A comprehensive account on phyto-hormones and pheromones in phycology literature: benchmarking of data-set, developing critical tools of biotechnological implications for commercial-aquaculture industry

Sachin G. Rathod [1,2], Satej Bhushan [1], Vaibhav A. Mantri [1,2,*]

[1] *Applied Phycology and Biotechnology Division, CSIR-Central Salt and Marine Chemicals Research Institute, Bhavnagar- 364021, India*

[2] *Academy of Scientific and Innovative Research (AcSIR), Ghaziabad- 201002, India*

*Corresponding author at: Tel: +91-278-2567760 Ext. 6131

E-mail address: vaibhav@csmcri.res.in (V. A. Mantri)

Supplementary Material

1. Biosynthetic pathways

1.1 Hormones

1.1.1. Auxin

The tryptophan-independent pathway for auxin biosynthesis has been shown in Supplementary Figure S1. It gets initiated either with indole or indole-3-glycerophosphate but ultimately joins the tryptophan biosynthetic pathway [225]. The production of indole-3-acetonitrile or indole-3-acetaldehyde and the enzymes involved in these steps have not yet been studied [226].

1.1.2. Brassinosteroids

Steroidal hormone brassinosteroids (BRs) regulate the growth and development of plants. The biosynthesis of brassinolide, the earliest discovered BR, involves two analogous pathways. Early and late C-6 oxidation pathways as presented in Figure S4, are connected at various steps and also to the C-22 oxidation pathway. The biosynthetic pathway of Brassinosteroids was discovered by the GC-MS technique which also helped in revealing the metabolic pathway and their reaction sequences. The earlier study by Bajguz demonstrated the seven brassinosteroids (BRs) compounds like teasterone (TE), 6-deoxoTE, typhasterol (TY), 6-deoxoTY, castasterone (CS), 6-deoxoCS and brassinolide (BL) occur in wild-type *Chlorella vulgaris* [132]. All the above compounds belong to the BR biosynthetic pathway, but the signal that initiates BR biosynthesis remains unidentified [227]. However, their possible physiological roles and the biosynthesis pathways are yet to be investigated [141].

1.1.2. Jasmonic acid (JA)

JA and MeJA biosynthesis take place by the Vick and Zimmermann pathway [228] as shown in Supplementary Figure S5. This pathway was first time revealed by Vick and Zimmerman

[229] and Hamberg and Hughes [230]. Variations occurring in this pathway are considered as the alternative routes of JA biosynthesis. The key enzymes in the synthesis of OPDA (Allene oxide cyclases) were isolated from the moss *Physcomitrella* patens [231]. The allene oxide cyclase (AOC), is not similar to that of other enzymes of the Vick and Zimmerman pathway, this situation makes this enzyme the bottleneck for DH-JA biosynthesis [232]. Primitive oxylipin-signalling components are also found in seaweeds. Oxylipins and two genes encoding for lipoxygenase were reported in *Chondrus crispus*. However, MeJA was identified in *C. crispus* [233]. Whereas, two enzymes, a 12-lipoxygenase, and a hydroperoxide isomerase were identified in *Gracilariopsis lemaneiformis* and involved in the biosynthetic pathway of JA [234].

1.1.3. Polyamines

The biosynthetic pathway for polyamines is conserved in bacteria, plants, and animals with minor variations [136]. Polyamine biosynthesis takes place through two alternative pathways (Supplementary Figure S6), which were identified by getting the genes involved in the biosynthetic pathway characterized in numerous plant taxa [235,236]. The biosynthesis starts with the amino acids, L-arginine and L-methionine, that act as precursor molecules. In plants, polyamines are found in the cytoplasm as well as in vacuoles [237].

1.1.4. Salicylic acid

There are two possible pathways for SA biosynthesis; the ICS and PAL pathways (Figure S7). Chorismate is the substrate required to initiate both pathways. Both the pathways differ in different plant species [138]. The ICS pathway for the synthesis of SA in plants is comparatively new, while the PAL pathway was discovered much earlier. PAL is an upstream enzyme responsible for the biosynthesis of SA and has been known to lead to the synthesis of other defense compounds [238].

1.1.5. Strigolactone (SLs)

SLs display high chemical diversity as there are 30 SLs identified to date [239]. Substantial chemical diversity in SLs may be due to the product of co-evolution with other organisms [240]. SL biosynthesis initiates from isoprenoid pigment (β-carotene) [241]. β-Carotene synthesis takes place in plastids by methylerythritol-5-phosphate isoprenoid pathway. There are three key enzymes: one is isomerase DWARF27 (D27), rest two are carotenoid cleavage dioxygenases (CCD7 and CCD8) [242]. Further downstream reactions subsequently convert CL to various SLs (Figure S8).

2. Pheromones

2.1. Finavarrene

Finavarrene biosynthesis is achieved from the dodecatrienoic acid by administering deuterated dodeca-3,6,9-trienoic acid (3,4,6,7,9,10-2H6) to the freshly excised *Senecio isatideus* plantlets. Acyclic hydrocarbon finavarrene yield with the loss of C(1) and a single hydrocarbon from C(5) (Figure S9) [103].

2.2. Hormosirene

In *Gomphonema parvulum*, a freshwater diatom, hormosirene biosynthesis starts from eicosapentaenoic acid, followed by oxidative degradation catalyzed respectively by 9-lipoxygenase and hydroperoxide lyase as presented in supplementary figure S10 [103]. The freshwater diatom has been reported to produce the algal pheromones hormosirene ($C_{11}H_{16}$) and dictyopterene A ($C_{11}H_{18}$), from highly unsaturated eicosanoic acids like 20: s (~3) and 20:4 (w-6) [105].

3. Mode of action of plant hormones

The steroidal hormone, Brassinosteroid (BR), performs dual functions in plants such as promoting growth and providing protection against environmental stresses [141]. BR binds to plasma membrane-localized BRI1 (Brassinosteroid Insensitive1), which is a Leucine-Rich Repeat (LRR) receptor-like kinase and activates the signaling cascade [243]. Steroidal hormone brassinosteroids are present in algae, but their possible signaling pathways are yet to be investigated [141]. Similarly, the occurrence of Jasmonic acid in seaweeds is limited, and molecular data for the physiological responses support the concept that JA might perform a similar stress-relieving role in seaweeds as in vascular plants. Further investigations are required for the possible signaling pathways [141]. Strigolactones (SL) perception and downstream signaling is regulated by an F-box leucine-rich protein (MAX2/RMS4/D3) and α/β-fold hydrolases (D14 and D14-like) in plants [23, 244]. The binding sites of the SL receptor orthologs are well conserved. Wang et al. suggested that the SL signaling originated in the charophyte lineages [245]. Based on comparative genome analyses of the SL signaling components, the hormone SL was not found in green algae and was detected only in charophytes [23]. The lack of whole genome sequences for lower-order plants comprising seaweeds makes such inferences difficult [141].

There were numerous genes in the salicylic acid (SA) response pathway have been identified in higher plants, NPR1 (Nonexpresser of Pathogenesis Related Protein1) from the BTB domain family of proteins is known for its key role. It has been suggested that NPR3 and NPR4 (NPR1 related proteins) act as SA receptors, as they showed low and high affinity with SA, respectively [246]. Further investigations are required for the perception and downstream signaling of SA in algae. Polyamines also need to be investigated for their mode of action including perception and signaling. In the red algae, algal filament cells when wounded, a cascade of cellular events occurs in the adjacent cells. The repair process occurs through the somatic cell fusion and throughout this process, the repaired cells show attracted growth

towards each other. There are two different signaling molecules involved in this process in *Griffithsia monalis*. Rhodomorphin is one of them, and it is released from the rhizoidal cells and is responsible for the growth of the repair cells [163]. The proteinaceous nature of this hormone in marine red algae *Antithamnion sparsum*, evidenced by its thermal inactivation and its strong interaction with a transfer membrane has been proposed [247]. This substance has D-mannosyl residues at a sterically accessible position; which allows it to exhibit the specific binding by lectins such as ConA (Concanavalin A) and LCA (*Lens culinaris* agglutinin). Therefore, this substance is to be expected as a water-soluble glycoprotein with an a-D-mannosyl residue. It was also concluded that the labeled compound was glycoprotein and similar to the rhodomorphin found in *Griffithsia pacifica* Kylin as it has a-D-mannosyl residues [247].

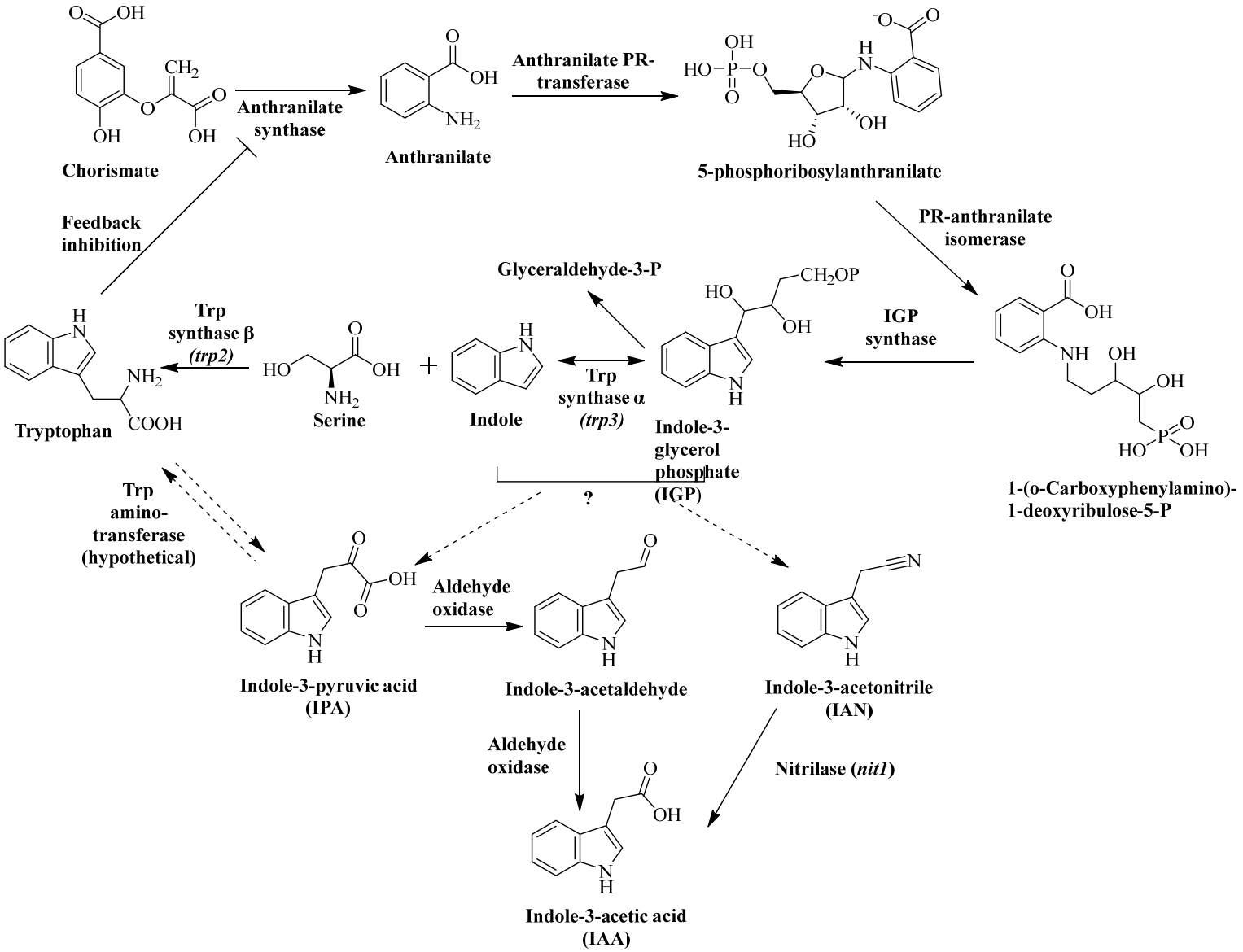
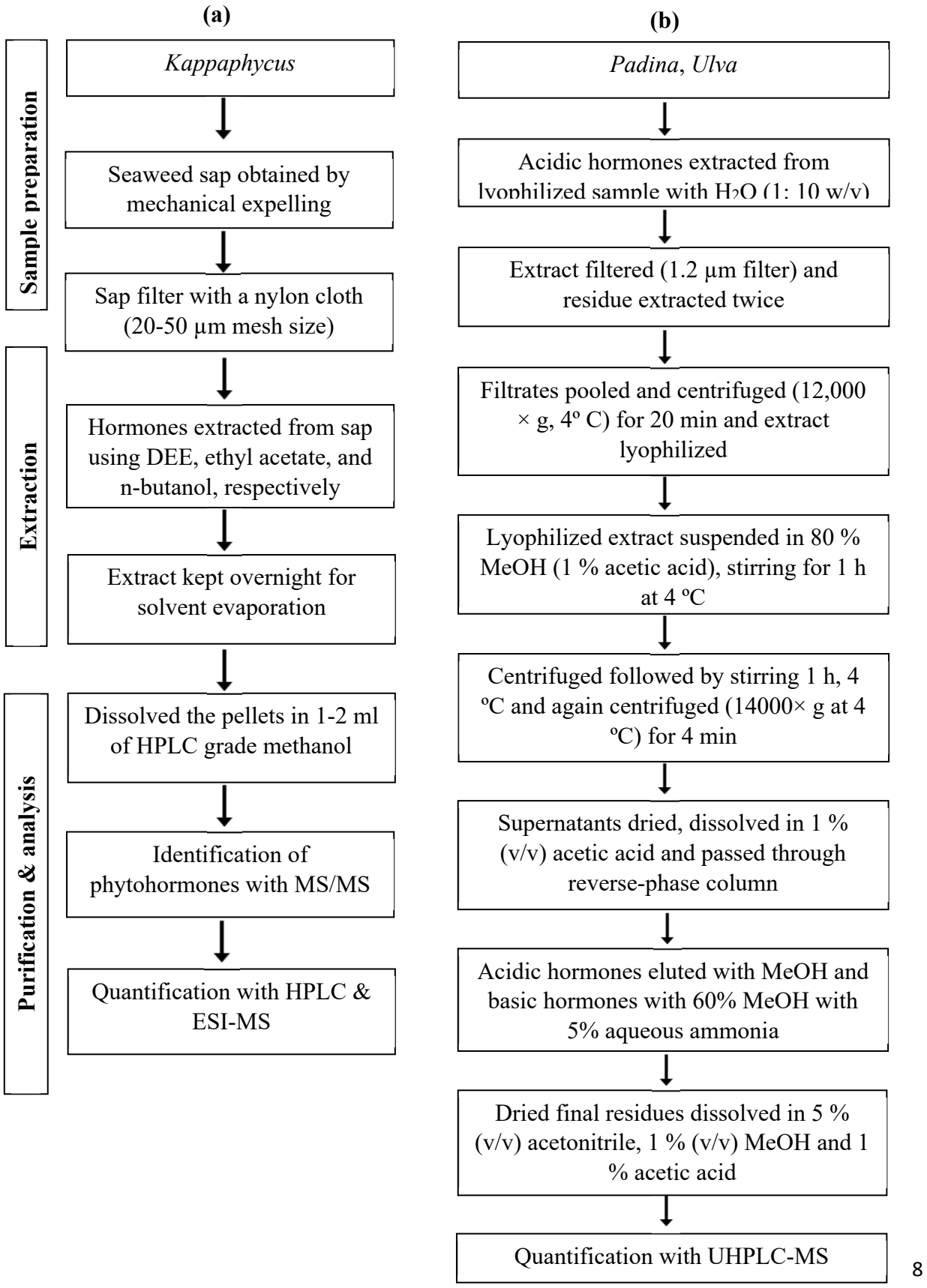


Figure S1. Tryptophan-independent pathway of auxin biosynthesis in higher plants [Modified from 78,84]. Solid arrows: genes responsible for the steps have been identified. Dashed arrows: proposed steps, but genes have not been conclusively determined.

Figure S2. Flow chart summarizing the protocols for identification and quantification of phytohormones from algae, (a) for auxins, gibberellins, and cytokinins [32], (b) for Acidic hormones like IAA, ABA, gibberellins, etc. [184], (c) for auxins, gibberellins, and cytokinins [187], (d) for gibberellins [186].



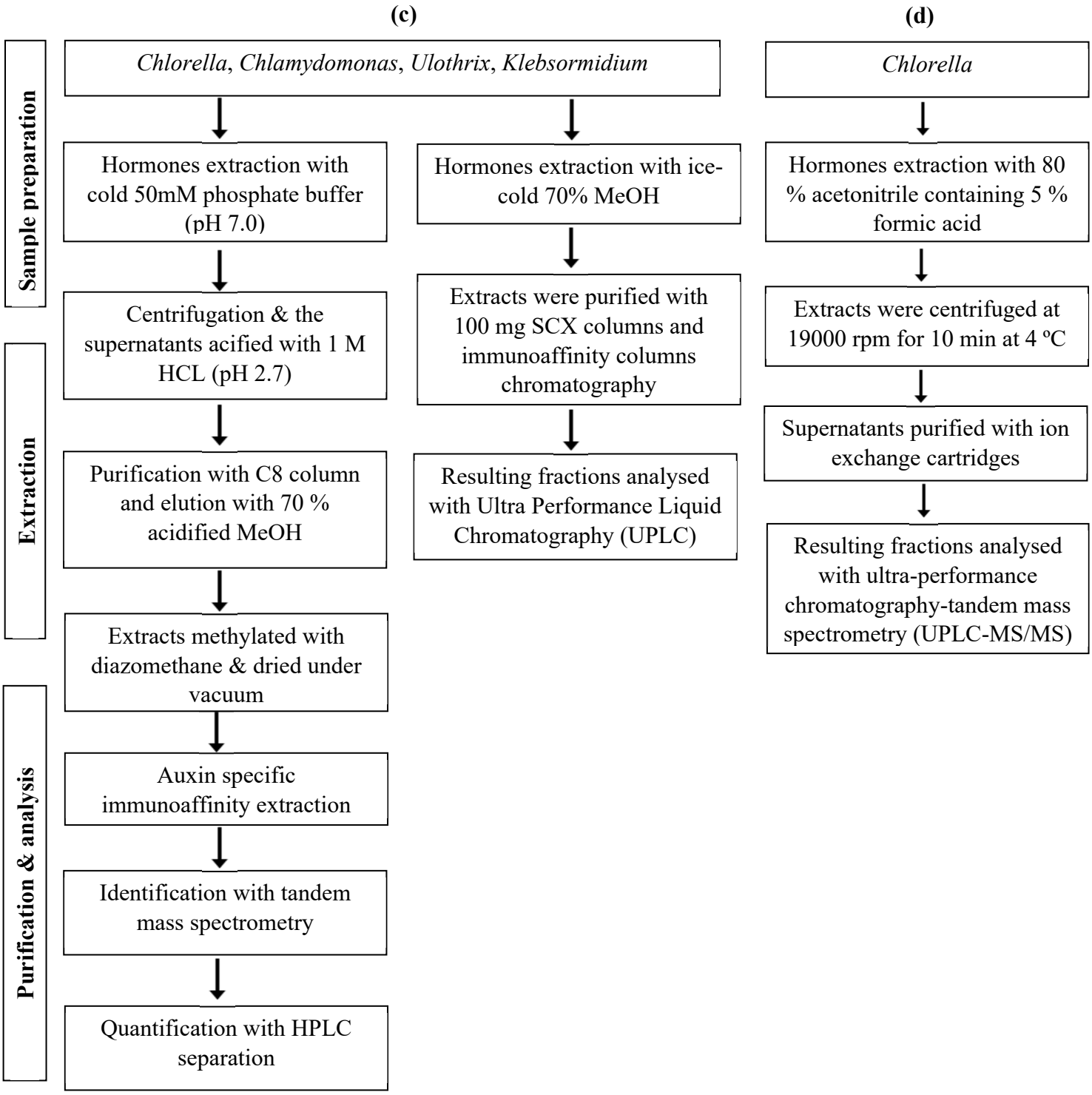
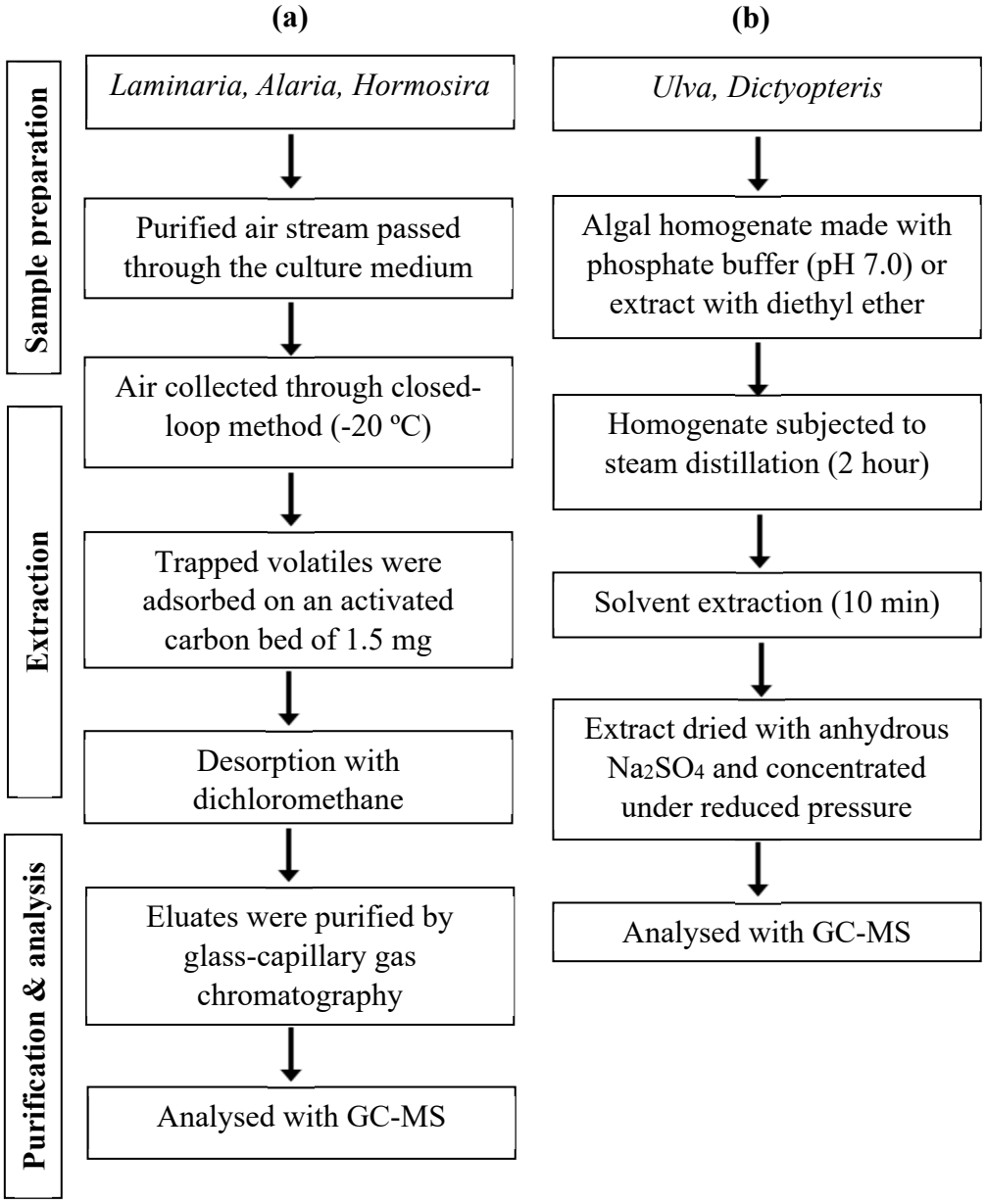
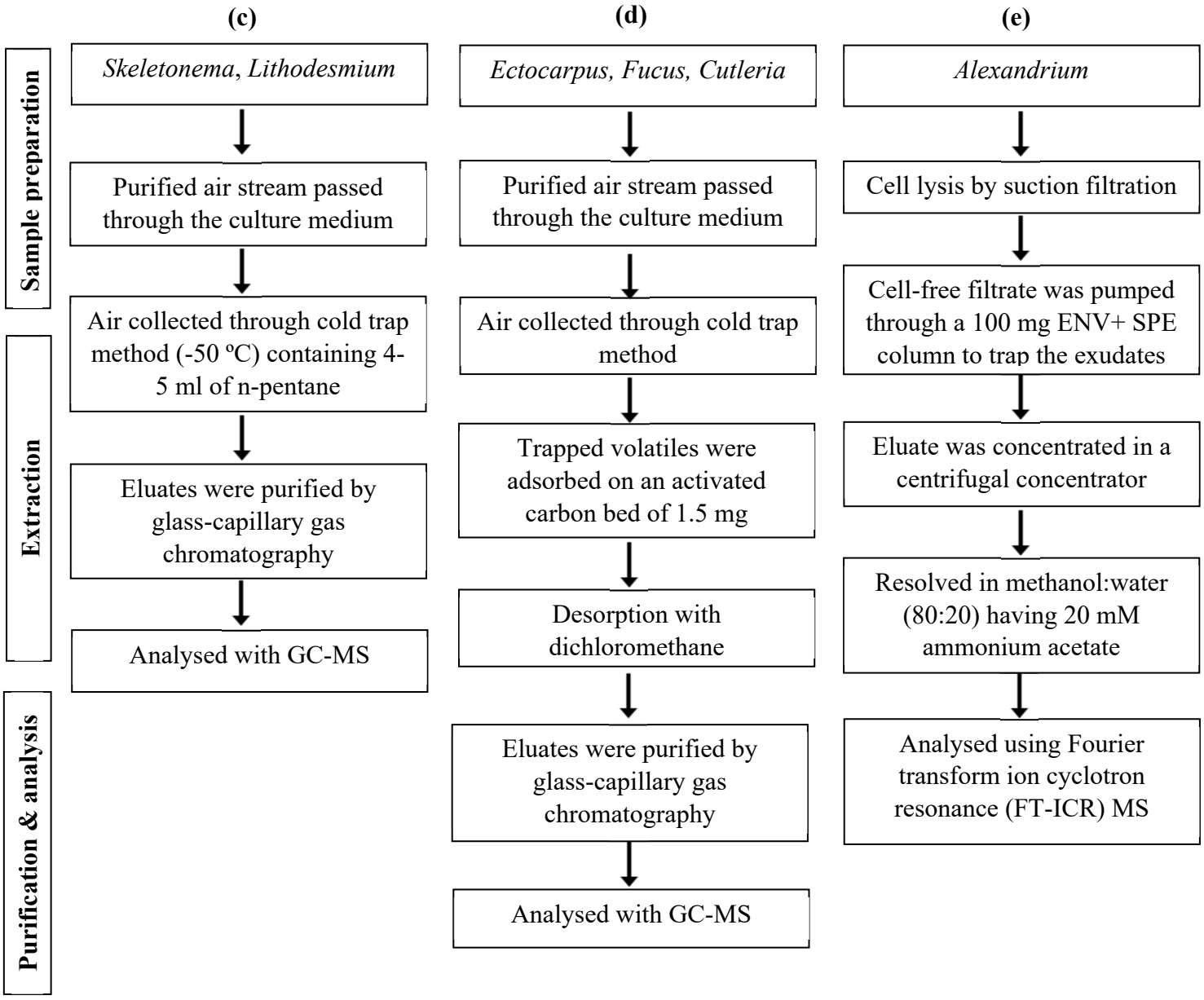

(c)
(d)
Sample preparation
Extraction
Purification & analysis
Chlorella, Chlamydomonas, Ulothrix, Klebsormidium
Chlorella
Hormones extraction with cold 50mM phosphate buffer (pH 7.0)
Centrifugation & the supernatants acified with 1 M HCL (pH 2.7)
Purification with C8 column and elution with 70 % acidified MeOH
Extracts methylated with diazomethane & dried under vacuum
Auxin specific immunoaffinity extraction
Identification with tandem mass spectrometry
Quantification with HPLC separation
Hormones extraction with ice-cold 70% MeOH
Extracts were purified with 100 mg SCX columns and immunoaffinity columns chromatography
Resulting fractions analysed with Ultra Performance Liquid Chromatography (UPLC)
Hormones extraction with 80 % acetonitrile containing 5 % formic acid
Extracts were centrifuged at 19000 rpm for 10 min at 4 ºC
Supernatants purified with ion exchange cartridges
Resulting fractions analysed with ultra-performance chromatography-tandem mass spectrometry (UPLC-MS/MS)

Figure S3. Flow chart summarizing the protocols for identification and quantification of pheromones from algae. (a) for hormosirene, lamoxirene [190,191], (b) for chemoattractants (allelochemicals), dictyopterene [192,248], (c) for Ectocarpene [194], (d) for ectocarpene, fucoserratene and multifidene [71], (e) for algal exudates (like sex pheromones, allelochemicals) [195].




(c)
(d)
(e)
Sample preparation
Extraction
Purification & analysis
Skeletonema, Lithodesmium
Purified air stream passed through the culture medium
Air collected through cold trap method (-50 ºC) containing 4-5 ml of n-pentane
Eluates were purified by glass-capillary gas chromatography
Analysed with GC-MS
Ectocarpus, Fucus, Cutleria
Purified air stream passed through the culture medium
Air collected through cold trap method
Trapped volatiles were adsorbed on an activated carbon bed of 1.5 mg
Desorption with dichloromethane
Eluates were purified by glass-capillary gas chromatography
Analysed with GC-MS
Alexandrium
Cell lysis by suction filtration
Cell-free filtrate was pumped through a 100 mg ENV+ SPE column to trap the exudates
Eluate was concentrated in a centrifugal concentrator
Resolved in methanol:water (80:20) having 20 mM ammonium acetate
Analysed using Fourier transform ion cyclotron resonance (FT-ICR) MS

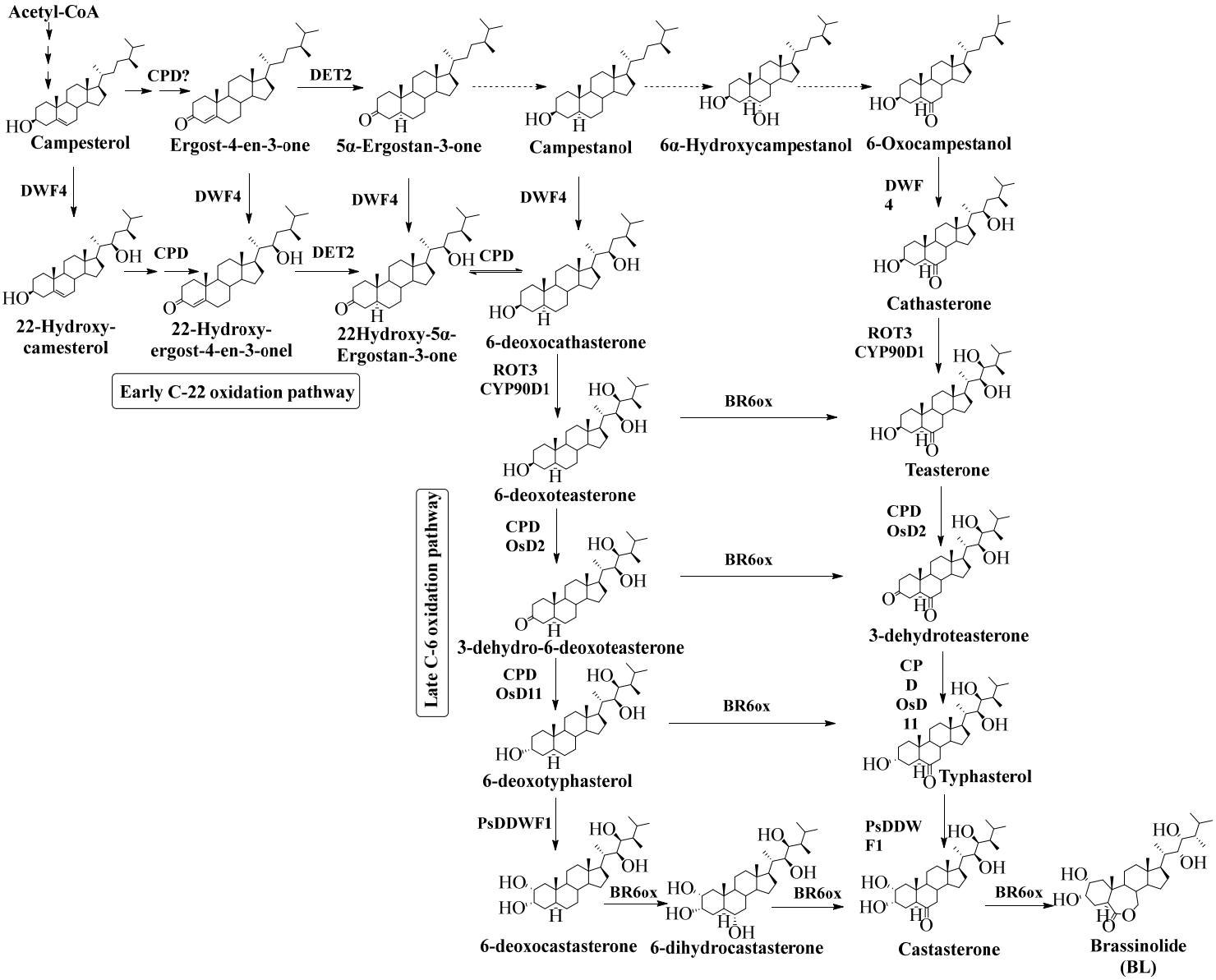


Figure S4. Biosynthesis of brassinosteroids in higher plants [Modified from 249,250]. Solid arrows: genes responsible for the steps have been identified. Dashed arrows: proposed steps, but genes have not been conclusively determined.

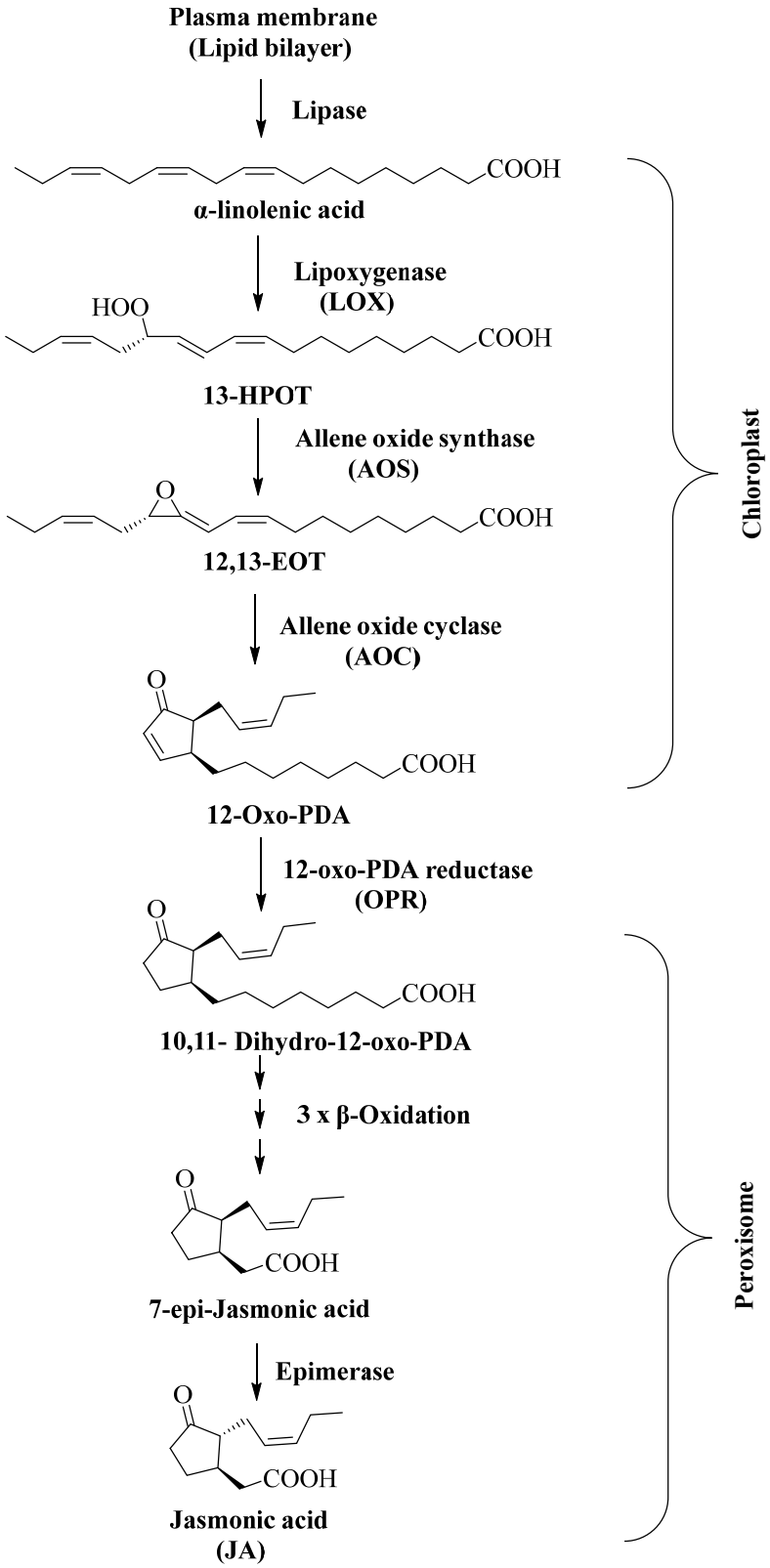


Figure S5. Biosynthesis of jasmonic acid (JA) in higher plant [Modified from 251,252] Some intermediates are indicated as 13-HPOT for 13S-hydroperoxy-9Z,11E,15Z-octadecatrienoic acid, 12,13-EOT for 12,13-epoxyoctadecatrienoic acid, 12-Oxo-PDA for 12-oxophytodienoic acid.

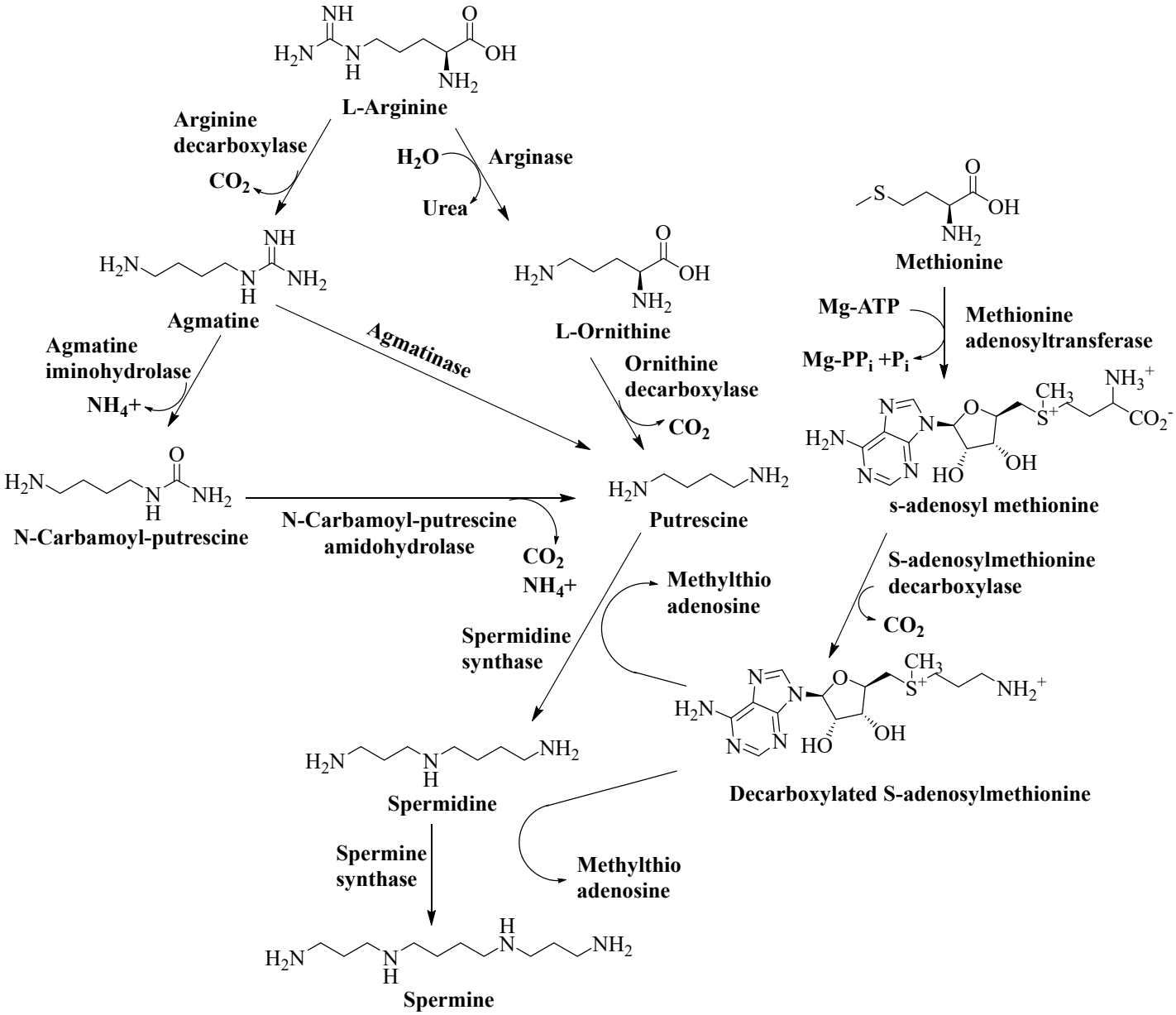


Figure S6. Biosynthesis of polyamines in higher plants [Modified from 253,254,255].

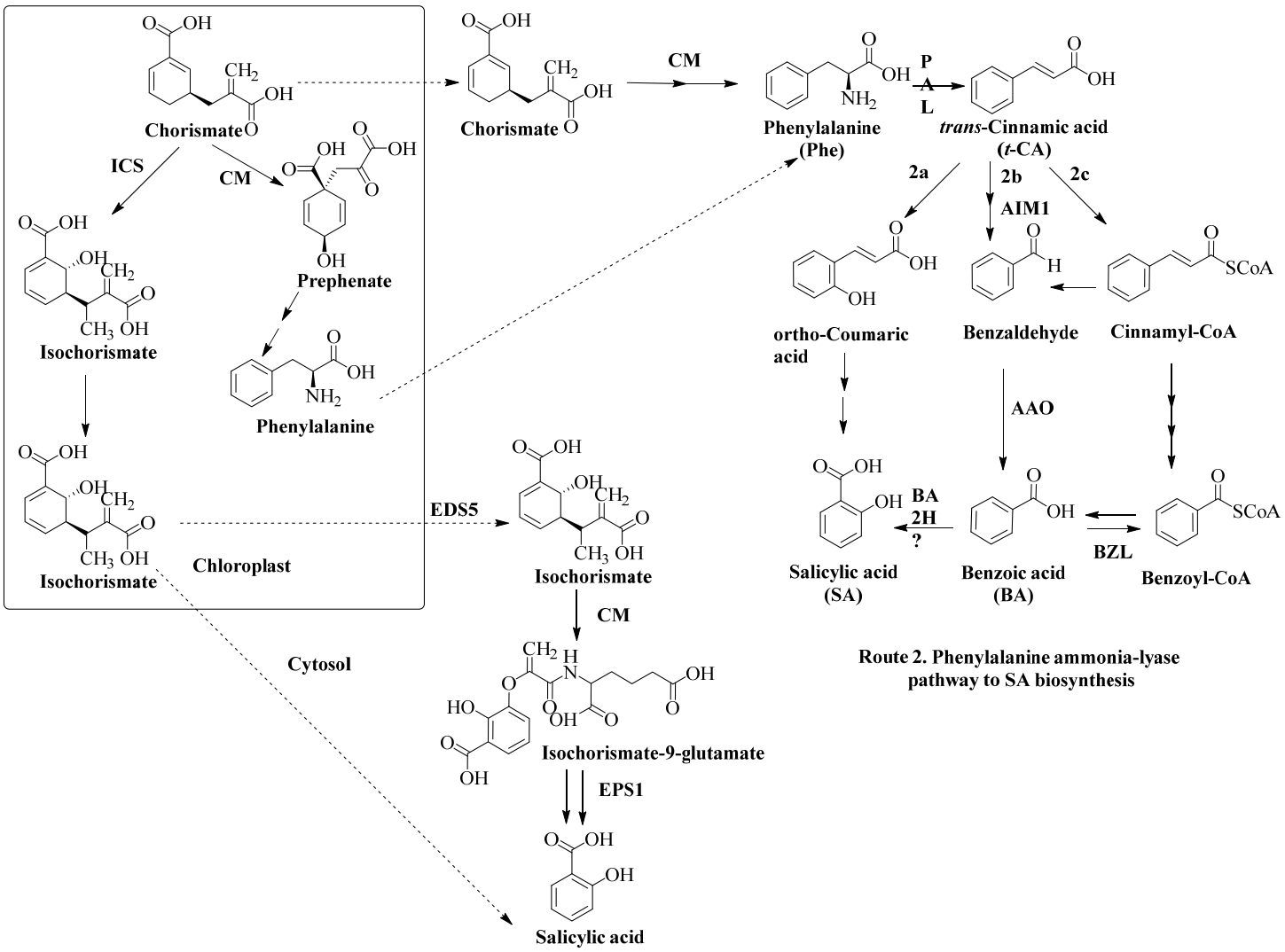


Figure S7. Biosynthesis of salicylic acid (SA) in plants [Modified from 139,256].

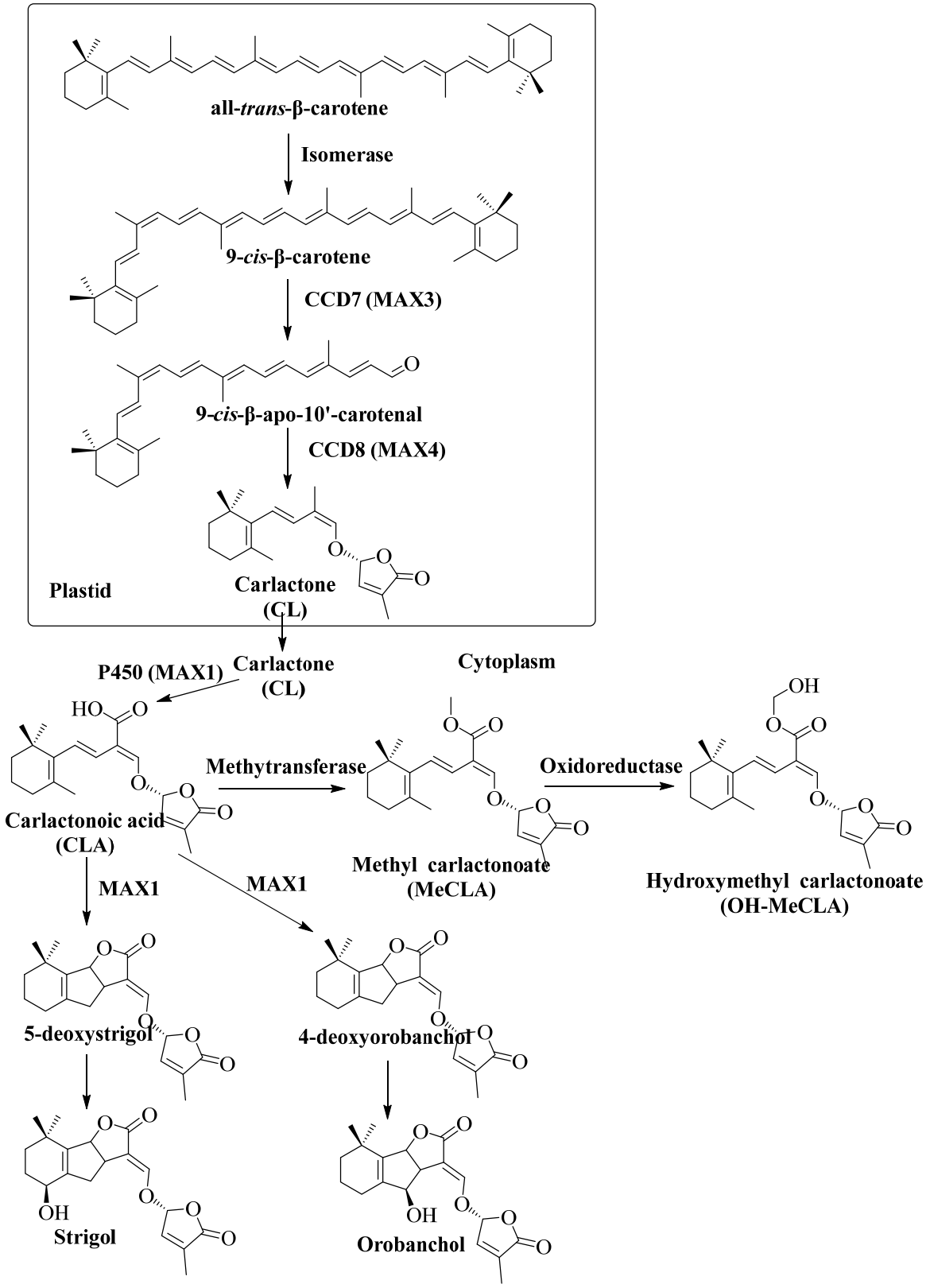


Figure S8. Biosynthesis of strigolactones (SL) in higher plants [Modified from 256,257].

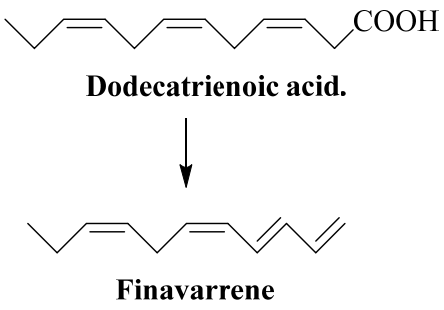


Figure S9. Biosynthesis of finavarrene from dodeca-3,6,9-trienoic acid in higher plants [105].

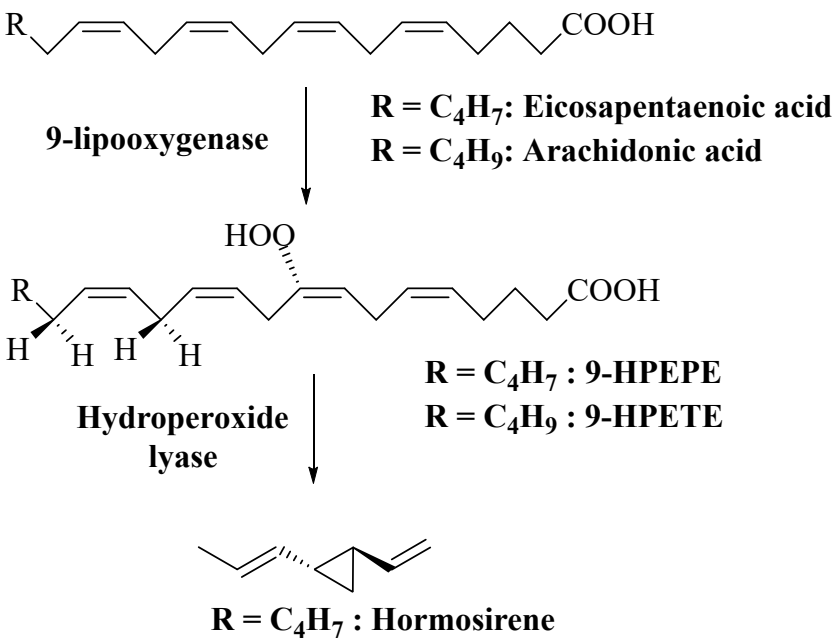


Figure S10. Biosynthesis of Hormosirene in higher plants [104]