used as the carrier gas and electron impact (EI) was used as ionization mode. Elemental analyses were conducted on a Carlo Erba Instruments CHNSO EA 1108 element analyzer. For the determination of optical

rotations a Perkin-Elmer 241 polarimeter was used. Solvents were dried using the following methods: dichloromethane was distilled from P_2O_5; ethyl acetate was distilled from K_2CO_3; diethyl ether was distilled from NaH; hexane was distilled from CaH_2; tetrahydrofuran was distilled from lithium aluminium hydride just before use. All other solvents were of analytical grade and used as purchased. Thin layer chromatography (TLC) was carried out on Merck precoated silica gel 60 F254 plates (0.25 mm). Spots were visualized with UV or using a 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 performed with Merck Kieselgel 60. Preparative HPLC separation of racemates **4** and **12** was carried out on a semi-preparative Chiralcel OD (10 μm) cellulose carbamate column (Daicel Chemical Industries Ltd., 1 x 25 cm) using mixtures of hexane and 2-propanol or ethanol as the eluent. Products were detected with a Merck-Hitachi L-4000 UV detector at 254 nm. Enantiomeric excess and purities of enantiopure isomers of **4** and **12** were determined by analytical HPLC using a Chiralcel OD (10 μm) cellulose carbamate column (Baker, 250 x 4.6 mm) and hexane/ethanol 85/15 (**12a**, **12b**) and 90/10 (**4a**, **4b**) as the eluent.

Nomenclature. Systematic names were generated using the ACD/Name programme, provided by Advanced Chemistry Development Inc. (Toronto, Canada).

8-Methyl-3-((*E*)-1-{[4'-methyl-5'-oxo-2',5'-dihydro-2'-furanyl]oxy}methylidene)-3,3a,4,8b-tetrahydro-2H-indeno[1,2-b]furan-2-one (4). To a cooled (0°C) and stirred solution of tricyclic lactone **10a** (200 mg, 1.06 mmol) in ethyl formate (10 mL) was added, under a continuous stream of nitrogen, 1.1 equiv. of metallic sodium (27 mg, 1.17 mmol). The mixture was allowed to warm to room temperature and stirred for 1.5 hours. When TLC analysis indicated complete formylation, excess ethyl formate was removed by evaporation *in vacuo*. The thus obtained sodium salt of formylated **10a** was suspended in THF (10 mL) and cooled to 0°C. Upon addition of chlorobutenolide **11** (212 mg, 1.6 mmol) the reaction mixture became clear. After 2 hours of stirring at room temperature, another 0.5 equiv. of **11** (70 mg, 0.53 mmol) was added and the mixture was stirred overnight. Then THF was removed *in vacuo*. The residue was dissolved in a mixture of brine and ethyl acetate. The aqueous phase was extracted with ethyl acetate (2x) and the combined organic layers were washed with satd. NH_4Cl (1x), dried ($MgSO_4$) and concentrated under reduced pressure. The crude product was purified using flash chromatography (SiO_2, hexane/ethyl acetate 2/1) to afford two diastereomeric products. Fast moving diastereomer **4a** (129 mg, 39%) and slow moving diastereomer **4b** (126 mg, 38%) were obtained as white solids, that were recrystallized from hexane/ethyl acetate.

4a: mp: 199-200°C. 300MHz ^{1}H NMR ($CDCl_3$): δ 2.03 (s, 3H, CH_3 D-ring); 2.44 (s, 3H, CH_3 A-ring); 3.08 (dd, 1H, $J_{4,3a,\ cis}$=3.7Hz, ^{2}J=16.9Hz, H4); 3.43 (dd, 1H, $J_{4,3a,\ trans}$=9.5Hz, ^{2}J=16.9Hz, H4); 3.93 (m, 1H, H3a); 6.00 (d, 1H, J=7.9Hz, H8b); 6.17 (m, 1H, H2'); 6.97 (m, 1H, H3'); 7.06 (m, 2H, H5 + H7); 7.24 (m, 1H, H6); 7.48 (d, 1H, $^4J_{3a,6'}$=2.5Hz, H6'). ^{13}C NMR ($CDCl_3$): δ 11.4 (CH_3 D-

ring); 19.2 (CH_3 A-ring); 38.4; 39.1 (CH_2, CH3a); 86.2 (CH8b); 101.2 (CH2'); 114.2 (Cq4'); 123.0; 129.1; 130.9; (3 x CH_{arom}); 136.6; 137.6; 138.2; 143.4 (3 x Cq_{arom}, Cq-C ring); 141.6 (CH3'); 151.5 (CH6'); 170.9; 172.0 (2 x C=O). IR (KBr): ν (cm^{-1}) 1787; 1733; 1681 (C=O, C=C); 1182 (lactone). MS [EI *m/z*, rel. intensity (%)]: 312 ($[M]^+$, 2.0); 215 ($[C_{13}H_{11}O_3]^+$, 39.7); 97 ($[C_5H_5O_2]^+$, 100). Anal. calcd. for $C_{18}H_{16}O_5$: C, 69.22; H, 5.16 found: C, 69.19; H, 5.20.

4b: mp: 178-179°C. 300MHz ^1H NMR ($CDCl_3$): δ 2.04 (s, 3H, CH_3 D-ring); 2.44 (s, 3H, CH_3 A-ring); 3.07 (dd, 1H, $J_{4,3a,\ cis}$=3.6Hz, ^2J=17.0Hz, H4); 3.42 (dd, 1H, $J_{4,3a,\ trans}$=9.5Hz, ^2J=17.0Hz, H4); 3.92 (m, 1H, H3a); 6.00 (d, 1H, J=7.9Hz, H8b); 6.18 (m, 1H, H2'); 6.97 (m, 1H, H3'); 7.06 (m, 2H, H5 + H7); 7.24 (m, 1H, H6); 7.48 (d, 1H, $^4J_{3a,6'}$=2.5Hz, H6'). ^{13}C NMR ($CDCl_3$): δ 11.4 (CH_3 D-ring); 19.2 (CH_3 A-ring); 38.4; 39.0 (CH_2, CH3a); 86.2 (CH8b); 101.3 (CH2'); 114.3 (Cq4'); 123.0; 129.0; 131.0; (3 x CH_{arom}); 136.6; 137.5; 138.1; 143.4 (3 x Cq_{arom}, Cq-C ring); 141.7 (CH3'); 151.5 (CH6'); 170.9; 172.1 (2 x C=O). IR (KBr): ν (cm^{-1}) 1785; 1738; 1678 (C=O, C=C); 1181 (lactone). MS [EI *m/z*, rel. intensity (%)]: 312 ($[M]^+$, 3.0); 215 ($[C_{13}H_{11}O_3]^+$, 52.8); 97 ($[C_5H_5O_2]^+$, 100). Anal. calcd. for $C_{18}H_{16}O_5$: C, 69.22; H, 5.16 found: C, 69.26; H, 5.19.

8-Methyl-3-((*E*)-1-{[4'-methyl-5'-oxo-2',5'-dihydro-2'(*R*)-furanyl]oxy}methylidene)-3,3a(*R*),4, 8b(*S*) -tetrahydro-2H-indeno[1,2-b]furan-2-one ((+)-4a) and its enantiomer **8-Methyl-3-((*E*)-1-{[4'-methyl-5'-oxo-2',5'-dihydro-2'(*S*)-furanyl]oxy} methylidene)-3,3a(*S*),4,8b(*R*)-tetrahydro-2H-indeno[1,2-b]furan-2-one ((-)-4a).** Fast moving 8-methyl-GR 24 diastereomer **4a** was separated into its enantiomers **(+)-4a** and **(-)-4a** using a semi-preparative Chiralcel OD HPLC column and hexane/2-propanol 90/10 as the eluent. Flow: 3.3 mL/min. Recrystallisation from hexane/dichloromethane afforded **(+)-4a** and **(-)-4a** as colourless needles. **(+)-4a:** mp: 156-157.5°C. e.e. >99% (determined by HPLC). $[\alpha]^{20}_D$=+435.4° (c=0.02, CH_2Cl_2). **(-)-4a:** mp: 157-158°C. e.e. >99% (determined by HPLC). $[\alpha]^{20}_D$=-430.0° (c=0.04, CH_2Cl_2). All other analytical data were identical with those of the racemate **4a**.

8-Methyl-3-((*E*)-1-{[4'-methyl-5'-oxo-2',5'-dihydro-2'(*S*)-furanyl]oxy}methylidene)-3,3a(*R*),4, 8b(*S*)-tetrahydro-2H-indeno[1,2-b]furan-2-one ((+)-4b) and its enantiomer **8-Methyl-3-((*E*)-1-{[4'-methyl-5'-oxo-2',5'-dihydro-2'(*R*)-furanyl]-oxy}methylidene)-3,3a(*S*),4,8b(*R*)-tetrahydro-2H-indeno[1,2-b]furan-2-one ((-)-4b).** Slow moving 8-methyl-GR 24 diastereomer **4b** was separated into its enantiomers **(+)-4b** and **(-)-4b** using a semi-preparative Chiralcel OD HPLC column and hexane/ethanol 90/10 as the eluent. Flow: 3.3 mL/min. Recrystallisation from hexane/dichloromethane gave **(+)-4b** and **(-)-4b** as colourless needles. **(+)-4b:** mp: 159-160°C. e.e. >99% (determined by HPLC). $[\alpha]^{20}_D$=+288.2° (c=0.03, CH_2Cl_2). **(-)-4b:** mp: 159-160°C. e.e. >99% (determined by HPLC). $[\alpha]^{20}_D$=-291.6° (c=0.04, CH_2Cl_2). All other analytical data were identical with those of the racemate **4b**.

Dimethyl 2-(3-methylbenzyl)malonate. Dimethyl malonate (17.2 mL, 150 mmol) was dissolved in tetrahydrofuran (100 mL). Potassium *tert.* butoxide (16.8 g, 150 mmol) was added

gradually and the resulting slurry was stirred at room temperature for 30 minutes. 1-(chloromethyl)-3-methylbenzene **5** (13.2 mL, 100 mmol) was added dropwise and the mixture was stirred overnight. After 24 hours, TLC analysis indicated complete conversion. THF was evaporated *in vacuo*. The residue was dissolved in ethyl acetate (100 mL) and washed with water (3x). Drying ($MgSO_4$) and concentration *in vacuo* gave the desired diester as a colourless oil, which was used in the next reaction without further purification. 300MHz ^{1}H NMR ($CDCl_3$): δ 2.29 (s, 3H, CH_3); 3.17 (d, 2H, J=7.8Hz, CH_2); 3.66 (s, 6H, 2 x OCH_3); 3.66 (t, 1H, J=7.8Hz, CH); 6.95-7.16 (m, 4H, H_{arom}). ^{13}C NMR ($CDCl_3$): δ 21.8 (CH_3); 35.2 (CH_2); 52.9 (CH); 54.0; 54.1 (2 x OCH_3); 126.1; 127.5; 128.9; 129.9 (4 x CH_{arom}); 138.1; 138.2 (2 x Cq_{arom}); 169.7 (2 x C=O). IR (KBr): ν (cm^{-1}) 1755; 1737 (C=O). GC-MS [EI *m/z*, rel. intensity (%)]: 236 ($[M]^+$, 50.8); 176 ($[C_{11}H_{12}O_2]^+$, 100); 145 ($[C_{10}H_9O]^+$, 88.3); 105 ($[C_8H_9]^+$, 31.4); 91 ($[C_7H_7]^+$, 27.5); 77 ($[C_5H_6]^+$, 18.5).

2-(3-Methylbenzyl)malonic acid. Crude dimethyl 2-(3-methylbenzyl)malonate (~150 mmol) was hydrolyzed in a mixture of dioxan (50 mL) and 5 M aqueous potassium hydroxide (100 mL). The solution was stirred overnight at room temperature and then concentrated to a volume of approximately 75 mL. Non ionic species were removed by extraction with ethyl acetate. The organic layer was discarded and the aqueous phase was acidified with sulfuric acid until the pH < 5. The acidic solution was extracted with ethyl acetate (3x). The combined organic extracts were washed with water (1x), dried ($MgSO_4$) and concentrated *in vacuo* to yield 2-(3-methylbenzyl)malonic acid as a white solid (28.7 g, 92% 2 steps). An analytical sample was obtained by recrystallisation from hexane/ethyl acetate. mp: 136-138°C. 300MHz ^{1}H NMR ($CDCl_3$): δ 2.31 (s, 3H, CH_3); 3.21 (d, 2H, J=7.6Hz, CH_2); 3.75 (t, 1H, J=7.6Hz, CH); 6.99-7.25 (m, 4H, H_{arom}); 9.63 (bs, 2H, 2 x COOH). ^{13}C NMR ($CDCl_3$): δ 22.0 (CH_3); 35.0 (CH_2); 54.1 (CH); 126.4; 128.5; 129.3; 130.1 (4 x CH_{arom}); 137.6; 139.0 (2 x Cq_{arom}); 175.0 (2 x C=O). IR (KBr): ν (cm^{-1}) 3019 (broad, OH); 1709 (C=O). MS [EI *m/z*, rel. intensity (%)]: 208 ($[M]^+$, 9.8); 164 ($[C_{10}H_{12}O_2]^+$, 37.8); 105 ($[C_8H_9]^+$, 100); 91 ($[C_7H_7]^+$, 19.9); 77 ($[C_5H_6]^+$, 15.3). Anal. calcd. for $C_{11}H_{12}O_4$: C, 63.46; H, 5.77 found: C, 63.39; H, 5.70.

3-(3-methylphenyl)propanoic acid (6). 2-(3-Methylbenzyl)malonic acid (20.8 g, 100 mmol) was dissolved in xylene (150 mL) and heated under reflux cooling for 4 hours. Evaporation of the solvent afforded acid **6** as a colourless oil that was sufficiently pure for use in the following cyclization reaction. Yield: 88%. 300MHz ^{1}H NMR ($CDCl_3$): δ 2.31 (s, 3H, CH_3); 2.65 (t, 2H, J=7.8Hz, Ar$\underline{CH_2}$); 2.90 (t, 2H, J=7.8Hz, $\underline{CH_2}$COOH); 6.98-7.19 (m, 4H, H_{arom}); 8.9 (bs, 1H, COOH). ^{13}C NMR ($CDCl_3$): δ 22.0 (CH_3); 31.1; 36.3 (2 x CH_2); 125.9; 127.8; 129.1; 129.7 (4 x CH_{arom}); 138.1; 140.7 (2 x Cq_{arom}); 180.3 (C=O). IR (KBr): ν (cm^{-1}) 3024 (broad OH) 1706 (C=O). GC-MS [EI *m/z*, rel. intensity (%)]: 119 ($[C_9H_{11}]^+$, 31.0); 105 ($[C_8H_9]^+$, 9.9); 91 ($[C_7H_7]^+$, 55.3); 77 ($[C_5H_6]^+$, 7.0).

7-Methyl-1-indanone (7a) and 5-methyl-1-indanone (7b). Polyphosphoric acid (PPA, 75 g) was stirred with an efficient mechanical stirrer and heated to 75°C. 3-(3-Methylphenyl)propanoic acid (**6**) (9.84 g, 60 mmol) was added at once and the mixture was stirred at 75°C during 1 hour. The appearance of a persistent bright red colour indicates completion of the reaction.[22] The mixture was cooled to room temperature and ice water (100 mL) was added to decompose the PPA. The acidic solution was extracted with diethyl ether (3x) and the combined ether fractions were subsequently washed with water, satd. $NaHCO_3$ and water. Drying ($MgSO_4$), followed by concentration *in vacuo* resulted in an oily mixture of 7-methyl-1-indanone (**7a**) and 5-methyl-1-indanone (**7b**) (8.15 g, 93%) in a ratio of 1:1 (determined by NMR).[23] Separation was achieved by column chromatography (SiO_2, dichloromethane/hexane 1/1). Recrystallisation from dichloromethane/hexane afforded pure **7a** (3.15 g, 36%) and **7b** (4.0 g, 46%) both as white needles.

7a: mp: 54-55°C. 300MHz ^{1}H NMR ($CDCl_3$): δ 2.62 (s, 3H, CH_3); 2.63 (t, 2H, J=6.0Hz, Ar$\underline{CH_2}$); 3.05 (t, 2H, J=6.0Hz, $\underline{CH_2}$C=O); 7.07 (d, 1H, J=7.3Hz, H6); 7.25 (d, 1H, J=7.3Hz, H4); 7.41 (m, 1H, H5). ^{13}C NMR ($CDCl_3$): δ 18.9 (CH_3); 25.9; 37.3 (2 x CH_2); 124.5; 129.6; 134.5 (3 x CH_{arom}); 135.0; 139.3; 156.4 (3 x Cq_{arom}); 208.4 (C=O). IR (KBr): ν (cm^{-1}) 2916 (arom. C-H); 1702 (C=O). GC-MS [EI *m/z*, rel. intensity (%)]: 146 ([M]$^+$, 90.1); 117 ([C_9H_9]$^+$, 100); 91 ([C_7H_7]$^+$, 15.3). Anal. calcd. for $C_{10}H_{10}O$: C, 82.16; H, 6.89 found: C, 81.63; H, 6.76.

7b: mp: 70-71°C. 300MHz ^{1}H NMR ($CDCl_3$): δ 2.44 (s, 3H, CH_3); 2.67 (t, 2H, J=5.9Hz, Ar$\underline{CH_2}$); 3.09 (t, 2H, J=5.9Hz, $\underline{CH_2}$C=O); 7.17 (d, 1H, J=7.8Hz, H6); 7.27 (s, 1H, H4); 7.65 (d, 1H, J=7.8Hz, H7). ^{13}C NMR ($CDCl_3$): δ 22.7 (CH_3); 26.3; 37.0 (2 x CH_2); 124.1; 127.6; 129.2 (3 x CH_{arom}); 135.5; 146.4; 156.3 (3 x Cq_{arom}); 207.2 (C=O). IR (KBr): ν (cm^{-1}) 2919 (arom. C-H); 1699 (C=O). GC-MS [EI *m/z*, rel. intensity (%)]: 146 ([M]$^+$, 80.1); 117 ([C_9H_9]$^+$, 100); 91 ([C_7H_7]$^+$, 18.6). Anal. calcd. for $C_{10}H_{10}O$: C, 82.16; H, 6.89 found: C, 82.23; H, 6.85.

Methyl 2-(2-methoxy-2-oxoethyl)-7-methyl-1-oxo-2-indanecarboxylate (8a). 7-Methyl-1-indanone 7a (1.6 g, 11.0 mmol) was alkylated according to the method of Mangnus *et al.*[9] Yield: 80%. An analytically pure sample was obtained by recrystallisation from diisopropyl ether/ethyl acetate. mp: 59-60°C. 300MHz ^{1}H NMR ($CDCl_3$): δ 2.62 (s, 3H, CH_3); 2.74 and 3.36 (AB, 2H, J=17.3Hz, $\underline{CH_2}$COOMe); 3.14 and 3.86 (AB, 2H, J=17.6Hz, Ar$\underline{CH_2}$); 3.66 and 3.68 (2 x s, 2 x 3H, 2 x OCH_3); 7.14 (d, 1H, J=7.4Hz, H6); 7.30 (d, 1H, J=7.6Hz, H4); 7.47 (m, 1H, H5). ^{13}C NMR ($CDCl_3$): δ 19.0 (CH_3); 37.9; 39.4 (2 x CH_2); 52.5; 53.6 (2 x OCH_3); 58.8 (Cq_{alif}); 124.3; 130.3; 135.5 (3 x CH_{arom}); 132.8; 140.6; 154.7 (3 x Cq_{arom}); 171.1; 172.1 (2 x $\underline{C}$OOMe); 207.2 (C=O). IR (KBr): ν (cm^{-1}) 2956 (arom. C-H); 1737 (C=O, ester); 1701 (C=O, ketone). GC-MS [EI *m/z*, rel. intensity (%)]: 276 ([M]$^+$, 16.4); 245 ([$C_{14}H_{13}O_4$]$^+$, 57.5); 216 ([$C_{13}H_{12}O_3$]$^+$, 100); 157 ([$C_{11}H_9O$]$^+$, 80.5); 77 ([C_6H_5]$^+$, 11.4). Anal. calcd. for $C_{15}H_{16}O_5$: C, 65.21; H, 5.84 found: C, 65.47; H, 5.65.

Methyl 2-(2-methoxy-2-oxoethyl)-5-methyl-1-oxo-2-indanecarboxylate (8b). Diester **8b** was prepared in 79% yield starting from ketone **7b** (2.0 g, 13.7 mmol) analogous to the procedure

described by Mangnus *et al.*[9] An analytically pure sample was obtained by recrystallisation from diisopropyl ether/ethyl acetate. mp: 94°C. 300MHz ^{1}H NMR ($CDCl_3$): δ 2.46 (s, 3H, CH_3); 2.76 and 3.35 (AB, 2H, J=17.4Hz, $C\underline{H}_2COOMe$); 3.15 and 3.85 (AB, 2H, J=17.6Hz, $ArC\underline{H}_2$); 3.66 and 3.67 (2 x s, 2 x 3H, 2 x OCH_3); 7.21 (d, 1H, J=7.9Hz, H6); 7.29 (s, 1H, H4); 7.67 (d, 1H, J=7.9Hz, H7). ^{13}C NMR ($CDCl_3$): δ 22.8 (CH_3); 38.2; 39.2 (2 x CH_2); 52.5; 53.6 (2 x OCH_3); 58.8 (Cq_{alif}); 125.4; 127.4; 129.8 (3 x CH_{arom}); 133.0; 147.7; 154.5 (3 x Cq_{arom}); 171.0; 172.1 (2 x $\underline{C}OOMe$); 201.0 (C=O). IR (KBr): ν (cm^{-1}) 2958 (arom. C-H); 1747, 1734 (C=O, ester); 1703 (C=O, ketone). GC-MS [EI *m/z*, rel. intensity (%)]: 276 ($[M]^+$, 16.5); 245 ($[C_{14}H_{13}O_4]^+$, 58.5); 216 ($[C_{13}H_{12}O_3]^+$, 100); 157 ($[C_{11}H_9O]^+$, 67.0); 77 ($[C_6H_5]^+$, 9.0). Anal. calcd. for $C_{15}H_{16}O_5$: C, 65.21; H, 5.84 found: C, 65.25; H, 5.75.

2-(7-Methyl-1-oxo-2,3-dihydro-1H-2-indenyl)acetic acid (9a). Carboxylic acid **9a** was obtained from diester **8a** (2.1 g, 7.6 mmol) in 97% using the procedure reported by Mangnus *et al.*[9] Recrystallisation from dichloromethane/diisopropyl ether gave colourless crystals. mp: 146-148°C. 300MHz ^{1}H NMR ($CDCl_3$): δ 2.63 (s, 3H, CH_3); 2.59 (dd, 1H, $J_{vic,\ trans}$=10.0Hz, ^{2}J=17.9Hz, $C\underline{H}_2COOH$); 2.85 (dd, 1H, $J_{vic,\ cis}$=4.6Hz, ^{2}J=17.0Hz, $ArC\underline{H}_2$); 3.00 (m, 1H, CH); 3.03 (dd, 1H, $J_{vic,\ cis}$=4.4Hz, ^{2}J=17.9Hz, $C\underline{H}_2COOH$); 3.42 (dd, 1H, $J_{vic,\ trans}$=7.8Hz, ^{2}J=17.0Hz, $ArC\underline{H}_2$); 7.11 (d, 1H, J=7.4Hz, H6); 7.26 (d, 1H, J=7.6Hz, H4); 7.46 (m, 1H, H5); 9.30 (bs, 1H, COOH). ^{13}C NMR ($CDCl_3$): δ 19.0 (CH_3); 33.2; 35.8 (2 x CH_2); 44.3 (CH); 124.4; 130.0; 135.0 (3 x CH_{arom}); 134.2; 139.8; 154.5 (3 x Cq_{arom}); 179.0; (COOH); 208.1 (C=O). IR (KBr): ν (cm^{-1}) 3041 (broad, OH); 1696 (C=O); 1596 (arom. ring). MS [EI *m/z*, rel. intensity (%)]: 204 ($[M]^+$, 100); 159 ($[C_{11}H_{11}O]^+$, 81.4); 91 ($[C_7H_7]^+$, 26.9); 77 ($[C_6H_5]^+$, 14.5). Anal. calcd. for $C_{12}H_{12}O_3$: C, 70.57; H, 5.92 found: C, 70.57; H, 5.82.

2-(5-Methyl-1-oxo-2,3-dihydro-1H-2-indenyl)acetic acid (9b). Acid **9b** was prepared in the same way as described for **9a**, starting from **8b** (1.75 g, 6.3 mmol). Recrystallisation from dichloromethane/diisopropyl ether gave colourless crystals. Yield: 98%. mp: 115-117°C. 300MHz ^{1}H NMR ($CDCl_3$): δ 2.44 (s, 3H, CH_3); 2.60 (dd, 1H, $J_{vic,\ trans}$=9.9Hz, ^{2}J=17.9Hz, $C\underline{H}_2COOH$); 2.85 (dd, 1H, $J_{vic,\ cis}$=4.3Hz, ^{2}J=17.1Hz, $ArC\underline{H}_2$); 3.01 (m, 1H, CH); 3.03 (dd, 1H, $J_{vic,\ cis}$=4.3Hz, ^{2}J=17.9Hz, $C\underline{H}_2COOH$); 3.42 (dd, 1H, $J_{vic,\ trans}$=7.6Hz, ^{2}J=17.1Hz, $ArC\underline{H}_2$); 7.19 (d, 1H, J=7.9Hz, H6); 7.26 (s, 1H, H4); 7.66 (d, 1H, J=7.9Hz, H7); 9.50 (bs, 1H, COOH). ^{13}C NMR ($CDCl_3$): δ 22.8 (CH_3); 33.6; 35.8 (2 x CH_2); 44.2 (CH); 124.6; 127.5; 129.6 (3 x CH_{arom}); 134.4; 147.1; 154.5 (3 x Cq_{arom}); 178.4; (COOH); 206.9 (C=O). IR (KBr): ν (cm^{-1}) 3023 (broad, OH); 1713, 1697 (C=O); 1612 (arom. ring). MS [EI *m/z*, rel. intensity (%)]: 204 ($[M]^+$, 76.0); 159 ($[C_{11}H_{11}O]^+$, 100); 91 ($[C_7H_7]^+$, 17.7); 77 ($[C_6H_5]^+$, 8.9). Anal. calcd. for $C_{12}H_{12}O_3$: C, 70.57; H, 5.92 found: C, 70.42; H, 5.85.

8-Methyl-3,3a,4,8b-tetrahydro-2H-indeno[1,2-b]furan-2-one (10a). Cyclization of carboxylic acid **9a** (1.36 g, 6.7 mmol) was effected by the method of House *et al.*[24] Recrystallisation from

hexane/ethyl acetate afforded tricyclic lactone **10a** as colourless plates in 80% yield. mp: 71-73°C. 300MHz ^{1}H NMR ($CDCl_3$): δ 2.43 (s, 3H, CH_3); 2.43 (dd, 1H, $J_{3,3a,\ cis}$=4.9Hz, ^{2}J=18.1Hz, C$\underline{H_2}$C=O); 2.90 (m, 1H, ArC$\underline{H_2}$); 2.92 (dd, 1H, $J_{3,3a,\ trans}$=6.6Hz, ^{2}J=18.1Hz, C$\underline{H_2}$C=O); 3.32 (dd, 1H, $J_{4,3a,\ trans}$=8.6Hz, ^{2}J=15.5Hz, ArC$\underline{H_2}$); 3.34 (m, 1H, H3a); 5.95 (d, 1H, J=7.0Hz, H8b); 7.08 (m, 2H, H5 + H7); 7.26 (m, 1H, H6). ^{13}C NMR ($CDCl_3$): δ 19.0 (CH_3); 36.4; 38.9 (2 x CH_2); 37.4 (CH3a); 87.9 (CH8b); 123.1; 129.1; 130.8 (3 x CH_{arom}); 137.4; 138.0; 143.4 (3 x Cq_{arom}); 177.7 (C=O). IR (KBr): ν (cm^{-1}) 1757 (C=O); 1180 (lactone). GC-MS [EI *m/z*, rel. intensity (%)]: 189 ([M]$^+$, 18.1); 144 ([$C_{11}H_{12}$]$^+$, 57.3); 129 ([$C_{10}H_9$]$^+$, 100); 77 ([C_6H_5]$^+$, 5.8). Anal. calcd. for $C_{12}H_{12}O_2$: C, 76.57; H, 6.43 found: C, 76.71; H, 6.34.

6-Methyl-3,3a,4,8b-tetrahydro-2H-indeno[1,2-b]furan-2-one (10b). Lactone **10b** was prepared from acid **9b** (918 mg, 4.5 mmol) in the same manner as reported for **10a**. Yield: 82%. An analytically pure sample was obtained by recrystallisation from hexane/ethyl acetate. mp: 62-63°C. 300MHz ^{1}H NMR ($CDCl_3$): δ 2.36 (s, 3H, CH_3); 2.36 (dd, 1H, $J_{3,3a,\ cis}$=5.4Hz, ^{2}J=18.0Hz, C$\underline{H_2}$C=O); 2.82 (dd, 1H, $J_{4,3a,\ cis}$=2.8Hz, ^{2}J=16.0Hz, ArC$\underline{H_2}$); 2.88 (dd, 1H, $J_{3,3a,\ trans}$=9.5Hz, ^{2}J=18.0Hz, C$\underline{H_2}$C=O); 3.27 (dd, 1H, $J_{4,3a,\ trans}$=8.4Hz, ^{2}J=16.0Hz, ArC$\underline{H_2}$); 3.33 (m, 1H, H3a); 5.84 (d, 1H, J=6.9Hz, H8b); 7.07 (s, 1H, H5); 7.09 (d, 1H, J=7.6Hz, H7); 7.35 (d, 1H, J=7.6Hz, H8). ^{13}C NMR ($CDCl_3$): δ 22.1 (CH_3); 36.4; 38.5 (2 x CH_2); 38.2 (CH3a); 88.2 (CH8b); 126.4; 126.6; 129.1 (3 x CH_{arom}); 136.6; 140.7; 143.4 (3 x Cq_{arom}); 177.6 (C=O). IR (KBr): ν (cm^{-1}) 1765 (C=O); 1170 (lactone). GC-MS [EI *m/z*, rel. intensity (%)]: 189 ([M]$^+$, 18.6); 144 ([$C_{11}H_{12}$]$^+$, 57.3); 129 ([$C_{10}H_9$]$^+$, 100); 77 ([C_6H_5]$^+$, 5.1). Anal. calcd. for $C_{12}H_{12}O_2$: C, 76.57; H, 6.43 found: C, 76.64; H, 6.26.

6-Methyl-3-((*E*)-1-{[4'-methyl-5'-oxo-2',5'-dihydro-2'-furanyl]oxy}methylidene)-3,3a,4,8b-tetrahydro-2H-indeno[1,2-b]furan-2-one (12). 6-Methyl GR 24 (**12**) was prepared in the same way as described for its isomer **4**. Fast moving diastereomer **12a** (130 mg, 39%) and slow moving diastereomer **12b** (122 mg, 37%) were obtained as white solids, that were recrystallized from hexane/ethyl acetate.

12a: mp: 205°C. 300MHz ^{1}H NMR ($CDCl_3$): δ 2.05 (s, 3H, CH_3 D-ring); 2.35 (s, 3H, CH_3 A-ring); 3.05 (dd, 1H, $J_{4,3a,\ cis}$=3.3Hz, ^{2}J=16.9Hz, H4); 3.39 (dd, 1H, $J_{4,3a,\ trans}$=9.3Hz, ^{2}J=16.9Hz, H4); 3.93 (m, 1H, H3a); 5.92 (d, 1H, J=7.8Hz, H8b); 6.17 (m, 1H, H2'); 6.96 (m, 1H, H3'); 7.04 (s, 1H, H5); 7.10 (d, 1H, J=7.8Hz, H7); 7.38 (d, 1H, J=7.8Hz, H8); 7.46 (d, 1H, $^4J_{3a,6'}$=2.5Hz, H6'). ^{13}C NMR ($CDCl_3$): δ 11.5 (CH_3 D-ring); 22.1 (CH_3 A-ring); 37.9; 39.8 (CH_2, CH3a); 86.5 (CH8b); 101.2 (CH2'); 114.3 (Cq4'); 126.2; 126.8; 129.2 (3 x CH_{arom}); 136.7; 136.8; 140.8; 143.5 (3 x Cq_{arom}, Cq-C ring); 141.5 (CH3'); 151.4 (CH6'); 170.8; 172.0 (2 x C=O). IR (KBr): ν (cm^{-1}) 1793; 1784; 1748; 1678 (C=O, C=C); 1186 (lactone). MS [EI *m/z*, rel. intensity (%)]: 312 ([M]$^+$, 3.6); 215 ([$C_{13}H_{11}O_3$]$^+$, 39.1); 97 ([$C_5H_5O_2$]$^+$, 100). Anal. calcd. for $C_{18}H_{16}O_5$: C, 69.22; H, 5.16 found: C, 69.09; H, 5.09.

12b: mp: 166°C. 300MHz ^{1}H NMR ($CDCl_3$): δ 2.04 (s, 3H, CH_3 D-ring); 2.35 (s, 3H, CH_3 A-ring); 3.05 (dd, 1H, $J_{4,3a,\ cis}$=3.3Hz, ^{2}J=16.9Hz, H4); 3.37 (dd, 1H, $J_{4,3a,\ trans}$=9.3Hz, ^{2}J=16.9Hz, H4); 3.92 (m, 1H, H3a); 5.92 (d, 1H, J=7.8Hz, H8b); 6.17 (m, 1H, H2'); 6.96 (m, 1H, H3'); 7.04 (s, 1H, H5);

7.09 (d, 1H, J=7.8Hz, H7); 7.38 (d, 1H, J=7.8Hz, H8); 7.47 (d, 1H, $^4J_{3a,6'}$=2.5Hz, H6'). ^{13}C NMR ($CDCl_3$): δ 11.5 (CH_3 D-ring); 22.1 (CH_3 A-ring); 37.9; 39.7 (CH_2, CH3a); 86.5 (CH8b); 101.3 (CH2'); 114.4 (Cq4'); 126.3; 126.7; 129.2; (3 x CH_{arom}); 136.6; 136.7; 140.9; 143.5 (3 x Cq_{arom}, Cq-C ring); 141.6 (CH3'); 151.5 (CH6'); 170.8; 171.9 (2 x C=O). IR (KBr): ν (cm^{-1}) 1792; 1783; 1738; 1680 (C=O, C=C); 1203 (lactone). MS [EI *m/z*, rel. intensity (%)]: 312 ($[M]^+$, 5.0); 215 ($[C_{13}H_{11}O_3]^+$, 47.8); 97 ($[C_5H_5O_2]^+$, 100). Anal. calcd. for $C_{18}H_{16}O_5$: C, 69.22; H, 5.16 found: C, 69.03; H, 5.10.

6-Methyl-3-((*E*)-1-{[4'-methyl-5'-oxo-2',5'-dihydro-2'(*R*)-furanyl]oxy}methylidene)-3,3a(*R*),4, 8b(*S*)-tetrahydro-2H-indeno[1,2-b]furan-2-one ((+)-12a) and its enantiomer **6-Methyl-3-((*E*)-1-{[4'-methyl-5'-oxo-2',5'-dihydro-2'(*S*)-furanyl]-oxy} methylidene)-3,3a(*S*),4, 8b(*R*)-tetrahydro-2H-indeno[1,2-b]furan-2-one ((-)-12a).** Fast moving 6-methyl-GR 24 diastereomer **12a** was separated into its enantiomers **(+)-12a** and **(-)-12a** using a semi-preparative Chiralcel OD HPLC column and hexane/2-propanol 80/20 as the eluent. Flow: 3.0 mL/min. Recrystallisation from hexane/dichloromethane yielded **(+)-12a** and **(-)-12a** as colourless needles. **(+)-12a:** mp: 162-163°C. e.e. >99% (determined by HPLC). $[\alpha]^{20}_D$=+417.2° (c=0.03, CH_2Cl_2). **(-)-12a:** mp: 160-161°C. e.e. >99% (determined by HPLC). $[\alpha]^{20}_D$=-428.4° (c=0.03, CH_2Cl_2). All other analytical data were identical with those of the racemate **12a**.

6-Methyl-3-((*E*)-1-{[4'-methyl-5'-oxo-2',5'-dihydro-2'(*S*)-furanyl]oxy}methylidene)-3,3a(*R*),4, 8b(*S*) -tetrahydro-2H-indeno[1,2-b]furan-2-one ((+)-12b) and its enantiomer **6-Methyl-3-((*E*)-1-{[4'-methyl-5'-oxo-2',5'-dihydro-2'(*R*)-furanyl]-oxy} methylidene)-3,3a(*S*),4,8b(*R*) -tetrahydro-2H-indeno[1,2-b]furan-2-one ((-)-12b).** Slow moving 6-methyl-GR 24 diastereomer **12b** was separated into its enantiomers **(+)-12b** and **(-)-12b** using a semi-preparative Chiralcel OD HPLC column and hexane/ethanol 85/15 as the eluent. Flow: 3.0 mL/min. Recrystallisation from hexane/dichloromethane furnished **(+)-12b** and **(-)-12b** as colourless needles. **(+)-12b:** mp: 159-161°C. e.e. >99% (determined by HPLC). $[\alpha]^{20}_D$=+275.6° (c=0.04, CH_2Cl_2). **(-)-12b:** mp: 158-160°C. e.e. >99% (determined by HPLC). $[\alpha]^{20}_D$=-267.9° (c=0.04, CH_2Cl_2). All other analytical data were identical with those of the racemate **12b**.

Biological Activity.

Plant material. Seeds of *Striga hermonthica* (Del.) Benth. were collected from Sorghum *(Sorghum bicolor* (L.) Moench) on Gezira Research Station, Sudan in 1994. *Orobanche crenata* Forsk. seeds were harvested from faba bean *(Vicia faba* L.) in Beheira, Egypt in 1993. The seeds were stored in glass vials in the dark at room temperature until use in germination tests.

Preparation of the test solutions. A compound to be tested was weighed out very accurately to the amount of 1.0 mg, dissolved in 5 mL acetone p.a. and diluted with demineralized water to 50 mL. These stock solutions of approximately 10^{-4} mol/L (the exact concentration depending on the molecular mass of the compound used) were further

diluted with demineralized water to obtain test solutions with concentrations ranging from 5 x 10^{-5} to 5 x 10^{-9} mol/L. All solutions were prepared directly before use.

Bioassays. For surface sterilization all seeds were exposed for 5 minutes to a 50% (v/v) aqueous solution of commercial bleach (2% hypochlorite). Subsequently, the seeds were thoroughly rinsed with demineralized water and air-dried. For conditioning the seeds were spread on glass fiber filter paper disks (8 mm diameter, approximately 60-100 seeds per disk) in Petri dishes, wetted with demineralized water and stored in the dark at 20 °C for *Orobanche* seeds and at 30 °C for *Striga* seeds. Thereafter the conditioning water was removed and conditioned seeds were placed in new Petri dishes and exposed to test solution. After incubation for 24 hours *(Striga)* and 7 days *(Orobanche)* in the dark at the indicated temperatures the percentages of germinated seeds were determined under a microscope. Seeds were considered to be germinated if the radicle protruded through the seed coat. In each test series an aqueous solution of 0.1% (v/v) acetone was included as a negative control. For full details of the bioassay, see Mangnus *et al.*[25]

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6

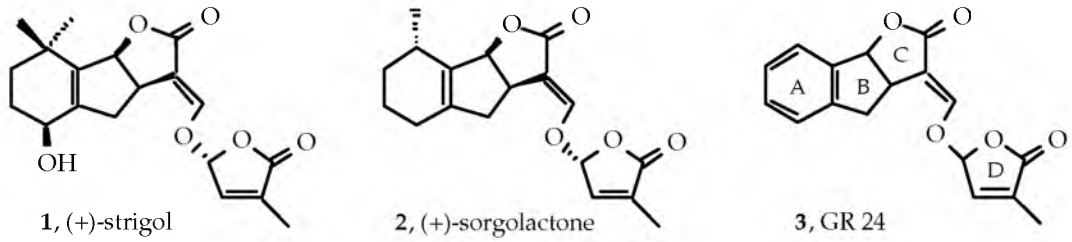
Synthesis and Biological Activity of Labelled Germination Stimulants for the Study of the Strigolactone Receptor

Abstract Strigolactones are highly potent germination stimulants for the parasitic weeds *Striga* and *Orobanche*. The induction of seed germination of is thought to proceed via a receptor-mediated mechanism. Three techniques are outlined for the study of the strigolactone receptor using suitably labelled strigolactone analogues. The synthesis of all enantiopure diastereoisomers of amino-GR 24 (**4**) is described. The amino group was used for the attachment of various labels to this synthetic germination stimulant. The biological activity of the labelled ligands was determined; they were inactive toward *O. crenata*, but did stimulate the germination of *S. hermonthica* seeds. Preliminary results of receptor studies using fluorescence microscopy and fluorescence correlation spectroscopy are reported. Although much more experimentation is needed, the preliminary results seem to indicate, that there is indeed a binding of the labelled stimulant with a protein.

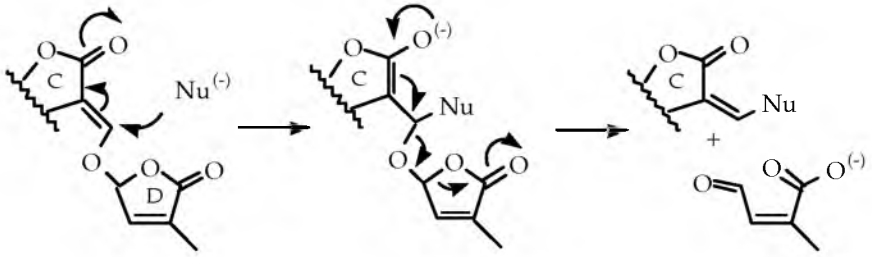
Introduction

The parasitic weeds *Striga* and *Orobanche* are serious agricultural pests in tropical and semitropical areas of the eastern hemisphere and in the Mediterranean area.[1,2] The parasitic process begins with the germination of the weed seeds, which is induced by a germination stimulant, present in the root exudate of host plants and also of several non-host plants.[3,4] In the absence of a germination stimulant, the parasite seeds do not germinate. As is adequately reviewed in chapter 1 and chapter 2 of this thesis, naturally occurring sesquiterpenes belonging to the class of the strigolactones, *e.g.* (+)-strigol (**1**)[5] and (+)-sorgolactone (**2**)[6] are highly potent germination stimulants for these parasitic weeds (Figure 1). Many strigolactone analogues have been synthesized (see chapter 1) *e.g.* GR 24 (**3**)[7,8] which is almost as active as the natural strigolactones.

Figure 1



Scheme 1

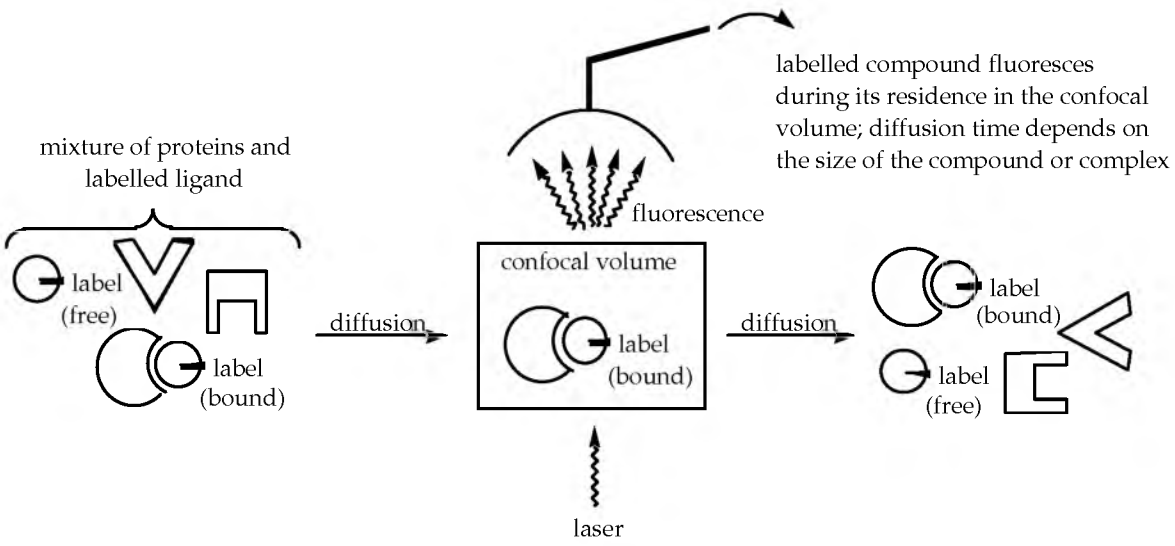


From structure-activity studies it was concluded, that the bioactiphore of strigolactones resides in the CD-part of these molecules.[9] It was hypothesized that the induction of *Striga* and *Orobanche* seed germination proceeds via a receptor-mediated molecular mechanism, which is depicted in scheme 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. In this manner a covalent bond is formed between the receptor and the germination stimulant. This chemical event is supposed to trigger the cascade of reactions that leads to germination.

Thus far, nothing is known about the protein structure of this hypothesized receptor, nor of its localization within the seeds. Detailed knowledge of the receptor protein would enable the design of a perfectly fitting substrate, that might be used to control parasitic weed pests in the suicidal germination approach (see chapter 1 and chapter 4).[10]

Among others, the following techniques can be used to obtain information about the strigolactone receptor: Fluorescence Correlation Spectroscopy (FCS), Scanning Force Microscopy (SFM) and Photo Affinity Labelling (PAL). The principle of FCS is outlined in figure 2. The protein mixture and a solution of the labelled substrate are placed in a small reaction vessel, *e.g.* a microtiter plate well. A specific part of the sample volume is illuminated by a focused laser beam. In this manner, a small open volume element, the confocal volume, is created. In this confocal volume, fluorescent molecules will be excited, leading to a burst of fluorescence photons. Due to Brownian motions, molecules will diffuse in and out of the confocal volume, which results in changes in the detected fluorescence intensity. These intensity fluctuations can be rapidly measured with a fast photon detector and autocorrelated on-line. The autocorrelation function provides information about the diffusion properties of fluorescent molecules.

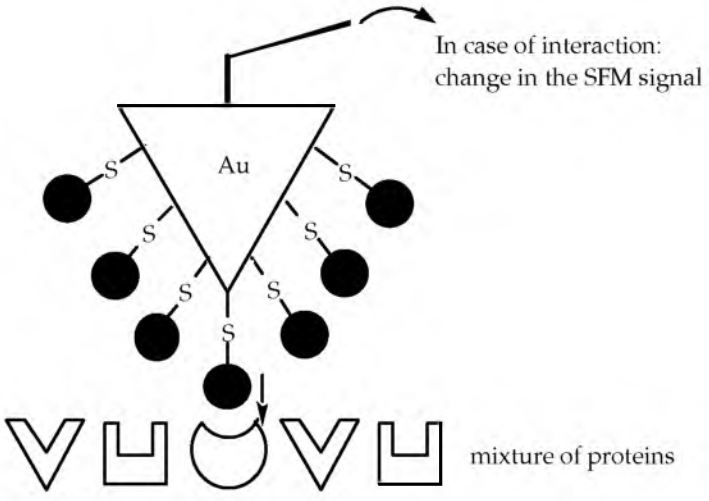
Figure 2



Small-sized molecules move more rapidly through the confocal volume element than large macromolecules such as proteins. When a small fluorescent ligand interacts with a protein, it can be immediately detected by a retarded diffusion time, and the interaction can be fully quantified. The main advantage of this technique is that it is highly sensitive in the low concentration range in which strigolactones are active (10^{-8}-10^{-11}M).[11,12] In addition, FCS does not require radioactively labelled ligands. Another advantage is the ease and speed with which FCS-measurements can be performed.

Scanning Force Microscopy can also be used to detect whether there is an interaction between a ligand and its receptor. The principle of the technique is shown in figure 3. The surface of a gold SFM-tip is covered with the ligand, which for this purpose requires a linker with a free SH-group to ensure attachment to the gold surface.

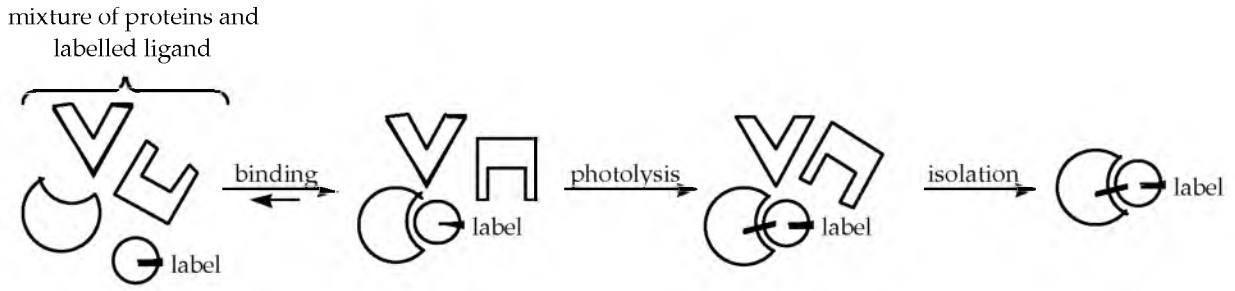
Figure 3



This tip is brought in close proximity of a surface, covered with the mixture of proteins under investigation. While scanning this surface, (specific) interaction can take place between the ligand and its receptor. This results in bending of the SFM probe toward the protein, which leads to a change in the electronic signal, indicating that an interaction has occurred.[13] Similar to FCS, this is a detection technique, which cannot be used for the actual isolation of a specific protein.

For the actual isolation of a receptor protein from a mixture, Photo Affinity Labelling has been successfully used.[14,15] It involves the formation of a covalent bond between a radio-labelled ligand and its receptor binding site, as is shown in figure 4. A photoreactive unit incorporated in the ligand, *e.g.* an azido group, is used to generate a very reactive, short-lived intermediate upon irradiation (photolysis), which will immediately form a covalent bond with the molecule in its nearest vicinity.[16] If the concentration of the photo affinity ligand is correctly chosen, and a certain time is allowed for binding, the substrate will be mainly located at the receptor site. Upon irradiation, the covalent bond is then formed between the ligand and this protein. The radiolabel allows isolation of the complex from the mixture. In principle, a fluorescent tag instead of a radiolabel can also be used for this purpose.

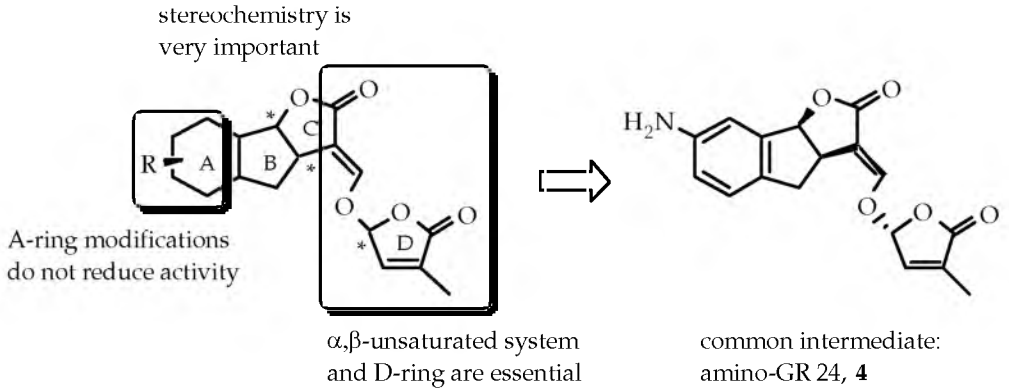
Figure 4



In the case of the strigolactone receptor, incorporation of a photoreactive unit may not strictly be necessary. If the molecular mechanism (Scheme 1) is correct, treatment of the seeds with a radioactive or fluorescent strigolactone analogue may directly lead to covalent attachment of the ligand to the receptor protein, which obviates the need for a photoreactive unit. Conventional fluorescence microscopy, may then be a suitable method to detect whether fluorescence has concentrated on the seed surface, thus indicating the presence of a receptor site. However, due to the low concentrations at which strigolactones are active, the fluorescence of the ligands may be below the detection limit of the microscope.

The position of the various labels in the ligand should be such, that the biological activity of the ligand is not affected. Preliminary research on this topic[17] (Figure 5) revealed, that a large substituent on the A-ring of GR 24 does not diminish the germinating activity toward *Striga hermonthica* seeds. In contrast, *Orobanche crenata* seeds did not germinate when exposed to a strigolactone analogue with a large A-ring substituent.

Figure 5



Furthermore, the stereochemistry of labelled germination stimulants should preferably be the same as that of the natural strigolactones, in order to achieve optimal binding. Amino-GR 24 (**4**)[17] allows the attachment of a variety of substituents to the A-ring, and is therefore an ideal building block for the synthesis of labelled strigolactone analogues.

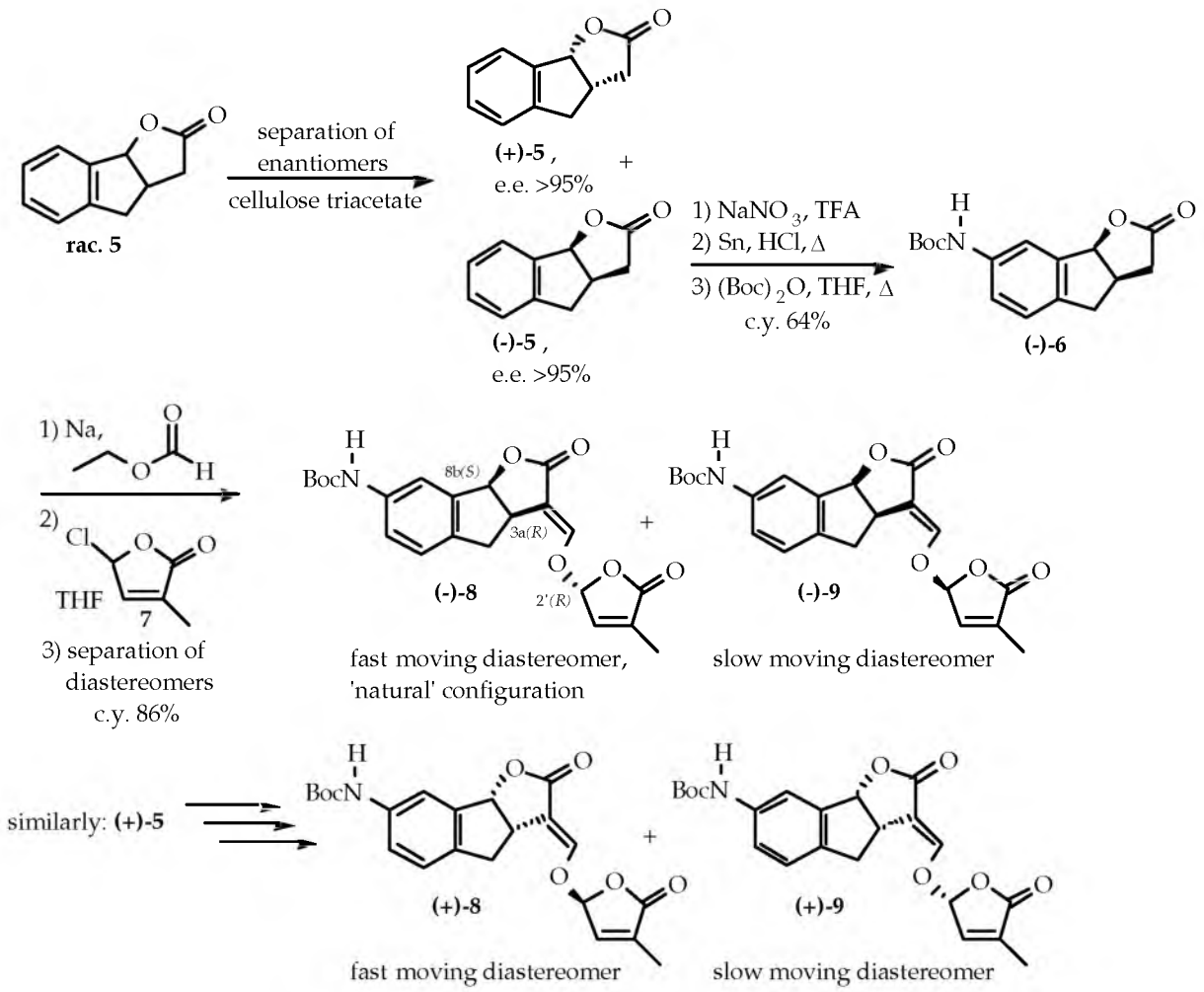
In this chapter the synthesis of optically active amino-GR 24 is described, as well as the preparation of three labelled strigolactone analogues, which might be used for the study of the strigolactone receptor with the aforementioned techniques. Preliminary results using FCS and conventional fluorescence microscopy are reported.

Results and Discussion

Synthesis

The synthetic strategy to obtain enantiopure amino-GR 24, involves coupling of racemic D-ring **7** to enantiopure amino-ABC-lactone. In this manner, two diastereoisomers of the strigolactone analogue are obtained, which can be separated by silicagel chromatography. In order to obtain the desired optically pure ABC-parts, racemic tricyclic lactone **5**[18,19] was chromatographically resolved using cellulose triacetate, as described previously[8] (Scheme 2). Nitration of **(+)-5** and **(-)-5**, and subsequent reduction of the nitro group to an amino substituent was performed according to the method of Thuring *et al.*[17] Prior to formylation and coupling with chlorobutenolide **7**, the amino substituent was protected with the acid labile *tert*-butyl oxycarbonyl (Boc) group. The benzylimine protective group that was used by Thuring *et al.* was rather difficult to remove, leading to low yields of **4**[17]; therefore preference was given to the Boc protective group in the synthetic sequence outlined in scheme 2. Formylation of **(-)-6** employing sodium in ethylformate as the solvent, followed by reaction of the resulting sodium enolate with chlorolactone **7**[7,20] in THF[21] gave protected enantiopure amino-GR 24 diastereoisomers **(-)-8** and **(-)-9** in good yield. In a similar manner, **(+)-8** and **(+)-9** were synthesized from tricyclic lactone **(+)-5**.

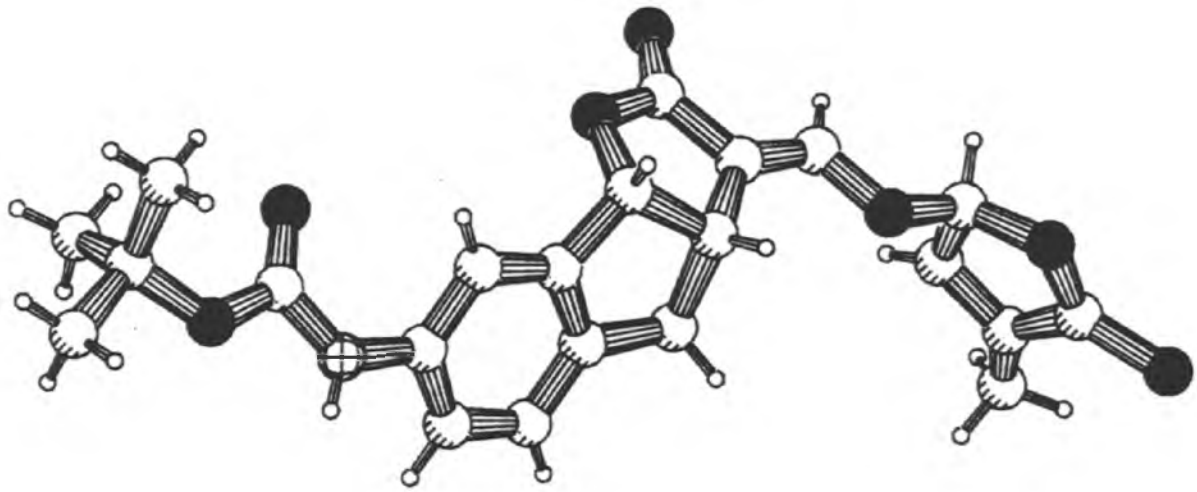
Scheme 2



In order to secure the absolute configuration of the protected amino-GR 24 diastereoisomers, X-ray diffraction analysis was undertaken of the slow moving Boc-amino-GR 24 diastereoisomer (based on its behaviour on TLC), resulting from the coupling of ABC-lactone **(+)-5**, with the D-ring. The absolute configuration of this diastereoisomer was established as **(+)-8**, which is the mirror image of the 'natural' diastereoisomer (see structure of (+)-strigol (**1**) in figure 1). A PLUTON[22] generated drawing of this X-ray crystal structure is depicted in figure 6. The rest of the decisions with regard to the stereochemistry of the substrates was based on this X-ray analysis, *i.e.* the slow moving BocNH-GR 24 diastereoisomer derived from **(-)-5** was considered to have the 'natural' absolute stereochemistry, and was therefore selected for further synthetic elaboration.

In a later stage of this study, when the synthesis and testing of the labelled analogues were already carried out, it was found - during the refinement process of the X-ray analysis of the slow moving BocNH-GR 24 diastereoisomer derived from **(+)-5** (see figure 6) - that the atom types in ring D were incorrectly assigned. For the determination of the orientation of ring D, it was assumed that the positions of the oxygen atoms were unambiguously defined with respect to the rigid part of the molecule.

Figure 6



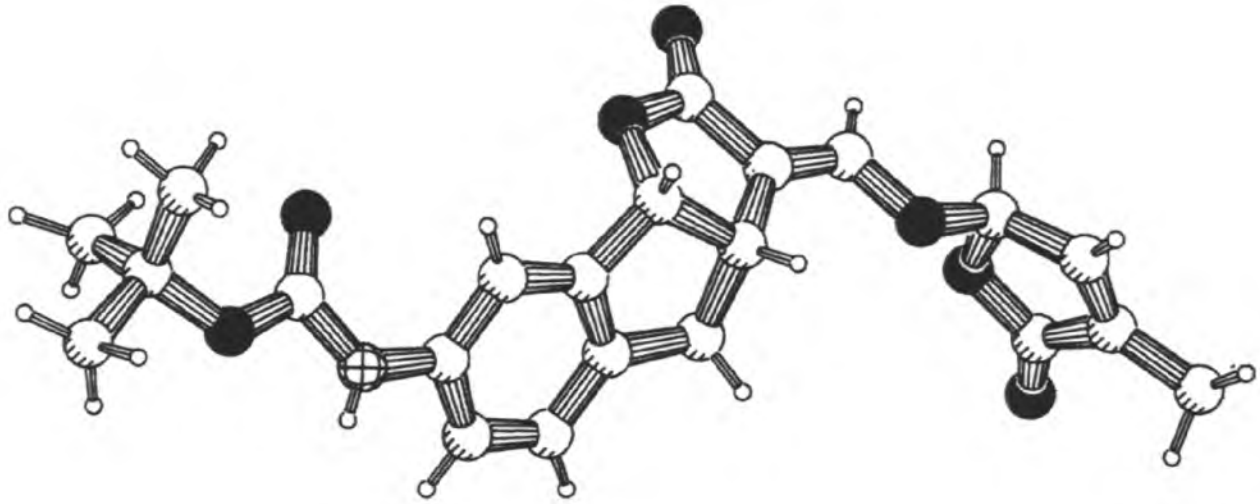
The assignment of the atom types to the atoms in the D-ring, was performed in accordance with the 2-dimensional projection of the molecule, however with an incorrect assumption for the absolute configuration *(i.e.* **(+)-8**).

During the initial refinement, with calculated positions for the hydrogen atoms, refinement converged to an R-value of 0.88. During those first refinements, the average displacement parameters showed no exceptional values, and did not point at any obvious problems in the model. Moreover, the preliminary model was not contradicted by chemical reasoning, since the skeleton of ring D contains twofold symmetry, apart from the atom types. However, during the final refinements of the model, the switch in atom type assignment was discovered, when the hydrogen atoms were freely refined. A switch of the atom types lowered the R-value considerably, and the resulting average displacement parameters, distances and angles showed more consistent values than those for the preliminary model. Therefore, the correct absolute structure of the slow moving BocNH-GR 24 diastereoisomer derived from **(+)-5** is **(+)-9**. A PLUTON generated drawing of the improved X-ray crystal structure is depicted in figure 7.

Due to a premature release (and the subsequent use) of the preliminary X-ray results, which were only based on the initial refinement of the model, a wrong selection was made for the BocNH-GR 24 diastereoisomer with the 'natural' stereochemical configuration for synthetic elaboration. This unfortunate event explains why the stereochemistry at C2' of the labelled strigolactone analogues, depicted in the following schemes, is unintentionally *(S)*, whereas the 'natural' strigolactones all have the C2'*(R)*-configuration.

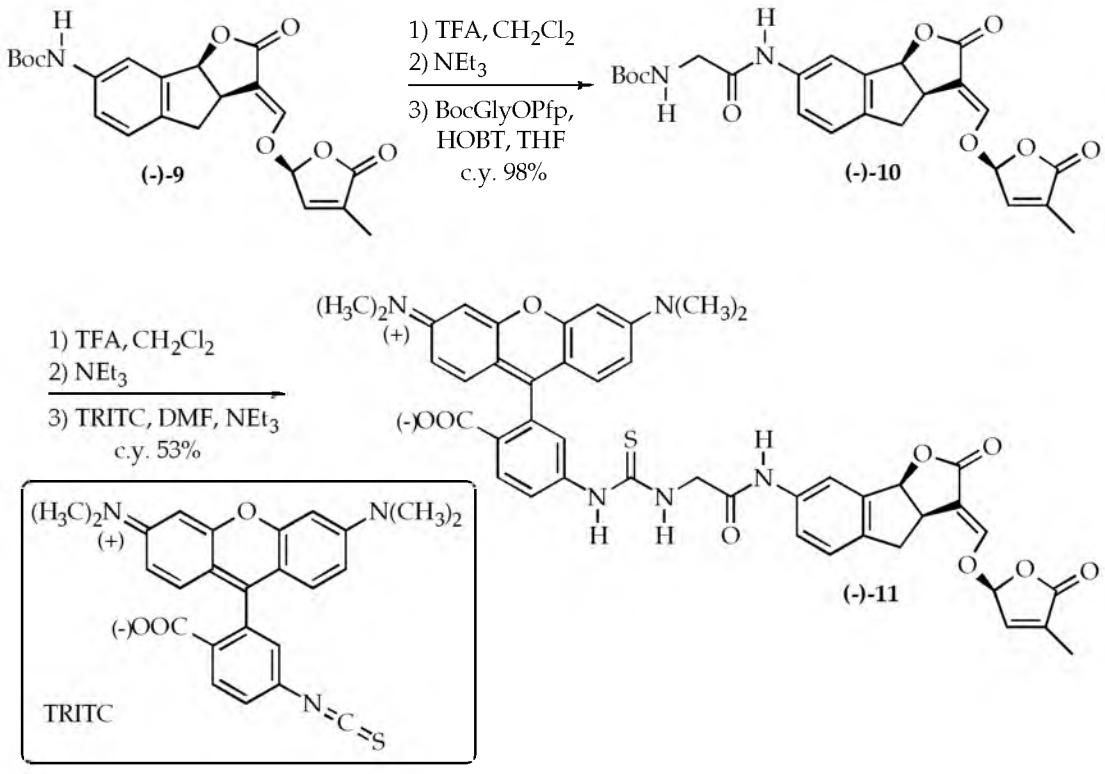
A labelled strigolactone analogue, suitable for Fluorescence Correlation Spectroscopy studies, requires a fluorescent label with an absorption maximum at the wavelength of the FCS-laser. Tetramethyl rhodamine (λ_{max}Abs = 543 nm), is perfectly suited for excitation at the 543 nm spectral line of the green Helium-Neon laser.[23] The reactive isothiocyanate of tetramethyl rhodamine, TRITC, was chosen for coupling to protected amino-GR 24 **(-)-9**. However, the deprotected aromatic amine group was not very reactive toward TRITC.

Figure 7

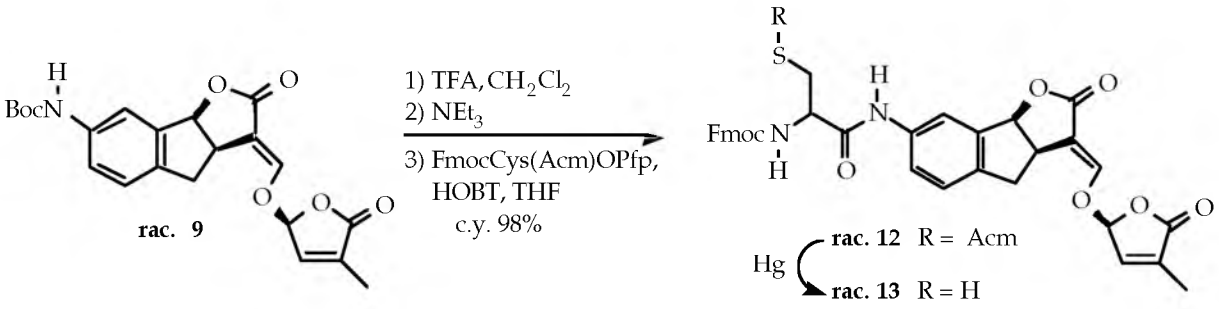


Therefore, a glycine spacer was linked to the aromatic amine, which was readily performed via coupling with the pentafluoro phenyl (PfP) ester of glycine[24] in the presence of HOBT (Scheme 3). Removal of the Boc protective group from **(-)-10**, gave a highly reactive primary amine, which smoothly reacted with TRITC. Isolation of the intermediate primary amine turned out to be impossible, because of its rapid conversion to the corresponding carbamic acid, by reaction with CO_2 in air. Fluorescently labelled strigolactone analogue **(-)-11** was purified by silicagel chromatography, and isolated as a bright pink powder in 53% yield.

Scheme 3

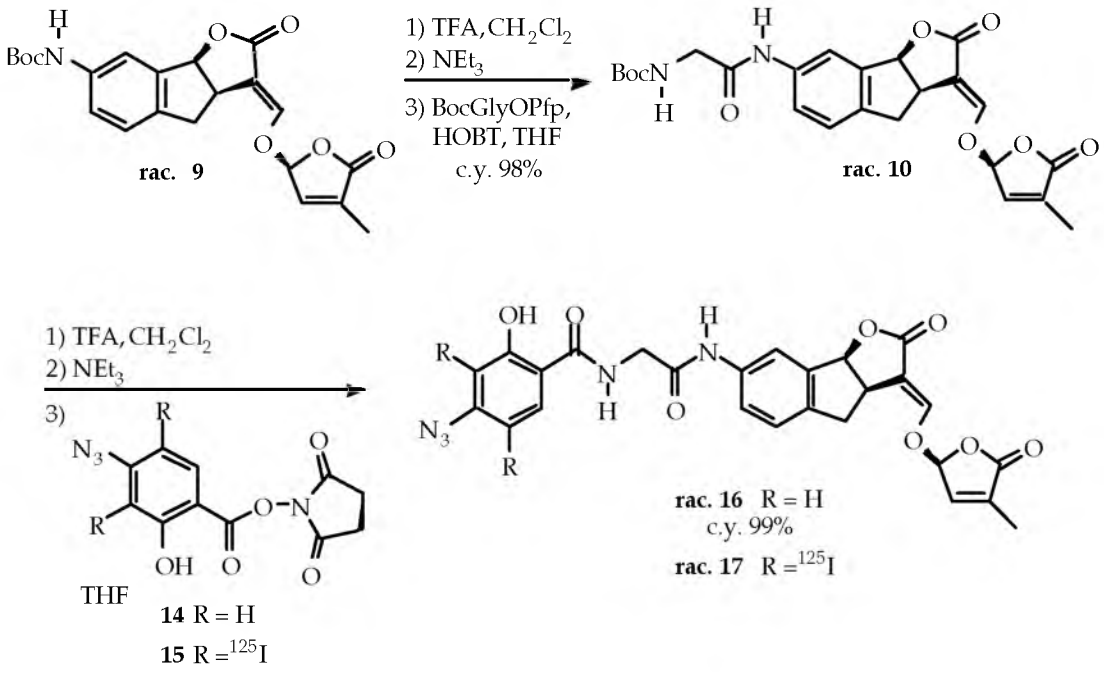


Scheme 4



A ligand that can be used as a probe in scanning force microscopy, requires the presence of an SH group. Since coupling of deprotected amine **9** with an amino acid PfP ester proceeded smoothly, this reaction was also chosen for the introduction of the SH moiety to the GR 24 derivative. Commercially available cysteine derivative FmocCys(Acm)OPfP was reacted *in situ* with deprotected amine **rac. 9** to give compound **rac. 12** (Scheme 4). The acetamido methyl (Acm) protective group can be removed under neutral conditions in the presence of mercury.[25] In principle, ligand **rac. 13** can be linked to a gold SFM tip. The position of the SH group in the ligand is such, that steric interference with the bioactiphore of the strigolactone analogue is unlikely. For protein-fishing studies, it is not strictly necessary to remove the Fmoc group, provided the molecule is sufficiently active toward the parasitic weed seeds. The synthetic scheme for **rac. 13** can also be used for enantiomerically pure material, which can subsequently be employed to coat the gold probe for the SFM-studies.

Scheme 5



The technique of photo affinity labelling necessitates a ligand equipped with a photo reactive group, as well as a radioactive atom. It is highly desirable to include these groups in the final synthetic step, to minimize the risk of premature activation of the light sensitive unit and to avoid unnecessary manipulations with radioactive material. The readily available salicylic acid derivative **15** is very useful in this respect, since it contains both the photoreactive azido group, as well as two radioactive iodine atoms.[26] To demonstrate the feasibility of the synthetic strategy, the commercially available precursor of **15**, *viz.* succinimidyl ester **14**, was reacted with strigolactone analogue **rac. 10** (Scheme 5). The coupling with the aliphatic primary amine of the spacer proceeded in quantitative yield, whereas the aromatic amine of amino-GR 24 (**4**) was much less reactive. The chemistry shown in scheme 5 can also be applied for the labelled materials **15** and **17**, respectively. A drawback of this labelling methodology is that the concentration of protein-bound label may escape detection, due to a too low concentration.

Biological studies

The germination stimulatory activity of labelled strigolactone analogues **(-)-11**, and **rac. 12** was assayed using seeds of *Striga hermonthica* and *Orobanche crenata*. In each bioassay, GR 24 was included as a positive control. In addition, an aqueous blank was assayed as a negative control. The results for *S. hermonthica* are collected in table 1. It was not possible to determine the biological activity of PAL-ligand **rac. 16**, because it was completely insoluble in an aqueous medium, even when a large amount of cosolvent was used to prepare the stock solution of this compound.

Stimulation of *O. crenata* seeds with solutions of 5 x 10^{-5}, 5 x 10^{-6} and 5 x 10^{-7} M GR 24 resulted in 39.1 ± 2.4; 42.4 ± 4.2 and 34.8 ± 1.4 % germinated seeds respectively. Strigolactone analogues **(-)-11** and **rac. 12** did not exhibit germination stimulatory activity toward *O. crenata*, which is in accordance with the results for dansyl-GR 24, reported by Thuring *et al.*[17] Obviously, a large substituent on the A-ring is not tolerated by the strigolactone receptor site of *O. crenata* seeds.

Table 1. Percentages of germinated seeds of S. hermonthica after exposure to solutions (5 x 10^{-6}, 5 x 10^{-7} and 5 x 10^{-9} mol/L) of **(-)-11**, **rac. 12** *and the control GR 24 (3).*[a]

		% germination ± SE at a concentration of		
entry	compound	5 x 10^{-6} mol/L	5 x 10^{-7} mol/L	5 x 10^{-9} mol/L
1	**(-)-11**	39.9 ± 4.9	44.4 ± 0.8	13.7 ± 0.7
2	**rac. 12**	22.8 ± 8.1	7.1 ± 2.0	7.7 ± 3.6
3	**GR 24**[b]	30.7 ± 2.3	66.8 ± 2.8	20.8 ± 5.3

[a]Data presented are the mean ± SE of one representative experiment. [b]Equimolar mixture of two racemic diastereoisomers.

For *S. hermonthica*, both labelled compounds **(-)-11** and **rac. 12** stimulated the germination of the weed seeds, confirming Thuring's result that a large A-ring substituent is indeed tolerated by the strigolactone binding site of *S. hermonthica* seeds.[17] It should be noted however, that **rac. 12** was also rather insoluble in aqueous medium. The germination values reported for this compound were difficult to reproduce, probably due to crystallisation of the stimulant in the aqueous solution. In all assays, the aqueous blanks had not induced germination of the seeds.

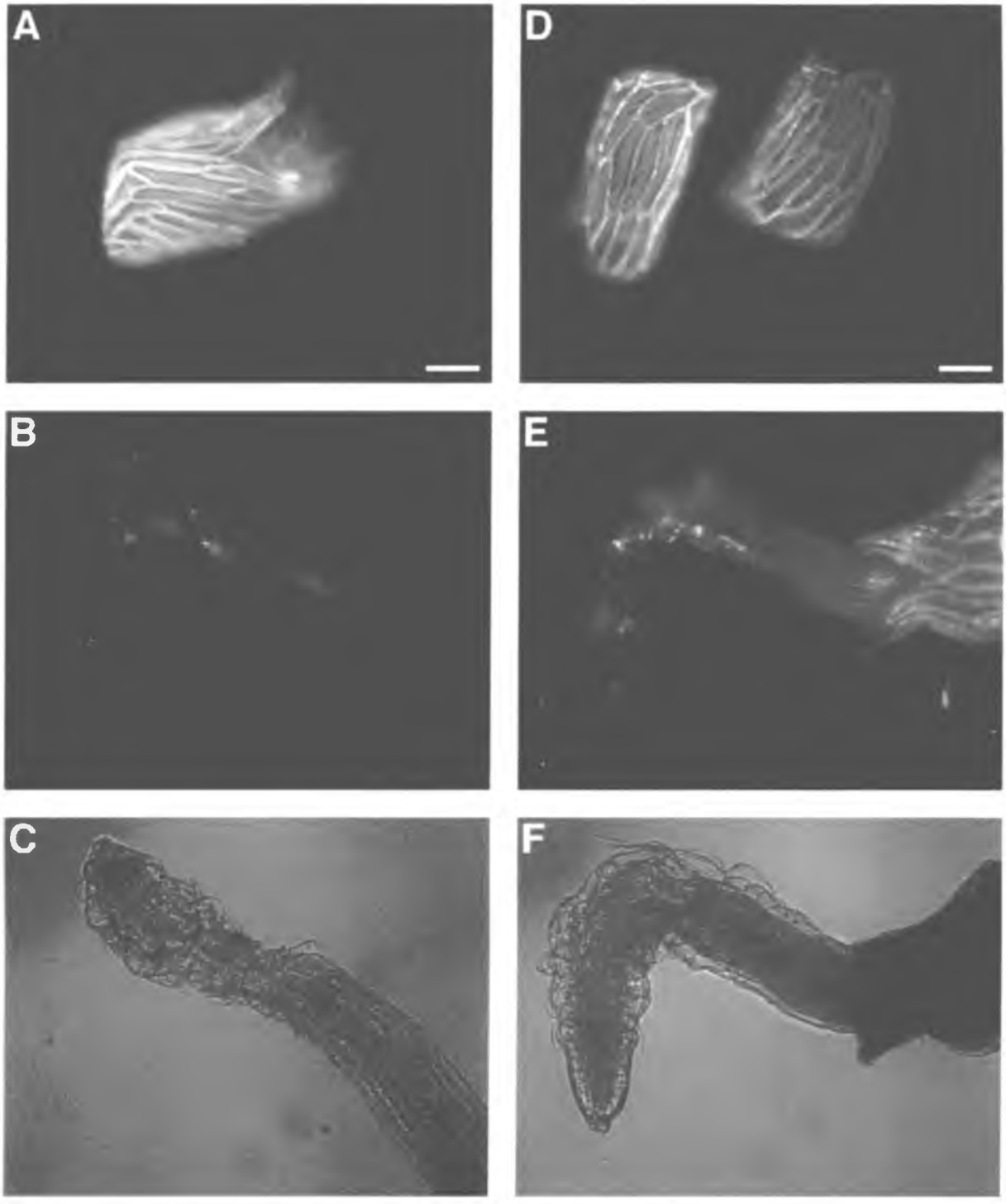
Fluorescent stimulant **(-)-11** dissolved well in water because of its ionic nature. Considering the fact, that **(-)-11** has the *(S)*-configuration at the D-ring stereogenic center, which is not the optimal steric arrangement, its biological activity is remarkably high. Therefore, tetramethyl rhodamine labelled GR 24 seems excellently suited for studies of the strigolactone receptor of *S. hermonthica* seeds. The C2'-epimer of **(-)-11**, which has the 'natural' stereochemical arrangement, is expected to give the best chance for good results in the preliminary experiments, that are described below.

Germinated *S. hermonthica* seeds, stimulated with 5 x 10^{-7} M tetramethyl rhodamine labelled stimulant **(-)-11**, were studied with a fluorescence microscope, using the 546 nm line of a mercury lamp for excitation. If the molecular mechanism depicted in scheme 1 is correct, a covalent bond should have been formed between the fluorescent ABC-part of the stimulant and its receptor, which might be visible as fluorescent spots on the seeds, indicating the presence of the receptor site. *S. hermonthica* seeds, germinated with 5 x 10^{-7} M GR 24 were used as a control. Prior to use under the fluorescence microscope, the seeds were washed with water to remove excess stimulant. The results of this study are shown in figure 6.

Figure 6 shows, that there is no clear difference in the fluorescence from a *S. hermonthica* seed stimulated with tetramethyl rhodamine labelled GR 24, **(-)-11** (6D and 6E), and that from a seed stimulated with unlabelled GR 24 (6A and 6B). The fluorescent spots in the radicles are not tetramethyl rhodamine, since they were also present in the radicles of seeds germinated with unlabelled stimulant. These spots are probably caused by metabolites produced in the radicle. These studies neither confirm nor disprove the hypothesized molecular mechanism for germination induction, because the amount of bound stimulant might be too small to exhibit detectable fluorescence. More experimentation is needed before firm conclusions can be drawn.

The aim of the fluorescence correlation spectroscopy studies, was to see whether an interaction could be observed between ligand **(-)-11** and a mixture of proteins extracted from *S. hermonthica* seeds. Binding of the fluorescent ligand to a large (receptor) protein would lead to elevated diffusion times. The concentration of 2,5 x 10^{-8} M of biologically active **(-)-11** gave an optimal signal. Proteins were extracted from conditioned *S. hermonthica* seeds with a standard lysis buffer. For these preliminary FCS-experiments, the crude protein extracts were used, to avoid loss of the essential protein fraction in subsequent purification steps.

Figure 6. Fluorescence micrographs of S. hermonthica seeds, germinated with 5 x 10^{-7} M GR 24. Picture A shows a germinated seed; B is a picture of the radicle of this seed; C is a transmitted light micrograph of the same radicle. Pictures D-F are similar micrographs of seeds germinated with 5 x 10^{-7} M ***(-)-11****. The bar represents 100 μm.*



The results of the FCS studies are collected in table 2. The mean diffusion times ($\tau_{diff.}$) observed for several mixtures of ligand and *Striga* proteins are given. The mean diffusion time of the proteins in the extract ($\tau_{diff.}$ = 400 μs) could be determined due to a small amount of autofluorescence. This diffusion time was consistent with values normally found for proteins.[27] Entry 2 shows that the mean diffusion time for unbound ligand was 52 μs. This value was fixed when a two component analysis of the autocorrelation curves was performed, to calculate the amount of free fluorescent ligand present in a mixture of ligand and protein. The mean diffusion time of a larger fluorescent molecular complex, that results upon addition of a solution of the ligand to *S. hermonthica* protein extract, was in all cases approximately 400 μs. This is an indication that the ligand has undergone complexation with a protein. Entries 3-7 show that the formation of the large fluorescent complex occurs instantly, and that the amount of complex formed does not increase in time.

Table 2. Sample contents, mean diffusion time, and the percentage of ligand bound to protein for various combinations of S. hermonthica protein extract and tetramethyl rhodamine labelled GR 24 ***(-)-11****. Several samples were followed in time (entries 3-7 and 8-9)*

entry	sample	time of measurement (min)	$\tau_{diff.}$ (μs)	% bound ligand ($\tau_{diff.}$ = 400 μs)
1	protein extract	0	400	-
2	2.5 x 10^{-8} M **(-)-11**	0	52	-
3	30 μL protein extract + 30 μL 5 x 10^{-8} M **(-)-11**	0	77	15
4	idem	10	75	14
5	idem	15	76	15
6	idem	20	76	15
7	idem	90	76	15
8	30 μL protein extract + 30 μL 2.5 x 10^{-8} M **(-)-11**	0	87	18
9	idem	10	89	19
10	30 μL protein extract + 30 μL 2.5 x 10^{-7} M **(-)-11/3** (1/1)	10	60	5.5
11	30 μL protein extract + 30 μL 2.5 x 10^{-7} M **(-)-11/3** (1/3)	10	64	7.5

Data presented are the mean of five representative FCS-measurements.

When the concentration of free ligand in the sample was lowered (entries 8 + 9), the percentages of free and bound ligand resulting from two-component analysis were expected to change: due to the presence of less free ligand, the relative amount of bound ligand should increase. Indeed this was the case, which is an indication that the ligand specifically complexes with a certain protein fraction. When the concentration of free ligand in the sample was lowered further, the amount of fluorescence was below the detection limit.

Competition studies, using a 1/1 mixture of tetramethyl rhodamine labelled ligand **(-)-11** and unlabelled GR 24 (**3**), seem to support the proposed specific binding, because the amount of fluorescent ligand protein complex with $\tau_{diff.}$ = 400 μs decreased (entry 10), which was expected because some of the binding sites would now be occupied by the unlabelled ligand. However, when a 1/3 mixture of **(-)-11** and **3** was added to the protein extract, a further decrease in the percentage of large fluorescent complex was not observed. It should be noted here, that the proposed covalent binding of the ligand (either **(-)-11** or **3**) represents an unusual situation. The relative binding kinetics are determining the ratio of binding with **(-)-11** and **3**. When there is no covalent binding, the ratio of complexation with **(-)-11** and **3** is the result of an equilibration process. Clearly, more experimentation is needed before unambiguous conclusions can be drawn. It should also explicitly be mentioned that the method of protein extraction from *S. hermonthica* seeds needs much more attention. Subtle differences in the extraction procedure may have a profound influence on the occurrence of the binding protein in the extract.

In conclusion, the preliminary results of the FCS-experiments seem to indicate that the fluorescently labelled strigolactone analogue **(-)-11** does bind to a protein present in *S. hermonthica* seed extract. The technique is perfectly suitable for studies of tetramethyl rhodamine labelled strigolactones at biologically active concentrations. On the basis of the preliminary results presented above, it is worthwhile to continue this research, using the 'natural' stereoisomer of **11**, which is optimally suited for binding to its receptor site. The method of protein extraction from *Striga* seeds needs explicit attention.

Experimental

Synthesis. General methods and instrumentation are the same as described in chapter 5. Enantiomeric excesses of compounds **8** and **9** were determined by analytical HPLC using a Chiralcel OD (10 mm) cellulose carbamate column (Baker, 250 x 4.6 mm) and mixtures of 2-propanol or ethanol and hexane as the eluent.

Nomenclature. Systematic names were generated using the ACD/Name programme, provided by Advanced Chemistry Development Inc. (Toronto, Canada).

7-Nitro-3,3a*(R)*,4,8b*(S)*-tetrahydro-2H-indeno[1,2-b]furan-2-one. Tricyclic lactone **(-)-5** (2.9 g, 16.7 mmol) was nitrated as described by Thuring *et al.*[17] Recrystallisation from toluene afforded pale yellow needles. Yield: 74%. mp: 118-119°C. $[\alpha]^{22}_{D}$=-197.2° (c=0.4, CH_2Cl_2). All

other analytical data were identical with those reported previously for the racemic compound.[17]

7-Nitro-3,3a*(S)*,4,8b*(R)*-tetrahydro-2H-indeno[1,2-b]furan-2-one. Tricyclic lactone **(+)-5** (3.25 g, 18.7 mmol) was nitrated as described by Thuring *et al.*[17] Recrystallisation from toluene gave pale yellow needles. Yield: 75%. mp: 119-120°C. $[\alpha]^{22}_D$=+207.8° (c=0.4, CH_2Cl_2). All other analytical data were identical with those reported previously for the racemic compound.[17]

7-Amino-3,3a*(R)*,4,8b*(S)*-tetrahydro-2H-indeno[1,2-b]furan-2-one. Reduction of (-)-nitro tricyclic lactone (2.66 g, 12.15 mmol) was accomplished in quantitative yield with tin and hydrochloric acid as described previously for the racemic compound.[17] mp: 128-130°C. $[\alpha]^{22}_D$=-180.8° (c=0.4, CH_2Cl_2). All other analytical data were in complete accordance with those reported for racemic 7-amino tricyclic lactone.[17]

7-Amino-3,3a*(S)*,4,8b*(R)*-tetrahydro-2H-indeno[1,2-b]furan-2-one. Reduction of (+)-nitro tricyclic lactone (2.35 g, 10.73 mmol) was accomplished in 97% yield with tin and hydrochloric acid as described previously for the racemic compound.[17] mp: 127-129°C. $[\alpha]^{22}_D$=+181.6° (c=0.4, CH_2Cl_2). All other analytic data were the same as reported for racemic 7-amino tricyclic lactone.[17]

***tert*-Butyl-*N*-[2-oxo-3,3a*(R)*,4,8b*(S)*-tetrahydro-2H-indeno[1,2-b]furan-7-yl]carbamate ((-)-6).** A mixture of 7-amino-3,3a*(R)*,4,8b*(S)*-tetrahydro-2H-indeno[1,2-b]furan-2-one. (2.0 g, 10.6 mmol) and di*tert*-butyl dicarbonate (3.5 g, 16 mmol) was heated under reflux in THF (25 mL). After 2.5 hours, the mixture was cooled and THF was removed under reduced pressure. The residue was dissolved in ethyl acetate, washed with tartaric acid (1M, 1x), dried ($MgSO_4$) and concentrated. Silicagel chromatography (hexane/ethyl acetate 1/1) yielded **(-)-6** (2.6 g, 85%) as a white solid. Recrystallisation from hexane/ethyl acetate afforded colourless needles. mp: 135-136°C. $[\alpha]^{22}_D$=-158.5° (c=0.4, CH_2Cl_2). 300MHz ^{1}H NMR ($CDCl_3$): δ 1.52 (s, 9H, 3 x CH_3 tBu); 2.37 (dd, 1H, $J_{3,3a,\ cis}$=5.8Hz, ^{2}J=18.0Hz, H3); 2.82 (dd, 1H, $J_{4,3a,\ cis}$=3.4Hz, ^{2}J=16.3Hz, H4); 2.88 (dd, 1H, $J_{3,3a,\ trans}$=9.8Hz, ^{2}J=18.0Hz, H3); 3.25 (dd, 1H, $J_{4,3a,\ trans}$=8.3Hz, ^{2}J=16.3Hz, H4); 3.37 (m, 1H, H3a); 5.83 (d, 1H, J=7.2Hz, H8b); 6.54 (bs, 1H, NH); 7.17 (d, 1H, J=8.2Hz, H5); 7.33 (d, 1H, J=8.2Hz, H6); 7.51 (s, 1H, H8). ^{13}C NMR ($CDCl_3$): δ 28.2 (3 x CH_3 tBu); 35.6; 37.3 (2 x CH_2); 37.7 (CH3a); 80.7 (Cq tBu) 87.5 (CH8b); 116.3; 120.8; 125.6 (3 x CH_{arom}); 136.8; 138.0; 139.6 (3 x Cq_{arom}); 152.7 (C=O, carbamate); 176.8 (C=O, lactone). IR (KBr): ν (cm^{-1}) 3338 (NH); 1756 (C=O, lactone); 1726 (C=O, carbamate); 1180 (lactone). MS [EI *m/z*, rel. intensity (%)]: 289 ($[M]^+$, 23.8); 233 ($[C_{12}H_{11}O_4N]^+$, 14.5); 189 ($[C_{11}H_{11}O_2N]^+$, 24.0); 57 ($[C_4H_9]^+$, 81.3); 28 ($[CO]^+$, 100). Anal. calcd. for $C_{16}H_{19}O_4N$: C, 66.42; H, 6.62; N, 4.84 found: C, 66.20; H, 6.60; N, 4.87.

***tert*-Butyl-*N*-[2-oxo-3,3a(*S*),4,8b(*R*)-tetrahydro-2H-indeno[1,2-b]furan-7-yl]carbamate ((+)-6).** Boc-amino tricyclic lactone **(+)-6** was prepared in the same way as **(-)-6**, starting from 7-amino-3,3a(*S*),4,8b(*R*)-tetrahydro-2H-indeno[1,2-b]furan-2-one (3.0 g, 15.9 mmol). An analytically pure sample was obtained by recrystallisation from hexane/ethyl acetate. Yield 87%. $[\alpha]^{22}_{D}$=+160.2° (c=0.4, CH_2Cl_2). Anal. calcd. for $C_{16}H_{19}O_4N$: C, 66.42; H, 6.62; N, 4.84 found: C, 66.23; H, 6.57; N, 4.88. All other analytical data were the same as reported for carbamate **(-)-6**.

***tert*-Butyl-*N*-[3-((*E*)-1-{[4'-methyl-5'-oxo-2',5'-dihydro-2'(*RS*)-furanyl]oxy}methylidene)-2-oxo-3,3a(*RS*),4,8b(*SR*)-tetrahydro-2H-indeno[1,2-b]furan-7-yl]carbamate (rac. 8)** and its 2(S) diastereoisomer: ***tert*-Butyl-N-[3-((*E*)-1-{[4'-methyl-5'-oxo-2',5'-dihydro-2'(*SR*)-furanyl]oxy}-methylidene)-2-oxo-3,3a(*RS*),4,8b(*SR*)-tetrahydro-2H-indeno[1,2-b]furan-7-yl]carbamate (rac. 9).** Racemic Boc-amino-GR 24 diastereoisomers **rac. 8** and **rac. 9** were prepared in the same way as described for optically pure compounds **(-)-8** and **(-)-9**, *(vide infra)* starting from carbamate **rac. 6** (1.0 g, 3.46 mmol). Yield: fast moving diastereoisomer **rac. 8**: 39%; slow moving diastereoisomer **rac. 9**: 37%. All analytical data were the same as reported for the corresponding enantiopure carbamates.

***tert*-Butyl-*N*-[3-((*E*)-1-{[4'-methyl-5'-oxo-2',5'-dihydro-2'(*R*)-furanyl]oxy}methylidene)-2-oxo-3,3a(*R*),4,8b(*S*)-tetrahydro-2H-indeno[1,2-b]furan-7-yl]carbamate ((-)-8)** and its 2(S) diastereoisomer: ***tert*-Butyl-*N*-[3-((*E*)-1-{[4'-methyl-5'-oxo-2',5'-dihydro-2'(*S*)-furanyl]oxy}-methylidene)-2-oxo-3,3a(*R*),4,8b(*S*)-tetrahydro-2H-indeno[1,2-b]furan-7-yl]carbamate ((-)-9).** To a cooled (0°C) and stirred solution of Boc-amino tricyclic lactone **(-)-6** (650 mg, 2.25 mmol) in ethyl formate (10 mL) was added, under a continuous stream of nitrogen, 2.2 equiv. of metallic sodium (111 mg, 4.8 mmol). The mixture was allowed to warm to room temperature and stirred for 1.5 hours. When TLC analysis indicated complete formylation, excess ethyl formate was removed by evaporation *in vacuo*. The thus obtained sodium salt of formylated **(-)-6** was suspended in THF (10 mL) and cooled to 0°C. Upon addition of chlorobutenolide **7** (583 mg, 4.4 mmol) the reaction mixture became clear. The mixture was stirred overnight. Then THF was removed *in vacuo*. The residue was dissolved in a mixture of brine and ethyl acetate. The aqueous phase was extracted with ethyl acetate (2x) and the combined organic layers were washed with satd. NH_4Cl (1x), dried ($MgSO_4$) and concentrated under reduced pressure. The crude product was purified using flash chromatography (SiO_2, hexane/ethyl acetate 2/1) to afford two diastereoisomeric products. Fast moving diastereoisomer **(-)-8** (400 mg, 44%) and slow moving diastereoisomer **(-)-9** (380 mg, 42%) were obtained as white solids. Analytical samples were obtained by recrystallisation from hexane/ethyl acetate.
(-)-8: mp: 115-117°C. e.e. >99% (determined by HPLC). $[\alpha]^{22}_{D}$=-331.6° (c=0.1, CH_2Cl_2). ^{1}H NMR ($CDCl_3$): δ 1.51 (s, 9H, 3 x CH_3 tBu); 2.04 (s, 3H, CH_3 D-ring); 3.04 (dd, 1H, $J_{4,3a,\,cis}$=3.2Hz,

^{2}J=16.7Hz, H4); 3.37 (dd, 1H, $J_{4,3a,\ trans}$=9.3Hz, ^{2}J=16.7Hz, H4); 3.94 (m, 1H, H3a); 5.90 (d, 1H, J=7.9Hz, H8b); 6.17 (m, 1H, H2'); 6.50 (bs, 1H, NH); 6.96 (m, 1H, H3'); 7.15 (d, 1H, J=8.3Hz, H5); 7.35 (dd, 1H, J=8.3Hz, $^4J_{6,8}$=1.7Hz, H6); 7.47 (d, 1H, $^4J_{3a,6'}$=2.5Hz, H6'); 7.50 (d, 1H, ^{4}J= 1.7Hz, H8). ^{13}C NMR ($CDCl_3$): δ 11.3 (CH_3 D-ring); 28.9 (3 x CH_3 tBu); 37.3 (CH_2); 39.9 (CH3a); 81.1 (Cq tBu); 86.4 (CH8b); 101.3 (CH2'); 113.7 (Cq C-ring); 116.9; 121.6; 126.0; (3 x CH_{arom}); 136.3; 137.5; 138.6; 140.2 (3 x Cq_{arom}, Cq D-ring); 141.7 (CH3'); 151.7 (CH6'); 153.4 (C=O carbamate) 170.9; 171.8 (2 x C=O, lactones). IR (KBr): ν (cm^{-1}) 3345 (NH); 1777; 1750; 1712; 1681 (3 x C=O, C=C); 1180 (lactone). MS [CI *m/z*, rel. intensity (%)]: 413 ([M]$^+$, 12.5); 357 ([$C_{18}H_{15}O_7N$]$^+$, 88.8); 313 ([$C_{17}H_{15}O_5N$]$^+$, 9.3); 217 ([$C_{12}H_{11}O_3N$]$^+$, 21.4); 97 ([$C_5H_5O_2$]$^+$, 32.2); 57 ([C_4H_9]$^+$, 100). Anal. calcd. for $C_{22}H_{23}O_7N$: C, 63.92; H, 5.61; N, 3.39 found: C, 64.10; H, 5.73; N, 3.13.

(-)-9: mp: 226-228°C (decomp.). e.e. >99% (determined by HPLC). $[\alpha]^{22}_D$=-323.6° (c=0.1, CH_2Cl_2). ^{1}H NMR ($CDCl_3$): δ 1.53 (s, 9H, 3 x CH_3 tBu); 2.04 (s, 3H, CH_3 D-ring); 3.03 (dd, 1H, $J_{4,3a,\ cis}$=3.1Hz, ^{2}J=16.7Hz, H4); 3.35 (dd, 1H, $J_{4,3a,\ trans}$=9.2Hz, ^{2}J=16.7Hz, H4); 3.93 (m, 1H, H3a); 5.90 (d, 1H, J=7.8Hz, H8b); 6.16 (m, 1H, H2'); 6.48 (bs, 1H, NH); 6.96 (m, 1H, H3'); 7.13 (d, 1H, J=8.3Hz, H5); 7.30 (dd, 1H, J=8.3Hz, $^4J_{6,8}$=1.8Hz, H6); 7.46 (d, 1H, $^4J_{3a,6'}$=2.5Hz, H6'); 7.53 (d, 1H, ^{4}J= 1.8Hz, H8). ^{13}C NMR ($CDCl_3$): δ 11.4 (CH_3 D-ring); 29.0 (3 x CH_3 tBu); 37.5 (CH_2); 39.9 (CH3a); 81.3 (Cq tBu); 86.5 (CH8b); 101.3 (CH2'); 114.0 (Cq C-ring); 116.9; 121.7; 126.2; (3 x CH_{arom}); 136.6; 137.7; 138.6; 140.3 (3 x Cq_{arom}, Cq D-ring); 141.7 (CH3'); 151.6 (CH6'); 153.4 (C=O carbamate) 170.9; 171.9 (2 x C=O, lactones). IR (KBr): ν (cm^{-1}) 3315 (NH); 1780; 1749; 1720; 1688 (3 x C=O, C=C); 1180 (lactone). MS [CI *m/z*, rel. intensity (%)]: 413 ([M]$^+$, 15.8); 357 ([$C_{18}H_{15}O_7N$]$^+$, 44.7); 313 ([$C_{17}H_{15}O_5N$]$^+$, 25.0); 217 ([$C_{12}H_{11}O_3N$]$^+$, 73.4); 97 ([$C_5H_5O_2$]$^+$, 68.2); 57 ([C_4H_9]$^+$, 100). Anal. calcd. for $C_{22}H_{23}O_7N$: C, 63.92; H, 5.61; N, 3.39 found: C, 64.06; H, 5.70; N, 3.21.

***tert*-Butyl-*N*-[3-((*E*)-1-{[4'-methyl-5'-oxo-2',5'-dihydro-2'(*S*)-furanyl]oxy}methylidene)-2-oxo-3,3a(*S*),4,8b(*R*)-tetrahydro-2H-indeno[1,2-b]furan-7-yl]carbamate ((+)-8)** and its 2(R) diastereoisomer: ***tert*-Butyl-*N*-[3-((*E*)-1-{[4'-methyl-5'-oxo-2',5'-dihydro-2'(*R*)-furanyl]oxy}-methylidene)-2-oxo-3,3a(*S*),4,8b(*R*)-tetrahydro-2H-indeno[1,2-b]furan-7-yl]carbamate ((+)-9).** Boc-amino-GR 24 diastereoisomers **(+)-8** and **(+)-9** were prepared in the same way as described for their enantiomers **(-)-8** and **(-)-9**, starting from carbamate **(+)-6** (770 mg, 2.7 mmol). Yield **(+)-8**: 43%; **(+)-9**: 38%. Analytical samples were obtained by recrystallisation from 2-methyl-2-butanol.

(+)-8: mp: 116-118°C. e.e. >99% (determined by HPLC). $[\alpha]^{22}_D$=+338.7° (c=0.1, CH_2Cl_2). Anal. calcd. for $C_{22}H_{23}O_7N$: C, 63.92; H, 5.61; N, 3.39 found: C, 64.10; H, 5.73; N, 3.13. All other analytical data were the same as reported for **(-)-8**.

(+)-9: mp: 228-230°C (decomp.). e.e. >99% (determined by HPLC). $[\alpha]^{22}_D$=+331.5° (c=0.1, CH_2Cl_2). Anal. calcd. for $C_{22}H_{23}O_7N$: C, 63.92; H, 5.61; N, 3.39 found: C, 63.63; H, 5.62; N, 3.44. All other analytical data were the same as reported for **(-)-9**.

***tert*-Butyl-*N*-(2-{[3-((*E*)-1-{[4'-methyl-5'-oxo-2',5'-dihydro-2'*(SR)*-furanyl]oxy}methylidene)-2-oxo-3,3a*(RS)*,4,8b*(SR)*-tetrahydro-2H-indeno[1,2-b]furan-7-yl]amino}-2-oxoethyl)carbamate (rac. 10).** Protected Boc-amino-GR 24 **rac. 9** (150 mg, 0.36 mmol was dissolved in a mixture of trifluoro acetic acid (1mL) and dichloromethane (10 mL) and stirred at room temperature for 3 h. When TLC analysis indicated complete deprotection, solvents were evaporated *in vacuo*. The residue was suspended in dichloromethane (5 mL) and treated with triethylamine (300 µL). After washing with satd. NaCl (1x) and drying ($MgSO_4$), volatiles were evaporated and the free amine was dissolved in THF (10 mL). Then 1.05 equiv. of 2,3,4,5,6-pentafluorophenyl 2-[(*tert*-butoxycarbonyl)amino]acetate (BocGlyOPfP, 130 mg, 0.38 mmol) and 1.05 equiv. of hydroxy benzotriazole (HOBT, 52 mg, 0.38 mmol) were added and the mixture was stirred at room temperature for 3 h. THF was removed *in vacuo* and the residue was purified over silicagel (ethyl acetate/hexane 3/1) to give **rac. 10** as a white solid (161 mg, 95%) that was recrystallized from dichloromethane/ethyl acetate. mp: 206-208°C. ^{1}H NMR ($CDCl_3$): δ 1.47 (s, 9H, 3 x CH_3 tBu); 2.05 (s, 3H, CH_3 D-ring); 3.04 (dd, 1H, $J_{4,3a,\ cis}$=2.6Hz, ^{2}J=16.8Hz, H4); 3.36 (dd, 1H, $J_{4,3a,\ trans}$=9.3Hz, ^{2}J=16.8Hz, H4); 3.93 (m, 2H, H3a + CH_2 gly); 5.29 (bs, 1H, NH); 5.90 (d, 1H, J=7.9Hz, H8b); 6.18 (m, 1H, H2'); 6.97 (m, 1H, H3'); 7.17 (d, 1H, J=8.2Hz, H5); 7.49 (d, 1H, $^4J_{3a,6'}$=2.4Hz, H6'); 7.52 (dd, 1H, J=8.3Hz, $^4J_{6,8}$=1.5Hz, H6); 7.62 (d, 1H, ^{4}J= 1.5Hz, H8); 8.43 (bs, 1H, NH). ^{13}C NMR ($CDCl_3$): δ 11.4 (CH_3 D-ring); 29.0 (3 x CH_3 tBu); 37.6 ($CH_2$4); 39.9 (CH3a); 46.6 (CH_2 gly); 81.3 (Cq tBu); 86.5 (CH8b); 101.4 (CH2'); 113.9 (Cq C-ring); 118.4; 123.1; 126.3; (3 x CH_{arom}); 136.6; 137.7; 139.2; 140.2 (3 x Cq_{arom}, Cq D-ring); 141.7 (CH3'); 151.6 (CH6'); 153.4 (C=O carbamate) 168.6 (C=O gly); 170.9; 171.9 (2 x C=O, lactones). IR (KBr): ν (cm^{-1}) 3346; 3282 (NH); 1788; 1742; 1721; 1682 (4 x C=O, C=C); 1183 (lactone). MS [FAB *m/z*, rel. intensity (%)]: 493 ([M + Na]$^+$, 54.3); 470 ([M]$^+$, 12.4); 371 ([$C_{19}H_{19}O_6N_2$]$^+$, 59.3); 97 ([$C_5H_5O_2$]$^+$, 100). Anal. calcd. for $C_{24}H_{26}O_8N_2$: C, 61.27; H, 5.57; N, 5.95 found: C, 61.06; H, 5.43; N, 5.93.

***tert*-Butyl-*N*-(2-{[3-((*E*)-1-{[4'-methyl-5'-oxo-2',5'-dihydro-2'*(S)*-furanyl]oxy}methylidene)-2-oxo-3,3a*(R)*,4,8b*(S)*-tetrahydro-2H-indeno[1,2-b]furan-7-yl]amino}-2-oxoethyl)carbamate ((-)-10).** Compound **(-)-10** was prepared in the same way as described for **rac. 10** starting from carbamate **(-)-9** (200 mg, 0.48 mmol). Yield: 97%. Recrystallisation from dichloromethane/ethyl acetate gave a white fluffy solid. mp: 189-190°C. $[\alpha]^{22}_D$=-298.9° (c=0.1, CH_2Cl_2). Anal. calcd. for $C_{24}H_{26}O_8N_2$: C, 61.27; H, 5.57; N, 5.95 found: C, 61.03; H, 5.55; N, 5.92. All other analytical data were the same as reported for its racemate **rac. 10.**

4-[({[2-{[3-((*E*)-1-{[4'-Methyl-5'-oxo-2',5'-dihydro-2'*(S)*-furanyl]oxy}methylidene)-2-oxo-3,3a*(R)*,4,8b*(S)*-tetrahydro-2H-indeno[1,2-b]furan-7-yl]amino}-2-oxoethyl]amino}carbothioyl)amino]-2-[6-(dimethylamino)-3-(1,1-dimethylammonio)-3H-9-xanthenyl]benzoate((-)-11). The amine protecting group was removed from carbamate **(-)-10** (16 mg, 0.034 mmol) by stirring for 1 hour at room temperature in a mixture of dichloromethane (2 mL) and trifluoro acetic acid (0.25 mL). Subsequently the mixture was concentrated under reduced pressure and the

residue was dissolved in dichloromethane (0.5 mL) and triethylamine (100 µL) was added to liberate the free primary amine. Tetramethyl rhodamine isothiocyanate (15 mg, 0.034 mmol) was dissolved in DMF (2 mL) and added to the solution of deprotected **(-)-10.** The desired thiourea linkage was formed instantly, as indicated by TLC analysis. After stirring for 1 hour at room temperature, solvents were evaporated *in vacuo*, and the residue was purified by flash chromatography over silicagel (10→50% methanol in dichloromethane) yielding a bright pink solid (14 mg, 53%). mp: >300°C. $[\alpha]^{22}_D$=-270.6° (c=0.03, H_2O). ^{1}H NMR (DMSO): δ 1.92 (s, 3H, CH_3 D-ring); 2.88 (m 1H, H4); 2.94 (s, 12H 4 x CH_3 TRITC); 3.30 (m 1H, H4); 3.94 (m, 1H, H3a); 4.30 (bs, 2H, CH_2 gly); 5.97 (d, 1H, J=7.9Hz, H8b); 6.49-6.58 (m, 6H, 2 x NH thiourea + 4 x H_{arom} TRITC); 6.69 (m, 1H, H2'); 6.82 (d, 1H, J=8.3Hz, H_{arom} TRITC); 7.14 (d, 1H, J=8.3Hz, H_{arom} TRITC); 7.22 (d, 1H, J=8.3Hz, H5); 7.40 (m, 1H, H3'); 7.46 (d, 1H, J=8.3Hz, H6); 7.70 (d, 1H, $^4J_{3a,6'}$=2.4Hz, H6'); 7.75 (bs, 1H, H8); 7.80 (d, 2H, J=7.9Hz, H_{arom} TRITC); 7.89 (d, 1H, 8.7Hz, H_{arom} TRITC); 10.20 (s, 1H, NH arom). ^{13}C NMR (DMSO): δ 10.2 (CH_3 D-ring); 36.3 (CH_2 4); 39.8 (4 x CH_3 TRITC) 38.6 (CH3a); 47.4 (CH_2 gly); 85.2 (CH8b); 101.3 (CH2'); 106.3 (2 x Cq TRITC); 111.7 (Cq C-ring); 97.9; 108.3; 113.8; 116.2; 121.1; 125.5; 120.6; 121.0; 124.9; 125.5; 127.4; 128.3 (12 x CH_{arom}); 133.7 (Cq D-ring); 137.3; 138.0; 139.7 (3 x Cq_{arom} GR24); 143.2 (CH3'); 151.8; 152.1 (6 x Cq TRITC); 152.8 (CH6'); 153.8 (Cq-COO^-); 167.9; 168.4; 170.6; 170. (4 x C=O); 180.1 (C=S). IR (KBr): ν (cm^{-1}) 3422 (COOH); 3299 (NH); 2989 CH_{arom}); 1783; 1749; 1680; 1648 (5 x C=O, C=C); 1536 (amide II). MS [FAB *m/z*, rel. intensity (%)]: 836 ($[M + Na]^+$, 7.3); 814 ($[M + H]^+$, 13.7); 414 ($[C_{20}H_{18}O_6N_2S]^+$, 100).

9H-9-Fluorenylmethyl-*N*-[2-{[3-((*E*)-1-{[4'-Methyl-5'-oxo-2',5'-dihydro-2'(*SR*)-furanyl]oxy}-methylidene)-2-oxo-3,3a(*RS*),4,8b(*SR*)-tetrahydro-2H-indeno[1,2-b]furan-7-yl]amino}-1-({[(acetylamino)methyl]sulfanyl}methyl)-2-oxoethyl]carbamate (rac. 12). Racemic, slow moving Boc-amino-GR 24 diastereoisomer **rac. 9** (829 mg, 2.0 mmol) was deprotected by stirring for 1 hour at room temperature in a mixture of dichloromethane (9mL) and trifluoro acetic acid (1 mL). Then solvents were evaporated under reduced pressure. The residue was dissolved in dichloromethane (10 mL) and triethylamine (1 mL) was added to liberate the free amine. The mixture was washed with brine (1 x), dried ($MgSO_4$) and concentrated. After dissolving the residue in THF (10 mL), 1.1 equiv. of the Fmoc- and Acm-protected pentafluorophenyl ester of cysteine (FmocCys(Acm)OPfP, 1.28 g, 2.2 mmol) and 1.1 equiv. of hydroxy benzotriazole (HOBT, 300 mg, 2.2 mmol) dissolved in THF (3 mL) were added. The mixture was stirred at room temperature for 3 h. THF was removed *in vacuo* and the residue was dissolved in ethyl acetate (20 mL), washed with brine (1 x), dried ($MgSO_4$) and concentrated *in vacuo*. Silicagel chromatography (dichloromethane/methanol 15/1) gave **rac. 12** as a white solid (1.39 g, 98%). An analytically pure sample was obtained by recrystallisation from ethyl acetate/diisopropyl ether. mp: 204-206°C. ^{1}H NMR (DMSO): δ 1.86 (s, 3H, CH_3 Acm); 1.92 (s, 3H, CH_3 D-ring); 2.77-2.99 (m, 3H, CH_2 cys + H4); 3.30 (m 1H, H4); 3.94 (m, 1H, H3a); 4.21-4.40 (m, 8H, CH cys + CH_2 cys + CH Fmoc + CH_2 Fmoc + CH_2 Acm); 5.98 (d, 1H, J=7.9Hz, H8b); 6.70

(m, 1H, H2'); 7.24 (d, 1H, J=8.3Hz, H5); 7.30-7.90 (m, 10H, NH cys + H3' + 8H_{arom} Fmoc); 7.70 (d, 1H, $^4J_{3a,6'}$=2.3Hz, H6'); 7.74 (d, 1H, J=8.3Hz, H6); 7.87 (bs, 1H, H8); 8.58 (t, 1H, J=6.2Hz, NH Acm); 10.10 (s, 1H, NH arom). ^{13}C NMR (DMSO): δ 10.2 (CH_3 D-ring); 22.6 (CH_3 Acm); 32.3 (CH_2 cys); 36.3 (CH_2 4); 38.6 (CH3a); 40.2 (CH_2 Acm); 46.6 (CH Fmoc); 65.8 (CH_2 Fmoc); 85.2 (CH8b); 101.3 (CH2'); 111.7 (Cq C-ring); 116.4; 121.1; 125.5; (3 x CH_{arom} GR24); 120.1; 125.3; 127.1; 127.6 (8 x CH_{arom} Fmoc); 133.7 (Cq D-ring); 137.4; 138.0; 139.7 (3 x Cq_{arom} GR24); 140.7; 143.8 (4 x Cq Fmoc); 143.2 (CH3'); 152.8 (CH6'); 156.0 (C=O Fmoc); 169.4; 169.8; 170.7; 170.8 (4 x C=O). IR (KBr): ν (cm^{-1}) 3327 (NH); 3066 CH_{arom}); 2942 (CH_{alif}); 1795; 1770; 1736; 1709; 1695; 1679 (5 x C=O, C=C); 1535 (amide II). MS [FAB *m/z*, rel. intensity (%)]: 732 ([M + Na]$^+$, 28.2); 710 ([M + H]$^+$, 15.3). Anal. calcd. for $C_{38}H_{35}O_9N_3S$: C, 64.30; H, 4.97; N, 5.92; S, 4.52 found: C, 63.83; H, 4.94; N, 5.70; S, 4.26.

***N*-1-(2-{[3-((*E*)-1-{[4'-Methyl-5'-oxo-2',5'-dihydro-2'*(SR)*-furanyl]oxy}methylidene)-2-oxo-3,3a *(RS)*,4,8b*(SR)*-tetrahydro-2H-indeno[1,2-b]furan-7-yl]amino}-2-oxoethyl)-4-azido-2-hydroxy-benzamide (rac. 16).** Carbamate **rac. 10** (39 mg, 0.083 mmol) was dissolved in a mixture of dichloromethane (2 mL) and trifluoro acetic acid (0.25 mL) and stirred at room temperature for 2 hours. When TLC analysis indicated complete removal of the Boc protective group, solvents were evaporated *in vacuo*. The residue was dissolved in THF (2 mL) and triethylamine (100 µL) was added, which was immediately followed by the addition of 1.1 equiv. of azido salicylic acid succinimidyl ester **14** (25 mg, 0.090 mmol), dissolved in THF (0.5 mL). The reaction takes place instantaneously, as was indicated by TLC analysis, and a white solid appeared in the solution. According to TLC analysis, this solid was excess ester **14**, which was removed by filtration. THF was evaporated *in vacuo*. The residue was dissolved in ethyl acetate, washed with aqueous $NaHCO_3$ (5%) to remove N-hydroxy succinimide, dried ($MgSO_4$) and concentrated. Upon concentration **rac. 16** (44 mg, 99%) was obtained as a white solid, which was recrystallized from ethyl acetate/hexane. mp: 200°C. ^{1}H NMR (DMSO): δ 1.92 (s, 3H, CH_3 D-ring); 2.87 (dd, 1H, $J_{4,3a,\ cis}$=2.5Hz, ^{2}J=16.7Hz, H4); 3.30 (m 2H, H4 + OH); 3.94 (m, 1H, H3a); 4.11 (m, 2H, CH_2 gly); 5.97 (d, 1H, J=7.9Hz, H8b); 6.63 (d, 1H, ^{4}J=2.1Hz, -CN_3C<u>H</u>COH-); 6.70 (m, 1H, H2'); 6.70 (dd, 1H, J=8.6Hz, ^{4}J=2.1Hz, -CN_3C<u>H</u>CH-); 6.97 (m, 1H, H3'); 7.23 (d, 1H, J=8.3Hz, H5); 7.49 (d, 1H, J=8.3Hz, H6); 7.70 (d, 1H, $^4J_{3a,6'}$=2.3Hz, H6'); 7.79 (bs, 1H, H8); 7.95 (d, 1H, J=8.6Hz, -CN_3CHC<u>H</u>-); 9.41 (bs, 1H, NH gly); 10.18 (S, 1H, NH arom). ^{13}C NMR (DMSO): δ 10.2 (CH_3 D-ring); 36.4 (CH_2 4); 38.5 (CH3a); 43.0 (CH_2 gly); 85.2 (CH8b); 101.3 (CH2'); 111.8 (Cq C-ring); 112.7 (Cq_{arom}); 107.0; 110.0; 116.3; 121.0; 125.5; 130.3 (6 x CH_{arom}); 133.7 (Cq D-ring); 137.3; 138.1; 139.7 (3 x Cq_{arom} GR24); 143.2 (CH3'); 144.5 (Cq_{arom}-N_3); 152.8 (CH6'); 160.7 (Cq_{arom}-OH); 167.3; 167.9; 170.6; 170.8 (4 x C=O). IR (KBr): ν (cm^{-1}) 3575 (OH); 3315 (NH); 1780; 1742; 1728; 1700; 1689 (4 x C=O, C=C);. MS [FAB *m/z*, rel. intensity (%)]: 554 ([M + Na]$^+$, 24.6); 532 ([M + H]$^+$, 32.3); 97 ([$C_5H_5O_2$]$^+$, 51.1). Anal. calcd. for $C_{26}H_{21}O_8N_5$: C, 58.76; H, 3.98; N, 13.18 found: C, 57.99; H, 3.99; N, 12.94.

X-ray crystallography. Crystals of **(+)-9**, suitable for X-ray diffraction studies, were obtained by slow evaporation from 2-methyl-2-butanol. A single crystal was mounted in air on a glass fibre. Intensity data were collected at room temperature. An Enraf-Nonius CAD4 single crystal diffractometer was used, CuK_α radiation, θ-2θ scan mode. Unit cell dimensions were determined from the angular setting of 25 reflections. Intensity data were corrected for Lorentz and polarization effects. Semi-empirical absorption correction (ψ-scans) was applied.[28] The structure was solved by the program CRUNCH[29] and was refined using standard methods (refinement against F^2 of all reflections with SHELXL-97[30]) with anisotropic parameters for the non-hydrogen atoms. All hydrogens were initially placed at calculated positions and were freely refined subsequently. Relevant numerical data are collected in table 3.

Biological studies

Plant material. Seeds of *Striga hermonthica* (Del.) Benth. were collected from Sorghum *(Sorghum bicolor* (L.) Moench) on Gezira Research Station, Sudan in 1994. *Orobanche crenata* Forsk. seeds were harvested from faba bean *(Vicia faba* L.) in Beheira, Egypt in 1993. The seeds were stored in glass vials in the dark at room temperature until use in germination tests.

Preparation of the test solutions. A compound to be tested was weighed out very accurately to the amount of 1.0 mg. GR 24 was dissolved in 1 mL acetone p.a. and diluted with demineralized water to 5 mL. **(-)-11** was dissolved in a mixture of 1 mL acetone p.a. and 1 mL DMF p.a. and diluted with demineralized water to 5 mL. **Rac. 12** was dissolved in 2 mL DMF and diluted with demineralized water to 5 mL. These stock solutions were further diluted with demineralized water to obtain test solutions with concentrations ranging from 5×10^{-5} to 5×10^{-9} mol/L. All solutions were prepared just before use.

Bioassays. For surface sterilization all seeds were exposed for 5 minutes to a 50% (v/v) aqueous solution of commercial bleach (2% hypochlorite). Subsequently, the seeds were thoroughly rinsed with demineralized water and air-dried. For conditioning the seeds were spread on glass fiber filter paper disks (8 mm diameter, approximately 60-100 seeds per disk) in Petri dishes, wetted with demineralized water and stored in the dark at 20 °C for *Orobanche* seeds and at 30 °C for *Striga* seeds. Thereafter the conditioning water was removed and conditioned seeds were placed in new Petri dishes and exposed to test solution. After incubation for 24 h *(Striga)* and 7 days *(Orobanche)* in the dark at the indicated temperatures the percentages of germinated seeds were determined under a microscope. Seeds were considered to be germinated if the radicle protruded through the seed coat. Aqueous solutions containing 1% (v/v) and 0.1% (v/v) DMF respectively, as well as an aqueous solution containing 0.1% (v/v) DMF and 0.1% (v/v) acetone were used as negative controls. For full details of the bioassay, see Mangnus *et al.*[30]

Table 3 Crystal data and structure refinement for slow moving BocNH-GR 24 diastereoisomer **(+)-9**

empirical formula	$C_{22}H_{23}NO_7$	index ranges	$0 \leq h \leq 11$, $0 \leq k \leq 15$, $0 \leq l \leq 21$
formula weight	413.41	reflections collected/unique	2282/2282
crystal colour	transparent colourless	reflections observed	1967 ($[I_0>2\sigma(I_0)]$)
crystal shape	regular rod	absorption correction	semi-empirical from ψ-scans
crystal size	0.42 x 0.12 x 0.11 mm	range of relat. transm. factors	1.055 and 0.960
temperature	293(2) K	refinement method	full-matrix least squares on F^2
radiation/wavelength	CuK_α (graphite monochrom.)/1.54184 Å	computing	SHELXL-97[31]
crystal system/space group	orthorhombic/$P2_12_12_1$	data/restraints/parameters	2282/0/364
unit cell dimensions (25 reflections $22.177<\theta<45.115$)	a = 9.3201(4) Å b = 12.5782(4) Å c = 17.9679(4) Å	goodness-of-fit on F^2	1.048
volume	2106.38(11) $Å^3$	SHELXL-97 weight parameters	0.077600 and 0.140500
Z/ calculated density	4/1.304 mg/m^3	final R indices [$I>2\sigma(I)$]	R_1 = 0.0398, wR_2 = 0.1093
absorption coefficient	0.815 mm^{-1}	final R indices (all data)	R_1 = 0.0485, wR_2 = 0.1169
diffractometer/scan	Enraf-Nonius CAD4/θ-2θ	extinction coefficient	0.0023(4)
F(000)	872	largest diff. peak and hole	0.192 and -0.178e. $Å^3$
θ range for data collection	4.29 to 69.94 °		

Protein extraction. Striga hermonthica seeds (2.0 g, dry weight) were conditioned for two weeks at 30 °C, in a closed 50 mL flask, containing 10 mL of sterilized, demineralized water. The conditioning water was removed by filtration and the seeds were air dried for 30 minutes. Subsequently, the conditioned seeds were frozen with liquid nitrogen and ground as finely as possible using a mortar and pestle. 250 mg of ground material was extracted with 1 mL lysis buffer (50 mM HEPES, pH 7.2; 140 mM NaCl; 10 mM EDTA, pH 8.0; 0.1%

deoxycholate; 1 mM PMSF; 0.1 mg/mL soybean trypsin inhibitor; 0.1% Triton and 0.1% Saponin).[32] The crude extract was centrifuged at 4 °C for 10 min. at 13000 rpm. The pellet was discarded and the supernatant was again centrifuged at 4 °C for 10 min. at 13000 rpm. In this manner a clear extract of *S. hermonthica* seeds was obtained. The protein concentration was determined by the Bradford dye binding procedure[33]; the extract contained approximately 0.5 mg protein per mL. For use in the FCS set up, the extract was diluted 5 times. [Instead of Triton and Saponin, also lysis buffers containing other surfactant combinations were used to extract *Striga* seeds *viz.* 0.1% Tween & 0.1% Triton, 0.1% Tween & 0.1% Digitonin and 0.1% Tween & 0.1% Saponin. Only the protein extract prepared with the lysis buffer containing Triton and Saponin as the surfactants, exhibited interaction with tetramethyl rhodamine labelled GR 24.]

Fluorescence microscopy. Fluorescence microscopy was performed with the system described by Gadella *et al.*[34] based on a Leica DMR microscope (Leitz, Wetzlar, Germany) with Leitz fluotar 10¥ NA 0.3 air or fluotar 40¥ NA 0.5-1.0 oil immersion objectives. Images were captured by a Quantix CCD-camera (Photometrics, Tucson, AZ, USA) interfaced through a PCI-card to an Apple Macintosh PowerPC 8500/180 computer and controlled by IPLab 3.1 software (Signal Analytics, Vienna, VA, USA). Fluorescence of tetramethyl rhodamine was acquired by excitation with a 100 W USH-102D mercury lamp (Fairlight, Rotterdam, The Netherlands) and an Omega 546DF10 nm bandpass filter. The emission was separated by an Omega 555 DRLP dichroic mirror and passed through a 580DF30 bandpass filter.

Fluorescence Correlation Spectroscopy. FCS measurements were performed with a Zeiss-EVOTEC ConfoCor inverted confocal microscope. An air cooled helium-neon laser supplied the excitation wavelength (543 nm), which was reflected by a Zeiss dichroic filter. The excitation light was focused by a water immersible objective (C-Apochromat 40 x, 1.2NA) into a 96 well microtiter plate with borosilicate bottom, containing the sample under investigation. The laser power was set with neutral density filters to 16 μW. The fluorescent light was collected by the same objective, passed through the dichroic mirror and a 560-620 nm bandpass filter (Omega). The light passed through a motor-controlled pinhole (40 μm diameter) in the image plane and was finally detected by an avalanche photodiode in single photon counting mode, which signal was processed by a hardware correlator (ALV-5000). The sampling time of the correlator was between 200 ns and 3400 s using 288 (logarithmic spaced) time channels. The samples were measured over 30 s at 293 K.

The FCS experiments were analyzed with the FCS ACCESS software package developed by EVOTEC/Zeiss, Inc. (version 1.0.10), using the one- and the two component fit model. The one component fit model determines, among other parameters, the average number of fluorescent molecules in the detection volume, as well as the characteristic diffusion time. The two component fit model, uses the characteristic diffusion time determined for the free ligand as a fixed value, and calculates the percentages of free and bound ligand in a

ligand/protein sample, as well as the characteristic diffusion time of the ligand-protein complex. For analysis, the curves were fitted with a fixed value for the structural parameter, which represents the ratio of ω_2/ω_1 of the detection volume. This value was obtained by calibration with a tetramethyl rhodamine solution.

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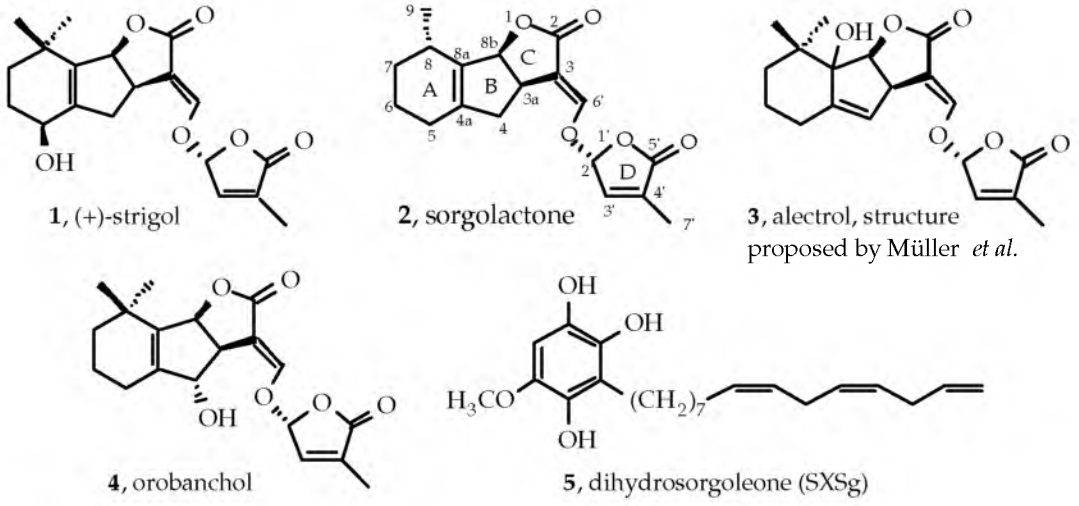
Summary

The root parasitic weeds *Striga* (witchweed) and *Orobanche* (broomrape) are serious pests in agriculture. *Orobanche* parasitizes dicotyledonous crops, such as faba bean, tomato and sunflower and predominantly occurs in the Mediterranean region. *Striga* mainly infects cereals such as sorghum, maize and millet in tropical and subtropical areas. The lives of millions of people in Africa, India and the Middle East are directly affected by the severe harvest reductions, due to heavy infestations of susceptible crops with these parasites. The lifecycle of the parasitic weeds is closely adapted to that of their host plants. The seeds of the parasites only germinate if they are exposed to specific chemicals (germination stimulants), which are present in the root exudate of a suitable host plant. The structures of several naturally occurring germination stimulants are depicted in figure 1.

Figure 1



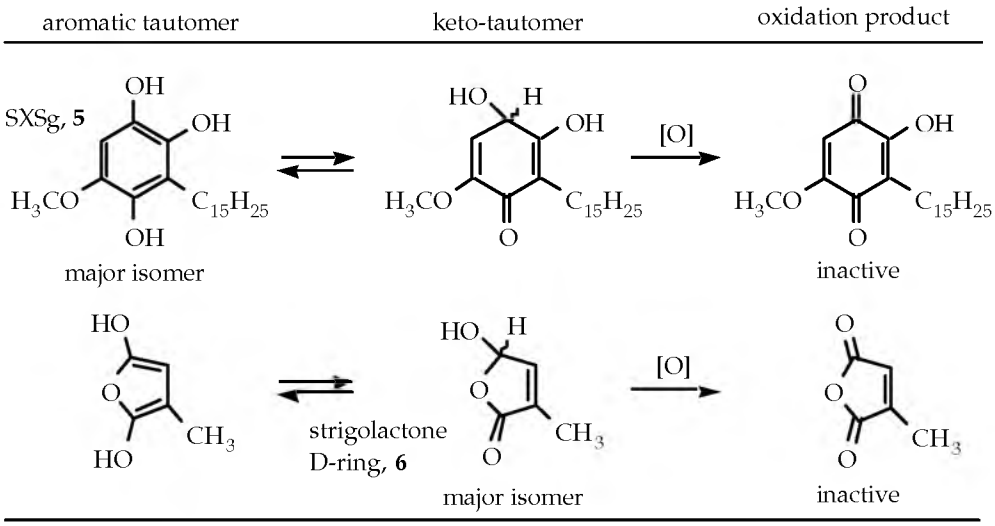
Strigol (**1**), sorgolactone (**2**), alectrol (**3**) and orobanchol (**4**) belong to the class of the strigolactones. The CD-part of strigolactones is essential for germination stimulatory activity. In addition, the importance of the stereochemistry of these molecules on their biological activity is well established. It is hypothesized that the induction of parasite seed germination by strigolactones proceeds via a receptor-mediated mechanism. In contrast, dihydrosorgoleone (**5**) is claimed to stimulate the germination of *Striga* seeds via a redox reaction. According to this hypothesis, the germination of parasitic weeds seeds by strigolactones is caused by oxidation of the D-ring, which supposedly has structural features similar to **5**.

A very specific method to control *Striga* and *Orobanche* infestations is the reduction of the amount of parasite seeds in the soil by suicidal germination, *i.e.* the application of a synthetic germination stimulant to the soil, before planting the desired crop. The *Striga* seeds will germinate, but, in the absence of a suitable host plant, the seedlings will die. In order to use this concept, a thorough understanding of the germination process is essential.

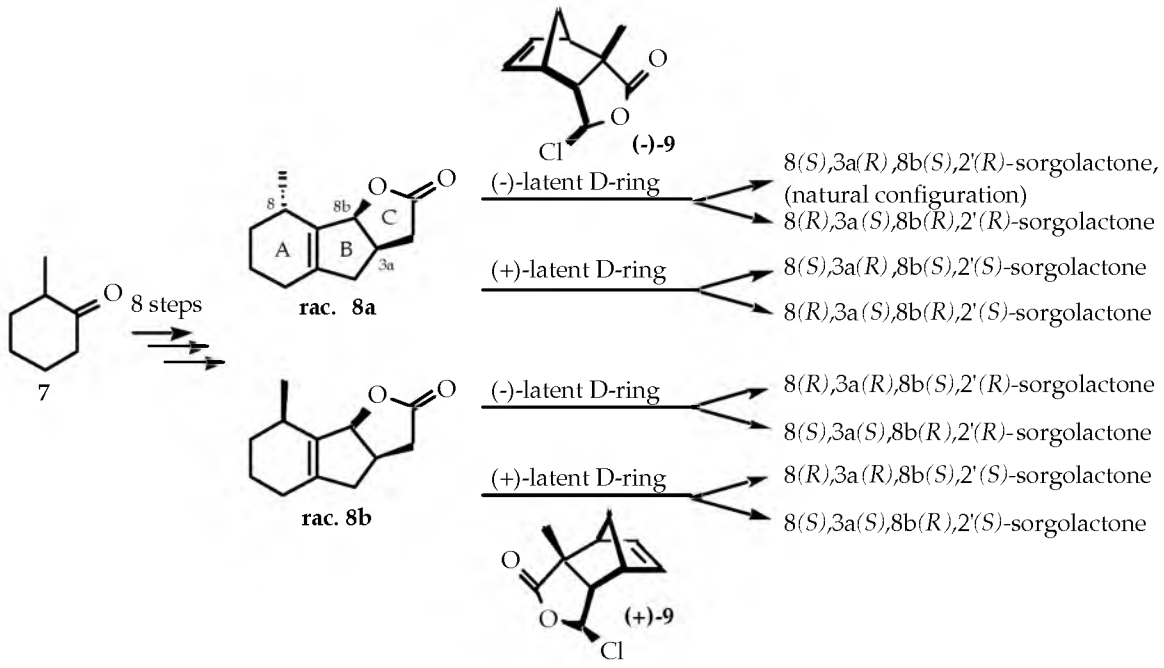
In **chapter 1** the background of the research is given. The problem of parasitic weed pests is described, as well as methods used to control these weeds. An overview of the synthetic achievements in this field of research is presented; special attention is given to the stereoselective synthesis of strigolactones and their analogues.

Chapter 2 deals with the discrepancies between the proposed redox mechanism for the induction of *Striga* seed germination, and the receptor mediated mechanism. It is shown that the mechanistic connection between SXSg (**5**) and the strigolactone D-ring, as was suggested by Boone and Lynn (Scheme1) is not justified. This study supports the hypothesis, that the germination of *Striga* and *Orobanche* seeds proceeds via a receptor-mediated mechanism.

Scheme 1. Comparison of the structural domains of SXSg and strigolactones that are responsible for germination inducing activity according to Boone and Lynn.



Scheme 2

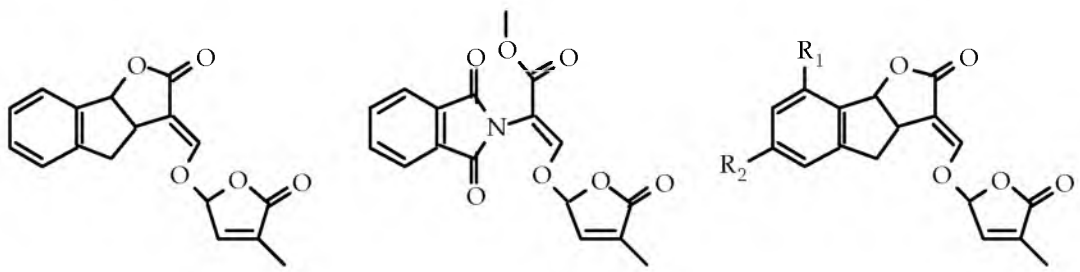


Chapter 3 is devoted to the synthesis and biological activity of the naturally occurring germination stimulant sorgolactone (**2**). This synthetic effort involved the preparation of all eight possible diastereoisomers of this germination stimulant as single stereoisomers. The strategy involved the stereoselective coupling of the enantiopure D-ring precursor **(-)-9** or **(+)-9**, with the diastereomerically pure racemic sorgolactone ABC-parts **rac. 8a** and **rac. 8b**, respectively (Scheme 2). In this manner, four diastereomeric mixtures of sorgolactone precursors were obtained, that could be separated over silica gel. Deprotection then furnished the eight single stereoisomers of sorgolactone. The proposed structure for the natural germination stimulant was confirmed by this total synthesis. Bioassays revealed that only those isomers possessing the same stereochemistry as natural sorgolactone at two adjacent chiral centers exhibit high biological activities.

In **Chapter 4**, dose-response curves of sorgolactone and the synthetic analogues GR24 (**10**) and Nijmegen-1 (**11**) are presented (Figure 2). It was shown that both synthetic analogues show appreciable activity at low concentrations towards a variety of parasite seeds, and have good promise in the application as suicidal germination agents for parasitic weed control.

Chapter 5 describes an expeditious synthesis of enantiopure strigolactone analogues. Separation of racemic germination stimulants by chiral HPLC over cellulose carbamate, allows the relatively fast collection of enough optically pure material for analysis and biological testing. All stereoisomers of 8-methyl GR 24 (**12**), which is the aromatic analogue of sorgolactone, and its regioisomer 6-methyl GR 24 (**13**) were synthesized in this manner (Figure 2). The presence of a methyl group on position 8 of GR 24 did not result in increased biological activity, whereas a 6-methyl substituent on GR 24 resulted in higher percentages of germinated *O. crenata* seeds, when compared with GR 24.

Figure 2



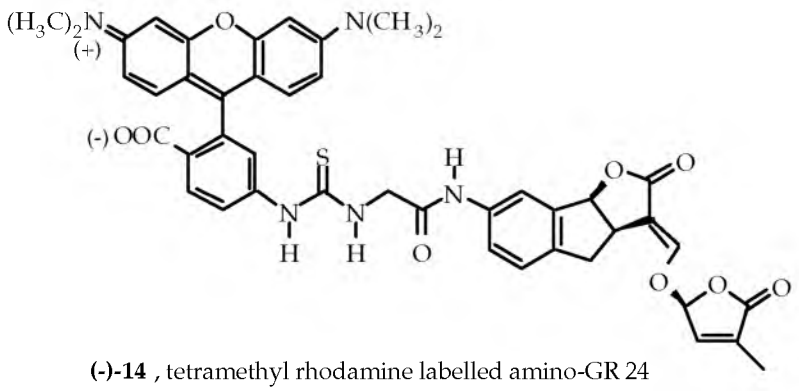
10, GR 24

11, Nijmegen-1

12, R_1=Me, R_2=H, 8-Me-GR 24
13, R_1=H, R_2=Me, 6-Me-GR 24

In **chapter 6,** the synthesis of all four diastereoisomers of 7-amino-GR 24 as single stereoisomers is described. The amino function was used for the attachment of various labels, that can be used to study the interaction between these strigolactone analogues and the hypothesized protein receptor. The biological activity of the labelled ligands was determined; they were inactive toward *O. crenata*, but did stimulate the germination of *S. hermonthica* seeds. Fluorescence Correlation Spectroscopy (FCS) experiments with tetramethyl rhodamine-labelled GR 24 (**(-)-14**) (Figure 3) at biologically active concentrations, and protein extracts of *S. hermonthica*, indicated that this technique is suitable for strigolactone receptor studies. Although much more experimentation is needed, the preliminary results seem to indicate that there is indeed a binding of the labelled stimulant with a protein.

Figure 3



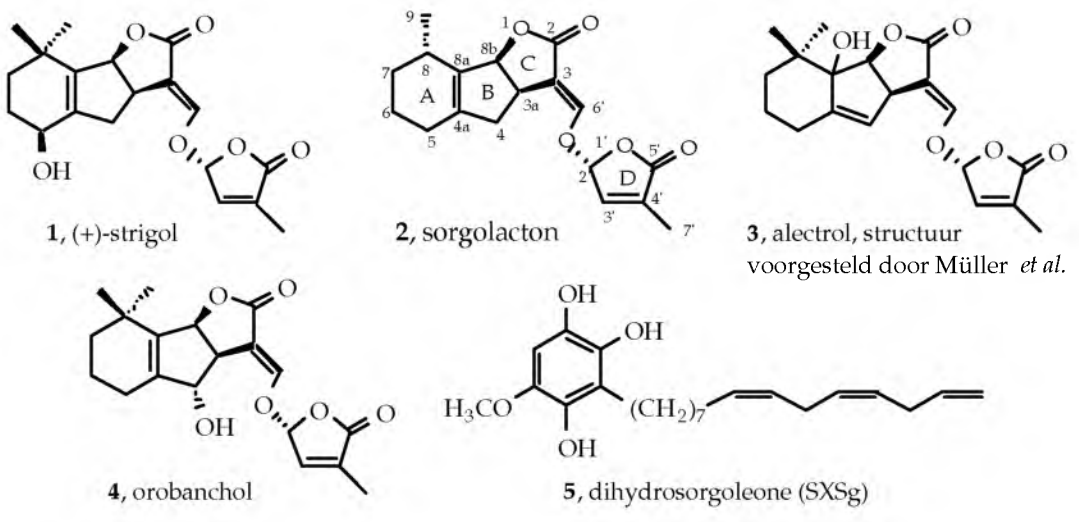
(-)-14 , tetramethyl rhodamine labelled amino-GR 24

Samenvatting

De parasitaire onkruiden *Striga* (heksenkruid) en *Orobanche* (bremraap) veroorzaken veel schade aan cultuurgewassen. *Orobanche* parasiteert voornamelijk tweezaadlobbige gewassen, zoals tuinbonen, tomaten en zonnebloemen en komt voornamelijk voor rondom de Middellandse Zee. *Striga* tast vooral granen aan, zoals sorghum, maïs en gierst in tropische en subtropische streken. Het leven van miljoenen mensen in Afrika, India en het Midden Oosten wordt direct beïnvloed door de ernstige oogstverliezen, die ontstaan ten gevolge van zware infecties van gevoelige gewassen met deze parasieten. De levenscyclus van de parasitaire onkruiden is geheel aangepast aan die van hun gastheer gewas. De zaden van de parasieten ontkiemen alleen na blootstelling aan een specifieke chemisch stof (kiemstimulant), die wordt uitgescheiden door de wortels van een gastheer plant. De structuren van enkele natuurlijk voorkomende kiemstimulanten zijn afgebeeld in figuur 1.

Strigol (**1**), sorgolacton (**2**), alectrol (**3**) en orobanchol (**4**) behoren tot de klasse van de strigolactonen. Het CD-gedeelte van strigolactonen is essentieel voor de kieming stimulerende activiteit van deze verbindingen. Bovendien is overtuigend aangetoond, dat de stereochemie van deze moleculen van grote invloed is op hun biologische activiteit.

Figuur 1



1, (+)-strigol

2, sorgolacton

3, alectrol, structuur voorgesteld door Müller *et al.*

4, orobanchol

5, dihydrosorgoleone (SXSg)

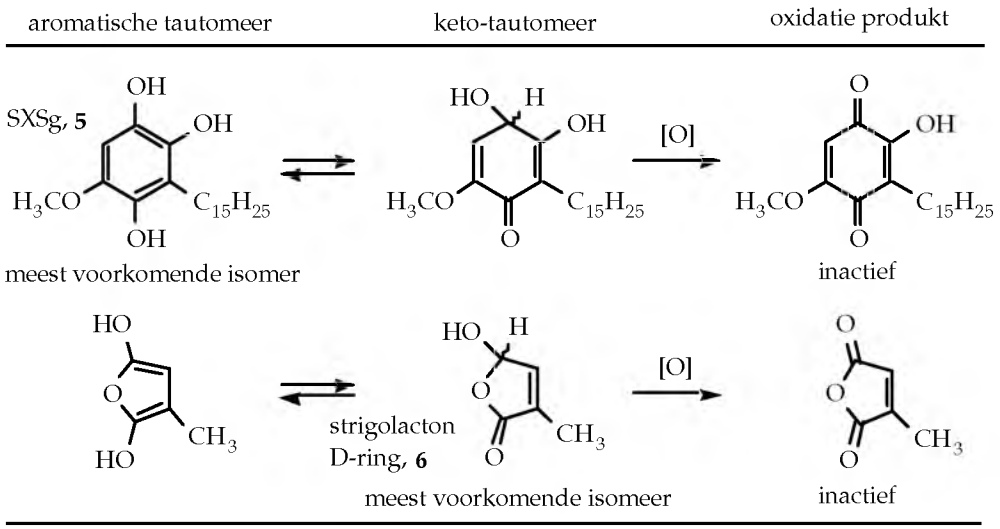
Het wordt verondersteld, dat de ontkieming van parasitaire onkruidzaden door strigolactonen via een receptor-gemedieerd mechanisme verloopt. In tegenstelling hiermee, wordt gesuggereerd dat de ontkieming van *Striga* zaden onder invloed van dihydrosorgoleone (**5**), verloopt via een redox reactie. Volgens deze hypothese, wordt de ontkieming van parasitaire onkruidzaden door strigolactonen veroorzaakt door de oxydatie van de D-ring, die structurele overeenkomsten zou vertonen met **5**.

Een heel specifieke methode om *Striga* en *Orobanche* plagen te bestrijden, is het terugbrengen van het aantal parasitaire onkruidzaden in de grond door middel van zelfmoord ontkieming. Dit houdt in de toepassing van een synthetische kiemstimulant in de landbouwgrond, voordat het gewenste gewas wordt geplant. De *Striga* zaden ontkiemen, maar kunnen niet overleven in de afwezigheid van een gastheer plant. Om dit concept te kunnen toepassen, is een diepgaand begrip van het ontkiemingsproces essentieel.

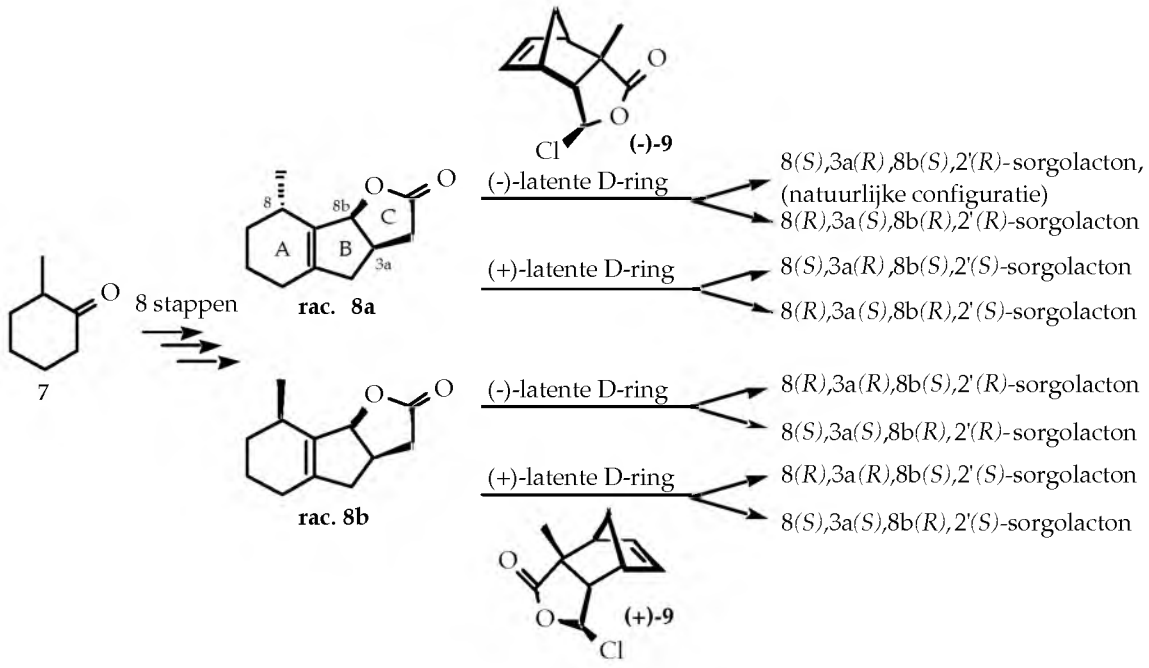
In **Hoofdstuk 1** wordt ingegaan op de achtergronden van het onderzoek. Het probleem van parasitaire onkruidplagen wordt beschreven, evenals methoden om deze onkruiden te bestrijden. Het bevat een overzicht van de synthetische prestaties in dit onderzoeksgebied, waarin speciale aandacht wordt besteed aan de stereoselectieve synthese van strigolactonen en hun analoga.

Hoofdstuk 2 gaat over de discrepanties tussen het voorgestelde redox mechanisme voor het begin van de ontkieming van *Striga* zaden, en het receptor-gemedieerde mechanisme. Het wordt aangetoond dat de mechanistische overeenkomst tussen SXSg (**5**) en de strigolacton D-ring, zoals gesuggereerd door Boone en Lynn (Schema 1) niet gerechtvaardigd is. Deze studie ondersteunt de hypothese dat de ontkieming van *Striga* en Orobanche zaden via een receptor-gemedieerd mechanisme verloopt.

Schema 1. Vergelijking van de structurele domeinen van SXSg en strigolactonen, welke volgens Boone en Lynn verantwoordelijk zijn voor de kieming stimulerende activiteit van de verbindingen.



Schema 2

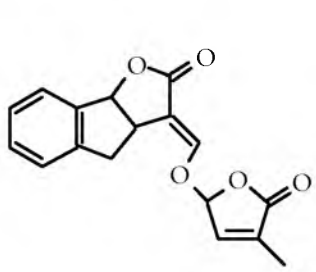


Hoofdstuk 3 is gewijd aan de synthese en biologische activiteit van de natuurlijk voorkomende kiemstimulant sorgolacton (**2**). De bereiding van alle acht mogelijke diastereoisomeren van deze kiemstimulant als afzonderlijke stereoisomeren is beschreven. De synthese strategie hield in: stereoselective koppeling van enantiomeer zuivere D-ring precursor **(-)-9** of **(+)-9**, met respectievelijk de diastereomeer zuivere racemische sorgolacton ABC-stukken **rac. 8a** en **rac. 8b** (Schema 2). Op deze manier werden vier mengsels van diastereomere sorgolacton precursors verkregen, die gescheiden konden worden op silicagel. Ontscherming gaf vervolgens de acht afzonderlijke stereoisomeren van sorgolacton. De structuur voorgesteld voor de natuurlijk voorkomende kiemstimulant kon worden bevestigd door deze totaalsynthese. Biologische testen lieten zien, dat alleen die isomeren met dezelfde stereochemie als natuurlijk sorgolacton op twee naburige chirale centra, hoge biologische activiteit vertoonden.

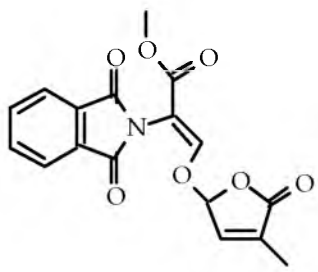
In **Hoofdstuk 4** worden dosis-respons krommes van sorgolacton en de synthetische analoga GR24 (**10**) en Nijmegen-1 (**11**) gepresenteerd (Figuur 2). Beide synthetische kiemstimulanten induceren in lage concentratie de ontkieming van verschillende soorten parasitaire onkruidzaden. Beide stoffen zouden toegepast kunnen worden als zelfmoordontkiemgsmiddel bij de bestrijding van parasitaire onkruiden.

Hoofdstuk 5 beschrijft een vlotte synthese van enantiomeer zuivere strigolacton analoga. Scheiding van racemische mengsels van kiemstimulanten met behulp van chirale HPLC over cellulose carbamate, leidt tot de relatief snelle verzameling van voldoende optisch zuiver materiaal voor analyse en biologische testen. Alle stereoisomeren van 8-methyl GR 24 (**12**), het aromatische analogon van sorgolacton, en zijn regioisomer 6-methyl GR 24 (**13**) zijn op deze manier gesynthetiseerd (Figuur 2).

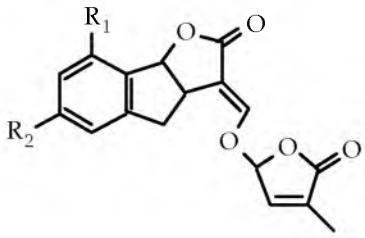
Figuur 2



10, GR 24



11, Nijmegen-1

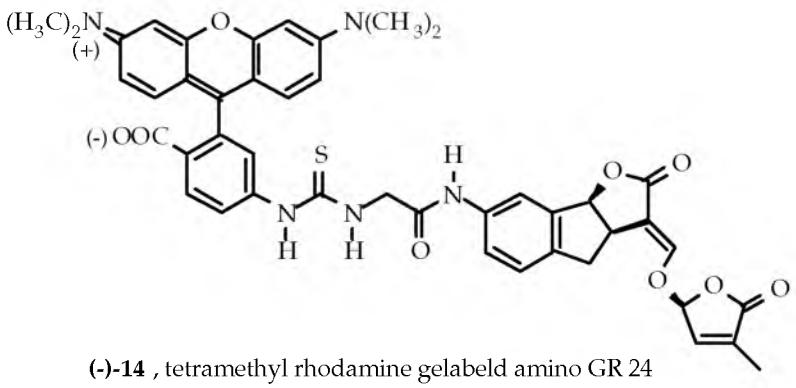


12, R_1=Me, R_2=H, 8-Me-GR 24
13, R_1=H, R_2=Me, 6-Me-GR 24

De aanwezigheid van een methyl groep op de 8-positie van GR 24 resulteerde niet in een toename van de biologische activiteit, terwijl een methyl substituent op positie C6 van GR 24 leidde tot hogere percentages ontkiemde *O. crenata* zaden, vergeleken met GR 24.

In **hoofdstuk 6** wordt de synthese van alle vier diastereoisomeren van 7-amino-GR 24 als afzonderlijke stereoisomeren beschreven. De amino functie is gebruikt om verschillende labels aan te koppelen, die gebruikt kunnen worden om de interactie tussen deze strigolacton analoga en hun voorgestelde eiwitreceptor te bestuderen. De biologische activiteit van de gelabelde ligenden is bepaald; de verbindingen waren inactief ten opzichte van *O. crenata*, maar ze stimuleerden wel de ontkieming van *S. hermonthica* zaden. Fluorescence Correlation Spectroscopy (FCS) experimenten met biologisch actieve concentraties van tetramethyl rhodamine gelabeld GR 24 (**(-)-14**) (Figuur 3), en eiwitextracten van *S. hermonthica*, lieten zien dat deze techniek geschikt is voor onderzoek naar de strigolacton receptor. Hoewel veel meer onderzoek noodzakelijk is, lijken de voorlopige resultaten erop te duiden, dat de gelabelde stimulant inderdaad een binding is aangegaan met een eiwit.

Figuur 3



(-)-14 , tetramethyl rhodamine gelabeld amino GR 24

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Y. Sugimoto, S.C.M. Wigchert, J.W.J.F. Thuring, B. Zwanenburg. Synthesis of All Eight Stereoisomers of the Germination Stimulant Sorgolactone. *J. Org. Chem.* **1998**, 64, 1259-1267.

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S.C.M. Wigchert and B. Zwanenburg. A Critical Account on the Inception of *Striga* Seed Germination. *J. Agric. Food Chem.* **1999**, *47*, 1320-1325.

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S.C.M. Wigchert and B. Zwanenburg. An Expeditious Preparation of All Enantiopure Diastereomers of Aromatic A-ring Analogues of Strigolactones, Germination Stimulants for Seeds of the Parasitic Weeds *Striga* and *Orobanche*. *J. Chem. Soc. Perkin Trans. 1* **1999**, *18*, 2617-2624.

Curriculum Vitae

Suzanne Wigchert werd geboren op 24 april 1971 te Velsen. Na het behalen van haar VWO diploma aan het Ichthus College te Driehuis in 1989, werd in september van dat jaar begonnen met de studie scheikunde aan de Universiteit Leiden. In juli 1990 werd het propaedeutisch examen behaald. Het studiejaar 1990-1991 studeerde zij tevens bio-farmaceutische wetenschappen aan het Leiden/Amsterdam Center for Drug Research. Het eerste semester van 1993 volgde zij colleges aan de Faculté des Sciences en de Faculté de Médecine et Pharmacie van de Université de Poitiers in Frankrijk, in het kader van het ERASMUS uitwisselingsprogramma. De studie scheikunde werd vervolgd in Leiden met een hoofdvakstage bij de werkgroep Synthese van Biopolymeren onder leiding van prof. dr. J.H. van Boom en een extra organisch chemische stage bij de afdeling Medicinal Chemistry III, van N.V. Organon in Oss, onder leiding van prof. dr. C.A.A. van Boeckel. Tijdens haar hoofdvakstage was zij studentlid van het bestuur van de vakgroep Organische Chemie. Op 28 april 1995 werd het doctoraal examen scheikunde behaald. Van mei 1995 tot mei 1999 was zij als assistent in opleiding verbonden aan de vakgroep Organische Chemie van de Katholieke Universiteit Nijmegen, die deel uitmaakt van de NSR-Onderzoeksschool. Onder leiding van prof. dr. B. Zwanenburg werd het in dit proefschrift beschreven onderzoek verricht. In deze periode was zij gedurende drie jaar bestuurslid van de AiO Organisatie van de faculteit Natuurwetenschappen (AON), en vertegenwoordigde zij de promovendi in de Faculteitsraad. In 1996/1997 was zij mede-organisator van een drie weken durende studiereis naar Zuid Afrika voor de staf en promovendi van de vakgroep Organische Chemie. Vanaf 1 juni 1999 is zij werkzaam als beleidsmedewerker bij het gebied Exacte Wetenschappen van de Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO) te Den Haag.