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The synthesis and biological evaluation of labelled karrikinolides for the elucidation of the mode of action of the seed germination stimulant

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ABSTRACT

Karrikins are a novel class of naturally occurring plant growth regulators that promote seed germination among a diverse range of species. Currently little is known about the mechanism by which these compounds overcome seed dormancy and initiate germination. The preparation of various karrikinolide derivatives provides an opportunity to investigate the karrikinolide mode of action at the cellular and molecular level. The first synthesis and biological evaluation of analogues suitable for use in metabolic labelling, affinity chromatography, photoaffinity labelling and NanoSIMS experiments are reported.

1. Introduction

Plant-derived smoke has a remarkable ability to increase the germination success of more than 1200 species from over 80 genera[1] located in various regions around the world including Australia,[2] South Africa,[3] North America[4] and Europe.[5] Interestingly, this response is not limited to species present in fire-prone regions as smoke-stimulated germination has also been observed for species from regions not frequented by fire.[6]

The key germination stimulant from smoke was first isolated and identified by Flematti et al. as 3-methyl-2*H*-furo[2,3-*c*]pyran-2-one **1**, now termed karrikinolide **1** (Fig. 1).[7] This naturally occurring germination stimulant displays activity in a variety of species at concentrations as low as one-part-per-billion.[1,8] Recently it has been revealed that at least five other analogues are present in smoke leading to the collective name 'karrikins'.[9]

The mode of action of karrikinolide remains unknown, however interactions with factors influencing germination such as light, gibberellic acid and abscisic acid have all been observed.[10,11]

Unlocking the secrets behind karrikinolide perception may result in a better understanding of the processes involved in regulating seed dormancy and germination, thus facilitating broader environmental and commercial applications. The potential for promoting a more uniform release from seed dormancy, improving germination rates and widening the range of species that respond, will allow for more effective plant regeneration and conservation in restoration programs. In addition, novel approaches to weed control through initiating germination followed by treatment with knockdown herbicides offers great potential for reducing weed seed levels in agricultural settings.[1]

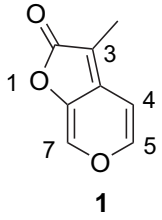

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Fig. 1. Karrikinolide.

Since the discovery of **1**, only four syntheses have been published. These include the preparation of **1** from pyromeconic acid **2**,[12] ethyl-4-methyl-2-oxo-2,5-dihydro-furan-3-carboxylate **3**,[13] 2-furfurylmethanol **4**[14] and D-xylose **5**[15] (Scheme 1).

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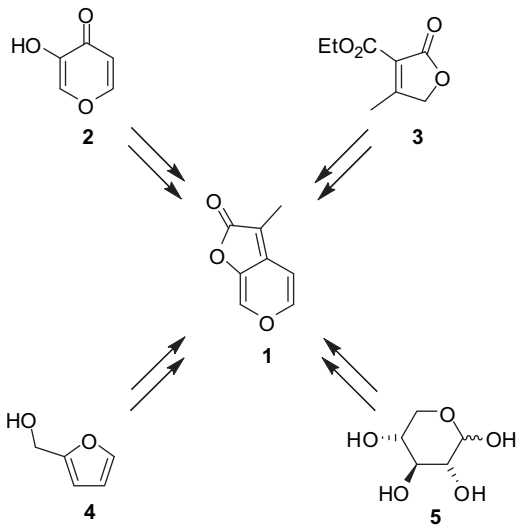
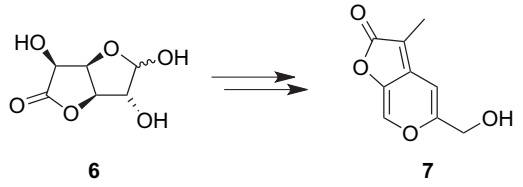


Scheme 1.

A variation on the synthesis of **1** from D-xylose **5** utilising the hexose D-glucuronic acid γ-lactone **6**, results in the addition of a hydroxymethyl moiety at the C5 position of **1** affording the alcohol **7** (Scheme 2).[15] Compound **7** provides an excellent scaffold for preparing analogues to investigate the karrikinolide mode of action as structure-activity relationships reveal that C5 derivatives retain a high level of bioactivity with test species *Solanum orbiculatum* compared with analogues substituted at other positions of **1**.[16]



Scheme 2.

The identification of the fundamental relationships between small bioactive molecules and their binding proteins, for example, a ligand and receptor, presents a challenging task that often relies on the nature of the interaction. As a result, a number of methods have been developed to recognise both covalent or non-covalent interactions including metabolic labelling,[17] affinity chromatography,[18] photoaffinity labelling[19] and NanoSIMS,[20] with each method requiring a uniquely prepared bioactive molecule.

Metabolic labelling, as the name suggests, involves the labelling of a cellular target with a modified bioactive molecule using the natural reaction mechanisms of the target biomolecule. The success of this method relies on the stable attachment of the bioactive molecule to a cellular target through a covalent linkage.[21] It has recently been proposed that **1** may adopt a similar mechanism to that proposed for strigolactone bioactivity based on their related methyl substituted butenolide rings and Michael acceptor characteristics.[22] If this proves to be the case, the interaction of **1** with a putative receptor would result in a covalent attachment detectable via metabolic labelling techniques.

One method of metabolic labelling that has attracted significant attention in recent years involves the incorporation of a non-evasive chemical reporter onto the bioactive molecule, for example, an azide or alkyne moiety, which is stable under most biological conditions. This allows for the identification of proteins that are covalently attached to a bioactive molecule of interest through highly specific bioorthoganol reactions between the reporter tag and a detection tag, for example, biotin. The Staudinger ligation[23,24] and Huisgen 1,3-dipolar cycloaddition[25,26] are examples of bioorthoganol reactions that have been successfully used in a number of systems. The Staudinger ligation involves the reaction between an azide and triphenylphosphine modified with a methyl ester to result in the formation of a stable amide linkage between the chemical reporter and detection tag. In a similar fashion, the Huisgen 1,3-dipolar cycloaddition also allows for the covalent attachment between an azide/alkyne containing chemical probe and an alkyne/azide modified detection tag through the formation of a triazole functionality, and has recently been used to label a papain-like cystine protease in *Arabidopsis thaliana*.[27]

Affinity chromatography is another common technique used for the separation of complex biological mixtures, and has previously been shown to be effective in the isolation of soluble and membrane bound abscisic acid binding proteins.[28] The identification of interactions between two molecules is made possible by the attachment of a molecular probe to a solid support. This is commonly achieved by tagging the molecular probe with biotin, usually via a polyethylene glycol linker to avoid steric hindrance and increase solubility, prior to the attachment to an avidin or streptavidin solid support. This methodology exploits the high affinity of avidin and streptavidin for biotin (K_d=10^{-14}–10^{-15} M).[29]

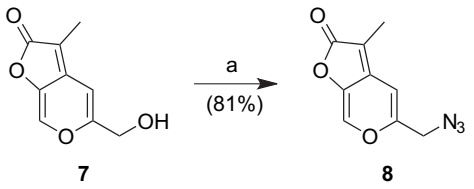
The use of photoaffinity labelling for the isolation of binding proteins requires the synthesis of a bioactive molecular probe containing a photoreactive group, which upon irradiation with a light source, will result in a covalent cross-link between the probe and a nearby protein.[19] Arylazides,[30] diazirines[31] and free radical generating ketones[32] upon irradiation, have all been shown to exhibit suitable photochemical properties for labelling experiments. Besides the attachment to a photoreactive group, bioactive probes must also contain a suitable label for downstream detection with the most common method involving the use of radioisotopes. However, recent reports have seen the development of radioisotope-free photoaffinity labelling by utilising alkyl azides and alkynes for subsequent Staudinger ligation and Huisgen cycloadditions with various detection tags.[33]

In addition to gaining information about the binding interactions of bioactive molecules at the molecular level, a number of techniques allow for visualising the location of bioactive molecules at the sub-cellular level. NanoSIMS is a modern technique that detects the difference in the isotope ratio of secondary ions generated by bombarding the surface of a sample with a primary ion source.[20] The use of an isotope-enriched molecular probe results in a different isotope ratio at the location of the probe. The isotope enrichment removes the need for structural modification of the bioactive probe, which may adversely affect the bioactivity, thus providing an advantage over other scanning techniques.

2. Results and discussion

A karrikinolide analogue suitable for use in metabolic labelling studies was investigated. Studies have shown that analogues modified at C3, C4 or C7 (Fig. 1) resulted in reduced germination activity, while derivatives substituted at C5 were better tolerated.[16] Thus, it was proposed that the incorporation of an azide moiety at C5 would yield an analogue that should retain good bioactivity in addition to one, that is, amenable to further functionalisation with detection tags. To this end, the alcohol **7**[15] was converted to the mesylate followed by displacement with azide to furnish the karrikin **8** (Scheme 3).

The germination activity of **8** was evaluated using *Brassica tournefortii* seeds that had low control germination levels (<10%) and were stimulated to over 90% germination with **1**. As expected, compound **8** showed excellent activity at concentrations as low as one hundred-parts-per-billion (Fig. 2).



Scheme 3. (a) (i) MsCl, Et_3N, CH_2Cl_2 (ii) NaN_3, DMF.

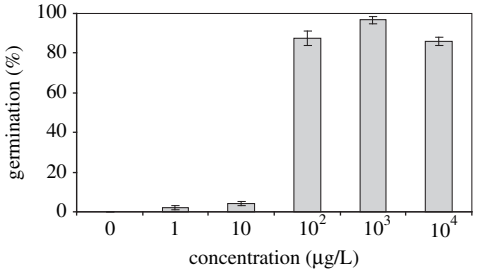
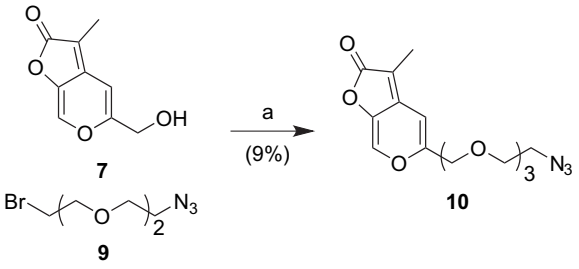


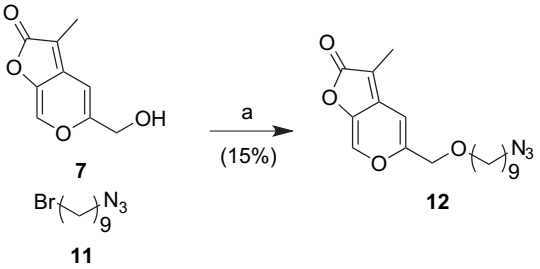
Fig. 2. Germination activity of **8**.

With the successful synthesis of an active karrikinolide derivative for metabolic labelling, attention was then directed towards the preparation of an analogue suitable for affinity chromatography. The attachment of a detection tag to the karrikinolide scaffold through a suitable linker appeared the most attractive method in achieving this goal. The alcohol **7**[15] was again chosen as the starting material, however, a conscious effort was made to reduce the number of subsequent synthetic steps given the relatively low overall yield in obtaining the alcohol **7**.[15] As a result, a triethylene glycol linker containing a halide leaving group for alkylation onto the karrikin backbone, along with an azido moiety for further functionalisation with biotin, was prepared.[34] With the bromide **9**[34] in hand, the alcohol **7**[15] was alkylated under standard conditions resulting in the azide **10** (Scheme 4). The bioactivity of **10** was assessed prior to the addition of biotin but no activity was observed with *B. tournefortii* seeds.



Scheme 4. (a) NaH, DMF.

Based on recent structure-activity relationships,[16] it was proposed that the loss of activity may be a result of the increased polarity of the triethylene glycol linker and that substitution with an alkyl chain may return some bioactivity. To test this theory, the alcohol **7**[15] was alkylated with 1-azido-9-bromononane **11** to furnish the azide **12** (Scheme 5).



Scheme 5. (a) NaH, DMF.

The germination response of **12** was screened using *B. tournefortii* seeds and revealed low activity, which was deemed sufficient at higher concentrations to proceed to labelling with biotin (Fig. 3).

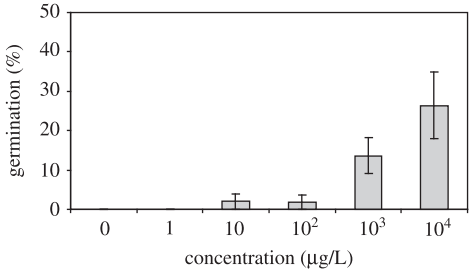
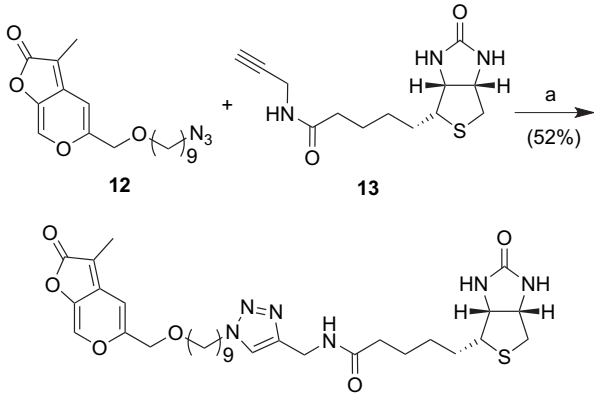


Fig. 3. Germination activity of **12**.

This was achieved via the treatment of azide **12** with biotin alkyne **13**[27] under Huisgen 1,3-dipolar cycloaddition conditions to return the triazole **14** in modest yield (Scheme 6).



Scheme 6. (a) $CuSO_4.5H_2O$, sodium ascorbate, EtOH, H_2O.

Unfortunately **14** did not stimulate the germination of *B. tournefortti* seeds under the range of concentrations tested. Although the lack of activity might be due to the additional steric elements of **14**, the possible low permeability properties could also contribute to this effect and as a result, the triazole **14** may still be suitable for in vitro affinity studies.

The design of a suitable photoaffinity probe relied on the highly active benzyl derivative **15** when tested against *S. orbiculatum* (Fig. 4).[16]

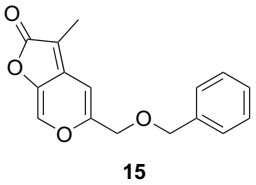
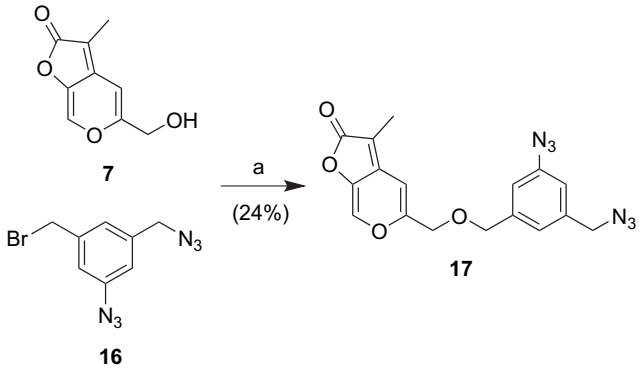


Fig. 4. Karrikinolide benzyl derivative.

The modification of the benzyl ring to include an aryl azide for photoactivation, in addition to a bioorthoganol alkyl azide for further functionalisation with a detection tag, would yield a compound with all the necessary requirements for a radioisotope-free photoaffinity probe. Hosoya et al. recently reported the modification of cervastatin with the bromide **16**[35] for use in photolabelling the catalytic domain of human 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA).[33] It was revealed that the alkyl azide moiety

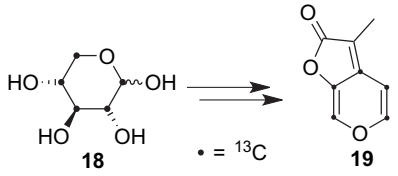
remained intact during photoactivation of the aryl azide thus allowing for chemoselective ligation with a fluorescent tag for detection. This methodology was adopted for the synthesis of **17** resulting from the alkylation of the alcohol **7**[15] with the bromide **16**[35] (Scheme 7).



Scheme 7. (a) NaH, DMF.

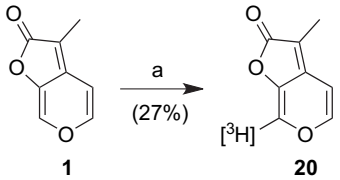
Once again the germination activity of the azide **17** was evaluated with *B. tournefortti* seeds with assay results indicating no stimulatory response. It was thought that any modification of **17** to render an active photoaffinity probe would result in limited success and therefore endeavours to prepare a bioactive photoaffinity probe were no longer pursued.

The preparation of karrikinolide from [^{13}C]$_5$-D-xylose **18** following the procedure of Goddard-Borger et al.[15] allowed for the isolation of an isotope-enriched karrikinolide **19** suitable for the use in NanoSIMS experiments (Scheme 8). As expected, the ability of compound **19** to stimulate the germination of *B. tournefortti* seeds was identical to that observed for **1**.



Scheme 8.

In addition to preparing analogues for binding protein elucidation and location, a compound suitable for identifying metabolism products and stability of **1** within the seed and during applications to soil was sought. Sun et al. recently demonstrated that the treatment of **1** with LiHMDS followed by the addition of an electrophile, results in the formation of C7 substituted analogues.[13] Following this methodology, the synthesis of a radiolabeled karrikinolide **20** was achieved through the use of [^{3}H]-enriched water as the electrophile source (Scheme 9).



Scheme 9. (a) (i) LiHMDS, THF (ii) [^{3}H]–H_2O.

3. Conclusion

In summary, the synthesis and biological evaluation of the first generation of labelled karrikinolides prepared from **7**[15] is reported. The diversity of labelling techniques allows the use of a variety of different methods for the potential localisation, isolation and identification of a karrikin binding protein. Various labelling experiments have now been made possible allowing the metabolic fate of karrikinolide to be investigated.

4. Experimental

4.1. General experimental

^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were obtained on a Bruker ARX500 (500 MHz for δ_H and 125.7 MHz for δ_C) or a Bruker AV600 (600 MHz for δ_H and 150.9 MHz for δ_C) spectrometer. Unless otherwise stated, deuterochloroform ($CDCl_3$) was used as the solvent with residual $CHCl_3$ (δ_H 7.26) or $CDCl_3$ (δ_C 77.0) being employed as internal standards. Spectrums run in tetradeuteromethanol (CD_3OD) used residual CH_3OH (δ_H 3.34) or CD_3OD (δ_C 49.0) as internal standards.

Melting points (mp) were determined on a Reichert hot stage melting point apparatus. High-resolution mass spectra (HRMS) were recorded with a VG-Autospec spectrometer using electron impact (70 eV) ionisation (EI) technique.

All experiments were carried out under an inert atmosphere and all solvents were dried prior to use. [^{13}C]$_5$-D-Xylose **18** was obtained from Omicron Biomedical Inc. and [^{3}H]-enriched water was acquired from Amersham. A 'usual workup' refers to dilution with water, repeated extraction into an organic solvent, sequential washing of the combined extracts with hydrochloric acid (1 M, where appropriate), saturated aqueous sodium bicarbonate and brine solutions, followed by drying over anhydrous magnesium sulfate, filtration and evaporation of the solvent by means of a rotary evaporator at reduced pressure.

Flash chromatography was performed on Merck silica gel 60 with the specified solvents. Thin-layer chromatography (TLC) was effected on Merck silica gel 60 F254 aluminium-backed plates that were stained by heating (>200 °C) with 0.25 M ceric sulfate in 2 M sulfuric acid.

Semi-preparative high pressure liquid chromatography (HPLC) was performed on an Agilent 1050 system using a 250×10 mm i.d., 5 μm, Apollo C_{18} reversed-phase column (Grace-Davison).

Percentage yields for chemical reactions as described are quoted only for those compounds that were purified by recrystallisation or by chromatography and the purity assessed by TLC or NMR spectroscopy.

4.2. Seed bioassay

All germination experiments were performed using *B. tournefortii* seeds collected in the Meckering region of Western Australia and stored at −80 °C until use. All assays were conducted using Millipore (MP) water obtained by filtration through a Milli-Q ultrapure water system (Millipore, Australia). A 1% acetone solution was used as a control.

Stock solutions of 100 ppm were prepared by dissolving 1.0 mg of compound in 100 μL of acetone prior to adding 9.9 mL of MP water. Subsequent dilutions gave concentrations of 10 ppm, 1 ppm, 100 ppb, 10 ppb and 1 ppb. The solutions were tested for germination activity by adding 3 mL to one piece of Filtech glass microfiber filter paper (8.2 cm) in plastic Petri dishes followed by approximately 20–30 seeds. The Petri dishes were sealed with a layer of plastic wrap and stored in a light proof container for 3 days at 20±1 °C. All experiments were conducted in triplicate.

4.3. Synthesis

*4.3.1. 5-Azidomethyl-3-methyl-2H-furo[2,3-c]pyran-2-one (**8**).* Triethylamine (55 mg, 0.5 mmol) in dichloromethane (0.5 mL) was added to the alcohol **7**[15] (28 mg, 0.2 mmol) in dichloromethane (2.0 mL) at 0 °C and the solution stirred (10 min). To this solution

was added, methanesulfonyl chloride (32 mg, 0.3 mmol) in dichloromethane (0.5 mL) and the solution left to stand (30 min). The reaction was subjected to a usual workup (dichloromethane) to leave a white powder that was dissolved in dimethylformamide (2.0 mL) followed by the addition of sodium azide (70 mg, 1.1 mmol). The mixture was stirred at room temperature (30 min) before being subjected to a usual workup (dichloromethane) and flash chromatography (ethyl acetate–hexane 3:7) to return the azide **8** (26 mg, 81%) as a tan powder. R_f (ethyl acetate–hexane 1:1) 0.8; mp 118–120 °C; δ_H (600 MHz) 7.41 (1H, s, H7), 6.52 (1H, s, H4), 4.17 (2H, s, H8), 1.95 (3H, s, H3b). δ_C (150.9 MHz) 171.03 (C2), 154.66 (C5), 141.69, 139.76 (C3a, C7a), 126.10 (C7), 101.80 (C3), 101.35 (C4), 51.39 (C8), 7.71 (C3b); HRMS (EI): *m/z* found 205.0488. $C_9H_7N_3O_3$ requires 205.0487.

*4.3.2. 5-(2-(2-(2-Azidoethoxy)ethoxy)ethoxy)methyl-3-methyl-2H-furo[2,3-c]pyran-2-one (**10**).* Sodium hydride (60% dispersion in mineral oil, 30 mg, 0.8 mmol) was added to a stirred solution of 1-(2-azidoethoxy)-2-(2-bromoethoxy)ethane **9**[34] (500 mg, 2.1 mmol) and the alcohol **7**[15] (75 mg, 0.4 mmol) in dimethylformamide (2.0 mL) at 0 °C and the solution stirred (10 min). The reaction was allowed to warm to room temperature (3 h) before being quenched with methanol (0.5 mL). A usual workup (dichloromethane) followed by flash chromatography (ethyl acetate–toluene 3:7) and semi-preparative HPLC (C18, acetonitrile-water 9:11) yielded the azide **10** (10 mg, 7%) as a colourless glass. R_f (ethyl acetate–dichloromethane 1:9) 0.6; δ_H (500 MHz) 7.43 (1H, s, H7), 6.62 (1H, s, H4), 4.37 (2H, s, H8), 3.76–3.67 (10H, m, H10, H11, H13, H14, H16), 3.40 (2H, t, $J_{16,17}$=5.2 Hz, H17), 1.62 (3H, s, H3b). δ_C (125.7 MHz) 171.46 (C2), 157.40 (C5), 141.82, 140.61 (C3a, C7a), 126.22 (C7), 100.57 (C4), 100.42 (C3), 70.74, 70.71, 70.66, 70.08, 69.29 (C10, C11, C13, C14, C16, C17) 50.67 (C8), 7.71 (C3b); HRMS (EI): *m/z* found 337.1274. $C_{15}H_{19}N_3O_6$ requires 337.1274.

*4.3.3. 5-(9-Azidononoxy)methyl-3-methyl-2H-furo[2,3-c]pyran-2-one (**12**).* Sodium hydride (60% dispersion in mineral oil, 90 mg, 2.2 mmol) was added to a stirred solution of 1-azido-9-bromononane **11** (850 mg, 3.4 mmol) and the alcohol **7**[15] (210 mg, 1.2 mmol) in dimethylformamide (5.0 mL) at 0 °C and the solution stirred (10 min). The reaction was allowed to warm to room temperature (3 h) before being quenched with methanol (1.0 mL). A usual workup (dichloromethane) followed by flash chromatography (ethyl acetate–dichloromethane 3:97) afforded the azide **12** (60 mg, 15%) as a light yellow powder. R_f (ethyl acetate–hexanes 1:1) 0.6; mp 28–30 °C; δ_H (600 MHz) 7.41 (1H, s, H7), 6.55 (1H, s, H4), 4.26 (2H, s, H8), 3.55 (2H, t, $J_{10,11}$=6.6 Hz, H10), 3.25 (2H, t, $J_{17,18}$=7.0 Hz, H18), 1.92 (3H, s, H3b), 1.65–1.55, 1.40–1.30 (14H, 2m, H11, H12, H13, H14, H15, H16, H17). δ_C (150.9 MHz) 171.37 (C2), 157.65 (C5), 141.72, 140.54 (C3a, C7a), 126.12 (C7), 100.25 (C4), 100.23 (C3), 71.76, 68.79 (C8, C10), 51.34 (C18), 29.44, 29.26, 29.17, 28.96, 28.70, 26.57, 25.89 (C11, C12, C13, C14, C15, C16, C17), 7.61 (C3b); HRMS (EI): *m/z* found 347.1853. $C_{18}H_{25}N_3O_4$ requires 347.1845.

*4.3.4. N-(1-(9-((3-Methyl-2-oxo-2H-furo[2,3-c]pyran-5-yl)methoxy) nonyl-1H-1,2,3-triazol-4-yl)methyl)-5-[(3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl]pentanamide (**14**).* Copper (II) sulfate pentahydrate (0.1 M solution, 50 μL, 5.0 μmol) was added to sodium ascorbate (0.1 M solution, 200 μL, 20 μmol), the azide **12** (18 mg, 52 μmol) and biotin alkyne **13**[27] (15 mg, 53 μmol) in a mixture of ethanol and water (1:1, 4.0 mL) and the reaction allowed to stand overnight at room temperature. The reaction was concentrated followed by flash chromatography (methanol–dichloromethane 1:19–1:9) and semi-preparative HPLC (C18, acetonitrile–water 1:1) to furnish the triazole **14** (17 mg, 52%) as a white powder. R_f (methanol–dichloromethane 3:17) 0.4; mp 108–110 °C; δ_H (500 MHz, CD_3OD) 7.83 (1H, s, H7′/H4), 7.78 (1H, s, H4/H7′), 6.88 (1H, s, H4′), 4.48 (1H dd, $J_{3a'',6a''}$=7.9 Hz, $J_{6'',6a''}$=5.0 Hz, H6a″), 4.41 (2H, s, H8′/H6), 4.32 (2H, s, H6/H8′), 4.37 (2H, t, $J_{17',18'}$=7.1 Hz, H18′), 4.28 (1H, dd, $J_{3a'',4''}$=4.4 Hz, H3a″), 3.55 (2H, t, $J_{10',11'}$=6.5 Hz, H10′), 3.20–3.15 (1H, m, H4″), 2.91 (1H, dd, $J_{6'',6''}$=12.7 Hz, H6″), 2.70 (1H, d, H6″), 2.23 (1H, t, $J_{9'',10''}$=7.3 Hz, H10″), 1.90 (3H, s, H3b′), 1.90–1.85, 1.75–1.53, 1.45–1.25 (20H, 3m, H11′, H12′, H13′, H14′, H15′, H16′, H17′, H7″, H8″, H9″). δ_C (150.9 MHz) 175.95, 173.65, 166.09 (C2′, C11″, C12″), 160.10 (C5′), 146.28 (C5), 143.16, 142.79 (C3a′, C7a′), 128.99 (C7′), 124.14 (C4), 101.92 (C4′), 100.52 (C3′)72.39, 69.84 (C8′, C10′), 63.33 (C3a″), 61.63 (C6a″), 56.99 (C4″), 51.34 (C18′), 41.05 (C6″), 36.55, 35.60, 31.27, 30.64, 30.46, 30.32, 29.99, 29.70, 29.44, 27.42, 27.09, 26.70 (C6, C11′, C12′, C13′, C14′, C15′, C16′, C17′, C7″, C8″, C9″, C10″), 7.44 (C3b′). HRMS (EI): *m/z* found 628.3049. $C_{31}H_{44}N_6O_6S$ requires 628.3043.

*4.3.5. 5-(3-Azido-5-(azidomethyl)benzyloxy)methyl-3-methyl-2H-furo[2,3-c]pyran-2-one (**17**).* Sodium hydride (60% dispersion in mineral oil, 55 mg, 1.4 mmol) was added to a stirred solution of the bromide **16**[35] (300 mg, 1.1 mmol) and alcohol **7**[15] (190 mg, 1.1 mmol) in dimethylformamide (5.0 mL) at 0 °C and the solution stirred (2 h). The reaction was quenched with methanol (1.0 mL) followed by a usual workup (ethyl acetate) and flash chromatography (ethyl acetate–hexane 1:3) yielded the azide **17** (92 mg, 24%) as a light yellow powder. R_f (ethyl acetate–hexane 1:2) 0.3; mp 60–63 °C; δ_H (600 MHz) 7.42 (1H, s, H7), 7.08, 7.02, 6.94 (3H, 3s, H12, H14, H16), 6.58 (1H, s, H4), 4.63 (2H, s, H10), 4.36, 4.33 (4H, 2s, H8, H17), 1.98 (3H, s, H3b). δ_C (150.9 MHz) 171.26 (C2), 156.66 (C5), 141.74, 141.08, 140.24, 139.71, 137.80 (C3a, C7a, C11, C13, C15), 126.11 (C7), 123.35, 118.12, 117.70 (C12, C14, C16), 100.86 (C4), 100.79 (C3), 72.27, 68.36 (C8, C10), 54.05 (C17), 7.64 (C3b); HRMS (EI): *m/z* found 366.1084. $C_{17}H_{14}N_6O_4$ requires 366.1077.

*4.3.6. 3a,4,5,7,7a-[^{13}C]$_5$-3-Methyl-2H-furo[2,3-c]pyran-2-one (**19**).* Compound **19** was prepared in a manner previously described for the preparation of karrikinolide **1** with the exception that $^{13}C_5$-D-xylose **18** was used as the starting material.[15] R_f (ethyl acetate–hexane 1:2) 0.3; mp 118–120 °C; δ^a_H (600 MHz) 7.61–7.59, 7.28–7.25 (1H, 2m, H7), 7.51–7.46, 7.18–7.13 (1H, 2m, H5), 6.68–6.63, 6.39–6.35 (1H, 2m, H4), 1.93 (3H, d, $J_{H3b,C3a}$=4.7, H3b). δ^b_C (150.9 MHz) 171.13 (m, C2), 147.86 (dddd, $J_{4,5}$=71.5 Hz, C5), 142.16 (dddd, $J_{7,7a}$=97.0 Hz, $J_{3a,7a}$=46.6 Hz, H7a), 139.60 (dddd, $J_{3a,4}$=57.3 Hz, H3a), 126.68 (br d, H7), 103.31 (dddd, C4), 100.19 (m, C3), 7.56 (C3b). HRMS (EI): *m/z* found 155.0489. $^{12}C_3^{13}C_5H_6O_3$ requires 155.0485. [a]Multiplets split by ^{13}C coupling. [b]Only one bond coupling assigned.

*4.3.7. 7-[^3H]-3-Methyl-2H-furo[2,3-c]pyran-2-one (**20**).* Lithium bis (trimethylsilyl)amide (1 M solution in tetrahydrofuran, 2.0 mL, 2.0 mmol) was added to karrikinolide **1** (150 mg, 1.0 mmol) in tetrahydrofuran (10 mL) at −78 °C and the resulting brown solution stirred (15 min). [^{3}H]–Water (5.0 mCi/mL, 50 μL, 2.5 mmol) was added to the solution and the resulting mixture allowed to warm to room temperature (1 h). The reaction was subjected to flash chromatography (ethyl acetate–hexane 1:3) to return an orange powder. This powder was treated with activated charcoal, filtered and concentrated. Recrystallisation from hexane returned the radiolabelled karrikinolide **20** (40 mg, 27%) as light yellow cyrstals. Specific activity 85 mCi/mol.

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References and notes

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