Supplementary Data

**On the Formation of Carlactone**

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Supplementary Figure 1. SLs structures and biosynthesis. Canonical SLs, such as orobanchol, consist of a tricyclic lactone (ABC ring) that is connected to a further cyclic lactone (D-ring) by an enol ether bridge. The numbering of C atoms is shown exemplarily for orobanchol. Canonical SLs are divided into the strigol- and orobanchol-like subfamilies, which differ in the stereochemistry of the C8b/C3a atoms (shown in the orobanchol structure). SLs biosynthesis starts with the reversible 9-cis/all-trans isomerization of β-carotene catalyzed by DWARF27 to yield 9-cis-β-carotene. CCD7 then cleaves the latter at the C9′-C10′ double bond in the trans-moiety of the substrate, to yield 9-cis-β-apo-10′-carotenal and β-ionone. The central enzyme is CCD8 that converts the shaded part of 9-cis-β-apo-10′-carotenal into carlactone [1] (s.
Fig. 1 and 2). In rice, carlactone is the substrate of the carlactone oxidase (CO) Os900, a CYP (711 clade), which catalyzes repeated oxygenation and ring closures to form 4-deoxyorobanchol (known as: ent-2’-epi-5-deoxystrigol), the parent molecule of the orobanchol-like SLs. A second CYP of the same clade, orobanchol synthase (OS, formerly designated as ent-2’-epi-5-deoxystrigol-4-hydroxylase) converts 4-deoxyorobanchol into orobanchol [2]. In Arabidopsis, MAX1, a CYP (711 clade), converts carlactone into carlactonic acid via the intermediate 19-hydroxy carlactone. Carlactonic acid can be converted into methyl carlactonate [3] and further hydroxylated by the Lateral branching oxidoreductase into a yet unidentified product [4]. In rice, carlactonic acid is likely a further precursor of 4-deoxyorobanchol. 5-Deoxystrigol, the parent of the strigol-like SLs is supposedly formed from carlactone by yet unidentified CYPs.

Supplementary Figure 2. Chemical synthesis of 9-cis-11-13C-β-apo-10’-carotenal (I).
(a). LDA (2 eq.), Diethylchlorophosphatate, CH₃¹³CN, THF, -60 °C;
(b). Dibal-H, separation isomers;
(c). Diethyl(3-cyano-2-methyl-2-propenyl)phosphonate, NaH/THF;
(d). Dibal-H, separation isomers;
(e). Diethyl(3-cyano-3-methyl-2-propenyl)phosphonate, NaH/THF;
(f). Dibal-H, separation isomers;
(g). Diethylcyanomethylphosphonate, NaH/THF;
(h). Dibal-H, separation isomers and crystallization.
Supplementary Figure 3. Structure of 9-cis-β-apo-10´-carotenal. $^{13}$C-natural abundance (1) and selectively $^{13}$C-labeled at position C-11, highlighted in blue (2*).

Supplementary Figure 4. 1D $^1$H NMR spectra of 9-cis-β-apo-10´-carotenal (1) and (1*). The Spectrum of labelled (1*) is shown in black and of native (1) in red, both in CD$_2$Cl$_2$, full spectral range. For experimental details, see Methods section of Supplementary Material.
Supplementary Figure 5. Expansion of the 1D $^1$H NMR spectra of 9-cis-β-apo-10'-carotenal (1) and (1*). Spectral region 0.8 – 2.3 ppm.

Supplementary Figure 6. Expansion of the 1D $^1$H NMR spectra of 9-cis-β-apo-10'-carotenal (1) and (1*). Spectral region 5.9 – 7.45 ppm.
Supplementary Figure 7. Expansions of the 2D $^1$H-$^{13}$C-HSQC spectra (with carbon type editing) of 9-cis-$\beta$-apo-10'-carotenal (I) and (1*). Compound (1) and (1*) showing $^{13}$C-$^1$H-one-bond ($^1$J$_{CH}$) coupling connectivities between protons and carbons in the corresponding spectral regions. Cross peaks of CH$_3$ and CH groups appear in blue, cross peaks of CH$_2$ groups appear in red.
Supplementary Figure 8. Expansions of the 2D $^1$H-$^{13}$C-HSQC spectra (with carbon type editing) of 9-cis-β-apo-10'-carotenal (1) and (1*). For the selectively labelled compound (1*) 2 intense cross peaks are visible for the $^{13}$C*-H-bond in position C-11, one of which (6.92 ppm/125.9 ppm) showing the same $^1$H- and $^{13}$C- shifts as the 9-cis isomer of (1) while the other 6.82 ppm / 127.3 ppm indicates the coexistence of the corresponding 9-trans isomer of (1).
Supplementary Figure 9. Structure of carlactone. $^{13}$C-natural abundance (8) and with $^{13}$C-label recovered at position C-11, highlighted in blue (8*).

Supplementary Figure 10. 1D $^1$H NMR spectra of carlactone (8) and (8). The Spectrum of labelled (8*) is shown in black and of native (8) in red, both in CD$_2$Cl$_2$, full spectral range. For experimental details, see Methods section of Supplementary Material.
Supplementary Figure 11. Expansion of the 1D $^1$H NMR spectra of (8) and (8*). Spectral region 0.8-2.1 ppm.

Supplementary Figure 12. Expansion of the 1D $^1$H NMR spectra of (8) and (8*). Spectral region 5.0 – 7.5 ppm. For (8*) the resonance of 11-H is split into a doublet due to one-bond coupling of 11-H with the $^{13}$C enriched 11-C* ($^2J_{\text{CH}} = 7.8$ Hz). In addition, $^{13}$C-enrichment at 11-C leads to long-range coupling interactions with the neighboring 12-CH group. This is evident from the $^1$H resonance of 12-H, which is a
poorly resolved pentet in (8) and is further split into a doublet in (8*) with a coupling constant of $^3J_{CH} = 7.8$ Hz.

Supplementary Figure 13. Expansions of the 2D $^1$H-$^{13}$C-HSQC spectra (with carbon type editing) of carlactone (8) and (8*). For compound (8*) a cross peak of enhanced intensity is visible for the $^{13}$C*-H-
bond in position C-11, while the remaining cross peaks become visible after increasing the scale by a factor of 64. The $^1$H-$^{13}$C-HSQC cross peak of 12-CH at 6.9 ppm/142.7 ppm of (8) was not visible in the 2D spectrum of (8*). This can be explained by a strong signal intensity loss of the 12-CH cross peak during the two delays (adjusted to 1/2 and 1/4 of $^1$J$_{CH}$, respectively, with $^1$J$_{CH}$ = 145 Hz) of the HSQC experiment (with multiplicity editing) due to coupling of the carbon nuclei at 12-C with their adjacent $^{13}$C enriched nuclei at C-11. To support this, a simulation of the same HSQC experiment was performed confirming the unwanted impact of C-C coupling between adjacent $^{13}$C carbons on the signal intensity of the HSQC cross peaks (Supplementary Figure 14).

Supplementary Figure 14. Simulation of $^1$H$^{13}$C-HSQC (hsqedetgpsy2.2) experiment. The «NMRsim» software (Bruker) was used for fictitious spin systems of two weakly coupled pairs of nuclei (11-CH, 12-CH) and one isolated (ref-CH) as reference. Overlay of 2D $^1$H-$^{13}$C-HSQC spectra with assumed $^1$J$_{CC}$-coupling constants of 0Hz (blue), 45 Hz (pink) and 72.5 Hz (red) slightly shifted to each other. The simulation demonstrates that the signal intensities of the C-H-cross peaks may become strongly reduced due to C-C coupling. Because of the low natural abundance of $^{13}$C isotopes (~1.1 %), this C-C coupling is usually negligible in the spectra of unlabeled compounds, but may no longer be neglected with $^{13}$C-enriched compounds.
Supplementary Figure 15. 3D-representation of chemical species proposed to be involved in the conversion of 9-cis-β-apo-10'-carotenal (1) into carlactone (8). The structures are as presented in Figure 1 (main text) in 2D. The numbering and labeling corresponds to that of Figure 1. The conformations were obtained with the Avogadro software [5] and the energy minimization algorithms included. The colored spheres (green and dark red) represent the molecule parts that are chemically not involved. They have been omitted from the intermediate species, for clarity. The curved arrow in red denotes the rotation around the C12-C13 bond required for converting the transoid conformation into the cisoid one as depicted in structure (2).
Supplementary Figure 16. Inverted reaction sequence. The inverted sequences of oxygenation ((b) and (e) in Figure 1) and rearrangement steps ((d) and (f)) would lead to a product lactone (analogous to 8 in Figure 1) linked to a molecule fragment derived from R₂.
References


