Bioorganic & Medicinal Chemistry Letters 22 (2012) 3743–3746

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Exploring the molecular mechanism of karrikins and strigolactones ☆

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ARTICLE INFO

Article history:
Received 16 February 2012
Revised 26 March 2012
Accepted 3 April 2012
Available online 10 April 2012

Keywords:
Karrikinolide
Strigolactones
Plant hormones
Smoke
Molecular mechanism

ABSTRACT

Karrikins and strigolactones are novel plant growth regulators that contain similar molecular features, but very little is known about how they elicit responses in plants. A tentative molecular mechanism has previously been proposed involving a Michael-type addition for both compounds. Through structure–activity studies with karrikins, we now propose an alternative mechanism for karrikin and strigolactone mode of action that involves hydrolysis of the butenolide ring.

Karrikinolide (KAR_1) **1** is a potent seed germination stimulant derived from burning plant material, and defines a family of related small molecules known as karrikins (Fig. 1).[1,2] These compounds are able to promote seed germination in numerous plant species native to both fire and non-fire prone regions around the world.[3] Recently, significant attention has been directed towards this intriguing class of small molecules due to their molecular similarity to the strigolactone group of germination and shoot-branching phytohormones (Fig. 1),[4,5] and the fact that both classes of compounds act through a common cell signalling pathway.[5,6] Although the molecular architecture of strigolactones is much more complex compared to the simpler planar karrikins, both compounds contain substituted methyl butenolide rings and it has been shown that this functionality is essential for strigolactone receptor recognition.[7,8] Both also contain enol ethers, yet despite these structural and functional similarities, very little is known about how these molecules interact with various biomolecules to elicit a response in plants.

A proposed mechanism for strigolactone activity has previously been reported based on the chemistry of the synthetic analogue GR24 **2** (Fig. 2a).[7,9] It was demonstrated that GR24 **2** undergoes a nucleophilic addition with thiophenol in a Michael fashion to release the D-ring and yield a covalent attachment of the nucleophile to the ABC portion of the molecule.[7] Furthermore, the saturation of the enol ether double bond renders the molecule inactive providing further evidence that a Michael type addition is required for bioactivity. Although various labelled derivatives have been prepared to explore this mechanism, no endogenous nucleophile or receptor molecule has been isolated to date.[10] Most recently, Zwanenburg et al. and Fukui et al. report that the enol ether moiety and ABC rings in strigolactones are not essential for activity, a conclusion based on active analogues lacking both these characteristics.[11,12] These analogues replace the enol ether and ABC rings with a leaving group suggesting that a general labile bond at C5 of the D-ring is sufficient to furnish activity. Furthermore, Zwanenburg et al. provide an alternative Michael acceptor mechanism

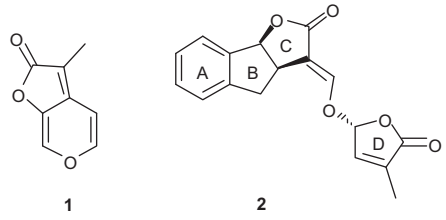

Figure 1. Chemical structures of karrikinolide (KAR_1) **1** and the synthetic strigolactone (GR24) **2**.

☆ The authors would like to thank the Australian Research Council (LP0882775, DP109671) for financial support, Lucy Commander for collecting *Solanum orbiculatum* seed and the Centre for Microscopy, Characterisation and Analysis at UWA.

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http://dx.doi.org/10.1016/j.bmcl.2012.04.016

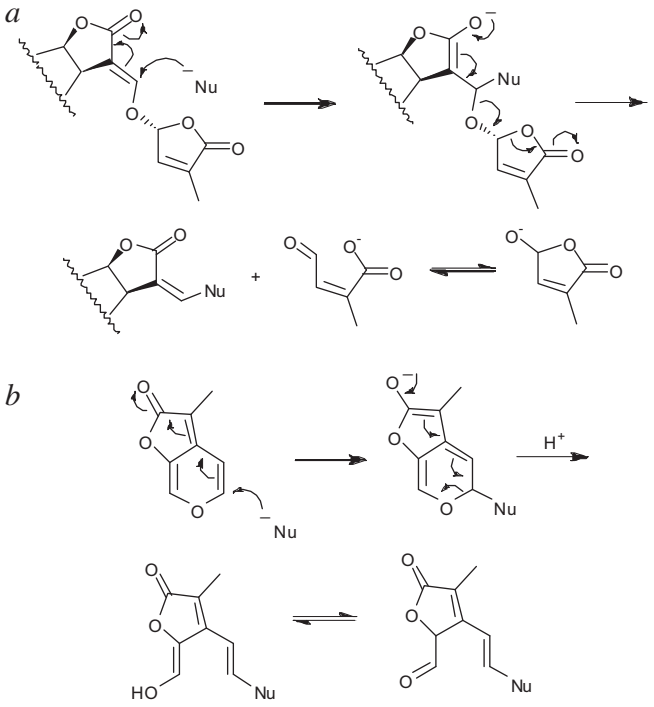

Figure 2. Previously proposed mechanisms for the Michael addition of a nucleophile to karrikins and strigolactones.

based solely on the D-ring.[12] In light of these results and given that the Michael acceptor mechanism has also been considered feasible for karrikins (Fig. 2b),[4,9] we investigated the ability of KAR_1 **1** to undergo nucleophilic addition, and explored the bioactivity of various saturated analogues.

To provide evidence for a nucleophilic addition mechanism *in vivo*, initially we sought to determine whether KAR_1 **1** is a suitable Michael acceptor. KAR_1 **1** contains two potential sites on the 4*H*-pyran ring (C5 and C7) that could undergo nucleophilic addition in a Michael type mechanism making outcome prediction difficult.[4,9] Our first approach followed the methods of Kupchan et al. who demonstrated the addition of cysteine to a number of alkyl substituted butenolides.[13] Although cysteine represents a biologically relevant nucleophile, no addition products were detected using this method. We then investigated whether the use of a stronger nucleophile, such as benzyl mercaptan, would result in an addition product. As expected, KAR_1 was consumed with the proposed enol trapped by treatment with acetic anhydride to furnish the acetate **3**. This confirmed the ability of KAR_1 **1** to undergo a Michael type addition exclusively at C5 (Fig. 3). To investigate whether this mechanism is required to stimulate germination, a number of reduced KAR_1 derivatives were prepared and their bioactivity evaluated.

The three primary targets were analogues containing various saturation patterns on the 4*H*-pyran ring (Fig. 4). All three

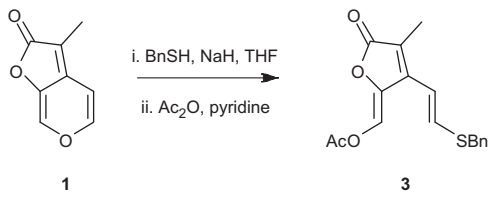

Figure 3. Nucleophilic addition to KAR_1 **1**.

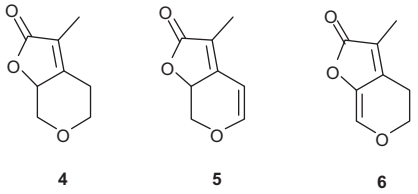

Figure 4. Chemical structures of saturated karrikin analogues.

analogues have recently been reported and were prepared according to published procedures, with the exception of the tetrahydropyran derivative **4**.[14] We found that catalytic hydrogenation of **1** resulted in clean conversion to **4** in good yield.

The biological evaluation of **5** as a germination stimulant has been reported,[15] but not in direct comparison with KAR_1 **1**. Therefore, the ability of **4**, **5**,[16] and **6**[17] to promote seed germination was evaluated using *Solanum orbiculatum* seeds that had low germination in water controls (<10%) and were stimulated to over 90% germination with **1**.[18] Our results show that in the range of 1–10 μM, both **5**[16] and **6**[17] were able to stimulate germination up to 100%, however, similar germination response was achieved with much lower concentrations of **1** (Fig. 5). In contrast, the fully saturated pyran analogue **4** only promoted germination at the highest concentration tested, and achieved only 60% germination. Hence, the removal of the conjugated enol ether functionality in KAR_1 still renders the molecule active albeit with reduced potency.[7]

To explore the functional importance of the 4*H*-pyran ring, we prepared a number of conjugated butenolide rings with either a terminal alkene or enol ether functionality (Fig. 6). All the monocyclic analogues **7**–**10** failed to stimulate germination at all concentrations tested (1 nM–10 μM, data not shown). This result implies that a 4*H*-pyran ring is essential for maximum karrikin activity and that the mode of action does not proceed through a Michael type addition at either C5 or C7. The exact function of the 4*H*-pyran ring remains unclear, but we postulate that the unique electronics associated with the extended conjugation present in the KAR_1 backbone are responsible for determining the extent to which the molecule stimulates germination. Evidence to support this hypothesis comes from the limited activity of the difluoromethyl derivative **11**[19] as previously observed[20] (Fig. 6). The electron-withdrawing capacity of the difluoromethyl group, which is similar in size to a methyl group, renders the compound inactive in promoting seed germination.

The above observations indicate an alternative mechanism is required to rationalise the activity of karrikins. Recently, we identified from *Arabidopsis thaliana* two genes, *KARRIKIN INSENSITIVE 2*

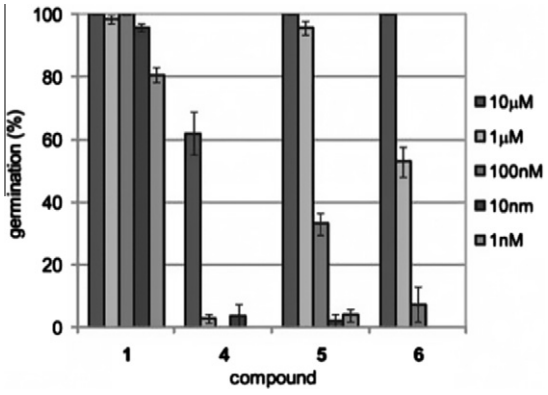

Figure 5. Germination of *Solanum orbiculatum* seeds tested with various saturated karrikin analogues.

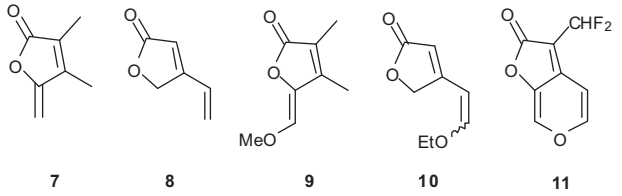

Figure 6. Chemical structures of butenolide analogues that possess a Michael acceptor similar to karrikinolide but are not active as seed germination stimulants.

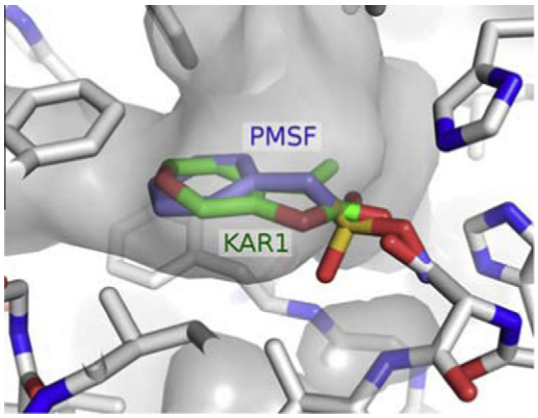

Figure 7. KAR_1 (green) is predicted to fit well into the large active site hydrophobic pocket of KAI2 (grey sticks and surface), in a similar manner to PMSF (blue) in RsbQ. The homology model of the active site of KAI2 (SWISS-MODEL)[24] is based on the structure of RsbQ complexed with PMSF (PDB entry 1WPR).[22] The active sites of KAI2 and RsbQ are almost identical, with only one of the amino acids pictured here being different (Ala to >Ile). Figure prepared with PyMOL.[25] KAR_1 has been manually posed over PMSF to illustrate similar relative positions of the electrophilic carbon and six-membered ring.

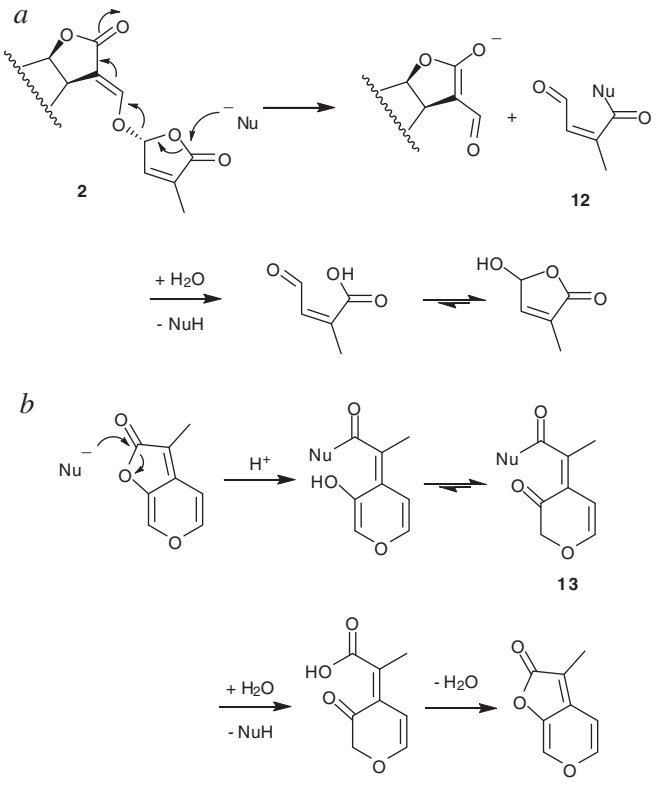

Figure 8. New proposed molecular mechanism for (a) strigolactones and (b) karrikins involving the hydrolysis of the butenolide ring.

(KAI2) and AtDWARF14 (AtD14), that mediate the distinct perception of karrikins and strigolactones, respectively.[6] Both encode proteins belonging to a diverse family of α/β hydrolases which are distinguished by a highly conserved catalytic triad composed of histidine, serine and aspartic acid residues.[6,21] The only member of this family to be well characterised is RsbQ from the bacterium *Bacillus subtilis*.[22,23] The crystal structure of RsbQ reveals the catalytic triad residing within a cavity that is predicted to bind a small hydrophobic molecule as a substrate for hydrolytic activity.[22] Furthermore, the serine protease inhibitor phenylmethanesulfonyl fluoride (PMSF) has been shown to label the catalytic triad, and it is possible to overlay KAR_1 **1** on PMSF to achieve a similar alignment in silico (Fig. 7).

Accordingly, we tentatively propose that KAI2 and AtD14 might hydrolyse the butenolide rings of karrikins and strigolactones, respectively. In the case of strigolactones, this would result in the cleavage of the ABC-rings from the D-ring analogous to the products resulting from a Michael addition (Fig. 8a). This single mechanism can account for all active strigolactone analogues including derivatives lacking the enol ether functionality, but possessing a leaving group attached to C5 of the D-ring. With regards to karrikins, the butenolide hydrolysis is difficult to rationalise as the hydrolysis product would furnish a ketone that would favour karrikin reformation upon dehydration with the resulting acid (Fig. 8b). However, given that both the ABC and D-rings of strigolactones themselves are not active,[26] we propose that the act of hydrolysis of the butenolide ring of both germination stimulants may provide an intracellular signal, potentially as a result of a change in protein tertiary structure rather than due to the formation of an active metabolite. Such a mechanism is consistent with observations from RsbQ, whereby upon binding of a small molecule in the active site, the cap domain of the protein is stabilised possibly permitting the RsbP phosphatase to bind.[22] If this scenario also applies to KAI2, the product of hydrolysis of the karrikin butenolide stabilised in the active site could trigger a new protein conformation that would only revert when the product is subsequently ejected from the active site. In this regard, it may be useful to note that the hydrolysis of karrikins generates the intermediate **13** which has similarity to the intermediate **12** that is generated upon strigolactone hydrolysis (Fig. 8).

In conclusion, significant attention has been directed towards strigolactone phytohormones given their ability to promote seed germination,[27] regulate shoot branching in plants[28,29] and stimulate arbuscular mycorrhizal fungi associations with plant roots.[30] Similarly, the ability of karrikins to promote seed germination is also attracting worldwide attention,[4,31] and it is clear that strigolactones and karrikins have structural and functional similarities.[5,32] Despite the importance of these bioactive molecules in multiple facets of plant development, very little is known about their mode of action. Based on karrikin structure–activity relationships, we propose a new mechanism for the activity of karrikins and strigolactones involving hydrolysis of the butenolide ring. This work will help direct future endeavours to elucidate the mode of action for both classes of bioactive compounds and will lead to the development of simpler more potent germination stimulants and plant growth regulators.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.04.016.

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