Bioorganic & Medicinal Chemistry 23 (2015) 22–32

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

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

Synthesis, structural characterization and effect on human granulocyte intracellular cAMP levels of abscisic acid analogs

Marta Bellotti [a,b,†], Annalisa Salis [b,c,†], Alessia Grozio [a], Gianluca Damonte [a,b], Tiziana Vigliarolo [a], Andrea Galatini [d], Elena Zocchi [a,b], Umberto Benatti [a,b], Enrico Millo [a,b,*]

[a] Department of Experimental Medicine, Section of Biochemistry, University of Genoa, Viale Benedetto XV 1, 16132 Genoa, Italy
[b] Center of Excellence for Biomedical Research (CEBR), University of Genoa, Viale Benedetto XV 5, 16132 Genoa, Italy
[c] Department of Hearth Environmental and Life Science (DISTAV), University of Genoa, Corso Europa 26, 16132 Genoa, Italy
[d] Department of Chemistry and Industrial Chemistry, University of Genoa, Via Dodecaneso 31, 16146 Genoa, Italy

ARTICLE INFO

Article history:
Received 1 July 2014
Revised 11 November 2014
Accepted 21 November 2014
Available online 27 November 2014

Keywords:
Abscisic acid
Abscisic acid analogs
cAMP
Granulocytes
Inflammatory disease

ABSTRACT

The phytohormone abscisic acid (ABA), in addition to regulating physiological functions in plants, is also produced and released by several mammalian cell types, including human granulocytes, where it stimulates innate immune functions via an increase of the intracellular cAMP concentration ([cAMP]i).

We synthesized several ABA analogs and evaluated the structure–activity relationship, by the systematical modification of selected regions of these analogs. The resulting molecules were tested for their ability to inhibit the ABA-induced increase of [cAMP]i in human granulocytes. The analogs with modified configurations at C-2′ and C-3′ abrogated the ABA-induced increase of the [cAMP]i and also inhibited several pro-inflammatory effects induced by exogenous ABA on granulocytes and monocytes. Accordingly, these analogs could be suitable as novel putative anti-inflammatory compounds.

1. Introduction

The phytohormone abscisic acid (ABA) coordinates important physiological functions involved in the response to abiotic stress (temperature, light, drought), such as regulation of seed dormancy and germination, control of stomatal closure and of gene transcription. ABA synthesis is also stimulated by thermal stress in marine sponges by light in hydroids. In these lower Metazoa, ABA activates an ADP-ribosyl cyclase (ADPRC) through a protein kinase A (PKA)-stimulated phosphorylation; ADPRC activation leads to an increase of the intracellular cyclic ADP-ribose (cADPR) concentration ([cADPR]i), which in turn induces intracellular calcium release. The increase of the intracellular calcium concentration $[Ca^{2+}]i$ stimulates oxygen consumption, water filtration and protein synthesis in sponges and tissue regeneration in hydroids.[1,2]

The discovery that ABA is involved in the response of lower Metazoa to environmental stress suggested that it may function as a 'stress hormone' also in higher animals. Granulocytes are the first line of defense of mammals against pathogens and an increase of the $[Ca^{2+}]i$ via cADPR is known to stimulate several functional activities of human granulocytes.[3]

Indeed, human granulocytes exposed to physical or chemical stimuli release ABA and the hormone stimulates migration, phagocytosis, reactive oxygen species and nitric oxide production in an autocrine manner. The signaling pathway activated by ABA in granulocytes sequentially involves binding to its G-protein-coupled receptor lanthionine synthetase C-like protein 2 (LANCL-2), activation of adenylate cyclase (AC), cAMP-dependent activation of protein kinase A (PKA), phosphorylation of the cADPR CD38 and consequent cADPR overproduction, leading to an increase of the intracellular Ca^{2+} concentration.[3–6]

Monocyte/macrophages also release ABA upon stimulation with activated platelets or quartz particles and autocrine ABA activates a signaling pathway involving AC and PKA, eventually stimulating cell migration, release of prostaglandin E2 (PGE2), of the cytokines monocyte chemoattractant protein-1 (MCP-1) and tumour necrosis factor-α (TNF-α) and of metalloprotease-9 (MMP-9).[7] These inflammatory mediators are known to participate in the atherosclerotic process.[6]

Abbreviations: ABA, abscisic acid; [cAMP]i, intracellular cAMP concentration; [cADPR]i, intracellular cADPR concentration; HATU, O-(7-azabenzotriazol-1-yl)-1,1,3,3 tetramethyluroniumhexafluorophosphate; $NaBH_4$, sodiumborohydride; DIBAL, diisobutylaluminium hydride.

* Corresponding author. Tel.: +39 (0)103533032 33; fax: +39 (0)103533024.
E-mail address: enrico.millo@unige.it (E. Millo).

† These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.bmc.2014.11.035
0968-0896/

Interestingly, ABA also stimulates insulin release from human and murine β-pancreatic cells, via a PKA-dependent activation of CD38, similarly to the signaling pathway of granulocytes. The dual activity of ABA in mammals, as a pro-inflammatory cytokine and as an insulin secretagogue, suggests that ABA may play a role in the metabolic syndrome (MS), a condition characterized by chronic adipose tissue inflammation, inducing insulin resistance and hyperinsulinemia.[8] The stimulatory effect of endogenous ABA on the activation of innate immune cells identifies this hormone as a new target of anti-inflammatory drugs. The possible role of ABA in the pathogenesis of the MS is a further incentive for the identification of ABA antagonists capable of preventing the pro-inflammatory actions of the hormone.

The scientific literature regarding the synthesis and functional characterization of ABA analogs is focused on the identification of the structural requirements for ABA action in plants,[9,10] with the aim of ameliorating plant response to abiotic[11–13] and other stresses.[14,15] Structure–activity studies performed in a variety of plant tissues have identified a number of determinants of the ABA molecule that need to be conserved in analogs for biological activity.

In plants, assessing the molecular requirements of ABA action from analog-bioassay studies is controversial, as metabolic pathways may activate or deactivate synthesized compounds.[16,17] Moreover, while previous studies have investigated synthesis of ABA analogs, the data concerning determination of their interaction and affinity with the ABA receptor are conflicting.[18–23]

A structure–activity study of ABA analogs on mammalian cells has not yet been attempted. In principle, it is possible that the structural determinants of the ABA molecule required for its biological activity are different in plant and animal cells. In plants, several proteins have been demonstrated to bind ABA and transduce its signal.[24] Among these proteins, GPCR2 shares a significant sequence homology with the only mammalian ABA receptor described so far, LANCL-2.[25] This homology suggests the evolutionary conservation of a protein fold capable of binding to conserved structural determinants of the ABA molecule.

The aim of this study was to design and synthesize ABA analogs and to test their biological activity as ABA agonists or antagonists on the [cAMP]i in human granulocytes. This screening assay was chosen because activation by ABA of AC, followed by cAMP-dependent activation of PKA and phosphorylation of CD38 appear to be the common feature of the ABA-signaling pathway in both innate immune cells and β-pancreatic cells.[3–6] Different regions of the ABA backbone were systematically modified. In particular, alterations were made to the pentadienoic side-chain (functionality at C-1), to the configuration at the C-2′–C-3′ bond, to the carbonyl group at C-4′ and to the cyclohexenic ring saturation. As a functional screening assay, all compounds were tested for their ability to affect the ABA-induced increase of the intracellular cAMP concentration ([cAMP]i).

2. Results and discussion

2.1. Synthesis

To identify possible ABA antagonists and to assess a possible structure–activity relationship, ABA analogs were synthesized by systematic modification of each of the three regions of the ABA molecule indicated in Figure 1. The ABA molecule contains a chiral center (C1′) and the racemic mixture was used as a starting material for analog synthesis, yielding the enantiomers (1′*S*)(+)ABA and (1′*R*)(−)ABA. First, the modifications were made in one region at a time, to determine the contribution of a particular structural attribute to bioactivity. Thus, the acidic functionality at C-1 was

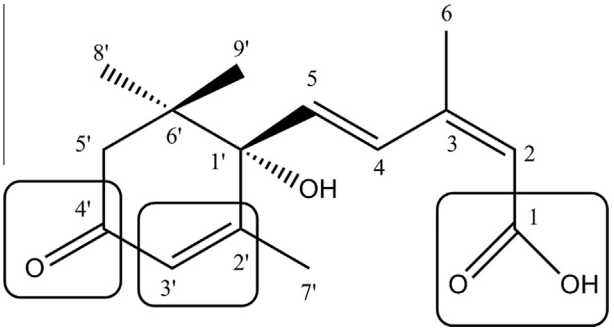

Figure 1. Structural formula of ABA, with the conventional carbon numbering. The regions of the molecule that were modified in the various analogs are boxed. Alterations were made to the functional group at C-1, to the configuration at the C-2′–C-3′ bond, to the carbonyl group at C-4′ and to the cyclohexenic ring saturation. Hydrogen atoms are omitted for clarity.

replaced with an ester, an amide or with carbon chains of different length, the double bond between C-2′ and C-3′ was replaced by an epoxidic bridge, the carbonyl group at C-4′ was reduced or substituted with an amino-group, and finally the cyclohexenic group was substituted with an aromatic ring (Fig. 2). To define the relative contribution of each structural modification to the ABA-agonist or -antagonist action of the compound, some ABA derivatives with simultaneous modifications on different parts of the molecule were also studied (Fig. 2). Another structural modification considered outside of this scheme was a two-carbon truncation of the normal 2-*cis*–4-*trans* pentadienoic side-chain, to yield the α- and β-ionone derivatives.

In the majority of the compounds, the following features were left unmodified: the C1′ hydroxyl group, the 7′ methyl group, the 2-*Z*-enoic acid on the side chain and the methyl group at position 3 (C6). Preliminary experiments suggested that modifications at these positions impaired the ability of the analogs to displace ABA from its binding to granulocyte membranes (not shown).

Some analogs were synthesized using methods reported by others or adapted from published protocols,[26–35] while others (analogs **1**, **2**, **3**, **4**, **6**, **7b**, **9**, **14b**, **17**) were synthesized for the first time in this study.

2.2. Effect of ABA analogs on the [cAMP]i in human granulocytes

In principle, the fact that chemical synthesis of the various ABA analogs yielded two enantiomers might complicate the evaluation of their biological activity, without prior separation.

However, in plants the (1′*S*) and the (1R′) enantiomers show similar biological activity in different assay systems,[36–38] indicating that in plants the synthetic isomer (1R′) can mimic at least some of the effects of the natural one (1′*S*). In addition, in mammalian cells, the functional effects elicited by ABA on different cell types were observed with either one of the enantiomers at the same concentration.[3] In our work, the ABA-triggered increase of the [cAMP]i in human granulocytes was similar with both ABA enantiomers. In line with previous results, the [cAMP]i increased by 35 ± 5% (n = 24) in human granulocytes incubated for 1 min at 25 °C in the presence of 1 μM (±)-ABA, as compared to untreated cells. The 20 synthetic ABA analogs shown in Figure 2 were tested on human granulocytes for their ability to induce an increase of the [cAMP]i, or to inhibit the ABA-triggered increase of the [cAMP]i. In order to favor the possible competition with ABA of analogs behaving as ABA-antagonists, each analog was tested at a concentration of 50 μM, 10 μM and 1 μM. Results are shown in Figure 3. Most

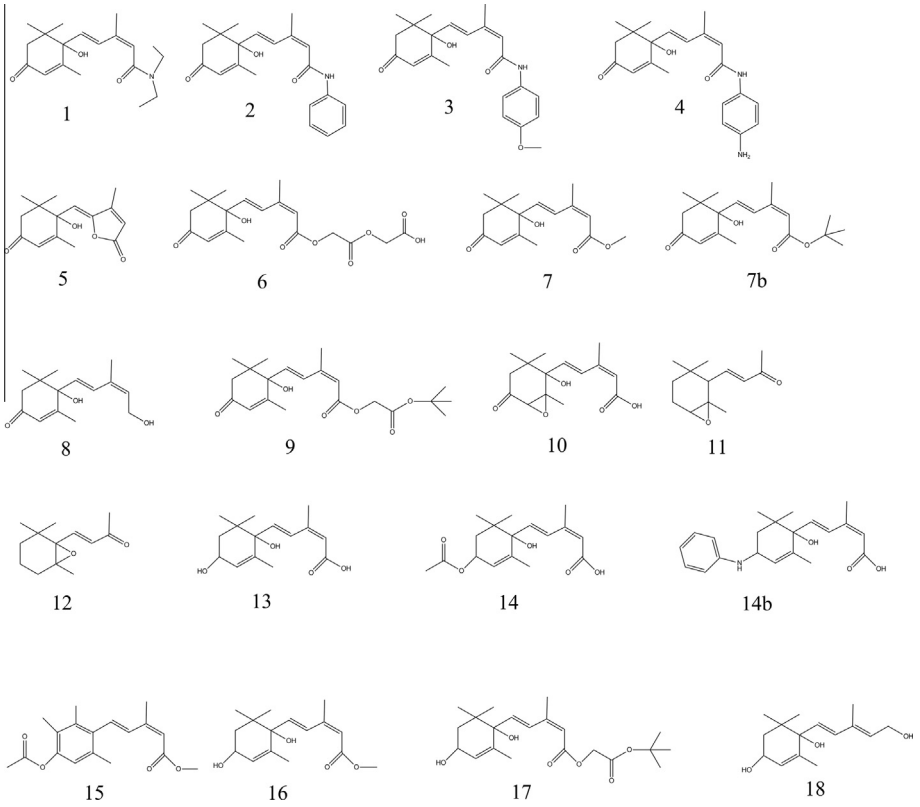

Figure 2. Structures of the ABA analogs tested in this study. Analogs were prepared by total synthesis, starting from the racemic mixture of (±)-ABA. Thus, all compounds were racemic mixtures.

analogs, when tested alone, behaved as ABA-agonists, inducing an increase of the [cAMP]i which was similar or even higher (e.g., analogs **1**, **2**, **3** and **9**) than that elicited by ABA, at all three concentrations tested. However, analogs **10** and **11** reduced or prevented the ABA-triggered increase of the [cAMP]i, behaving as ABA-antagonists.

2.3. SAR of ABA derivatives

2.3.1. Modifications of functionality at C-1

To evaluate the relevance of the carboxylic group of ABA to its biological function, we synthesized a panel of different analogs where the carboxylic group was modified. Most of the molecules synthesized and tested were modified only in the pentadienoic side-chain, without varying the cyclohexenoic ring.

In a first series of analogs, the carboxyl group was changed into an amide (Fig. 2, analogs **1**, **2**, **3**, **4**), introducing aliphatic or aromatic amino groups. The amide modification of the C1 carboxylic group of ABA has apparently never been tested on plant cells, unlike C1 derivatives with ester, alcohol or aldehydic groups.[18,19] These amide derivatives were synthesized with a simple reaction between the carboxylic group, activated with an uronium salt, and the amino group of the various amines. Collectively, the ABA-amide analogs behaved as ABA agonists (Fig. 3A), except for analog **4**, which did not affect the [cAMP]i per se, nor did it antagonize the increase of the [cAMP]i induced by ABA. In fact, the addition of ABA to the analog **4** at the concentration of 50 μM, 10 μM and 1 μM in the reaction mixture is able to restore the original effect of ABA (data not shown).

Moreover, our attention was focused on compounds **1**, **2** and **3**, which showed the strongest agonist activity at the concentration of 50 μM. For these molecules, the same effect was confirmed at 10 μM (data not shown) and at 1 μM (Fig. 3C), when tested in the absence of ABA. Interestingly, when compounds **1**, **2** and **3** were tested at the concentration of 1 μM in the presence of an equal concentration of ABA, the effect of compounds **2** and **3** on the [cAMP]i was not additive with that of ABA alone, while compound **1** exhibited a synergistic activity (Fig. 3C). This suggests that compounds **2** and **3** may act on the same receptor as ABA, while compound **1** may interact with a different target.

The methyl ester of ABA (analog **7**, Fig. 2) was obtained in near quantitative yield by using the direct reaction of ABA with dimethyl sulfate.[28] Analog **7** per se induced an increase of the [cAMP]i of granulocytes and appeared to have a slightly additive effect if administered together with ABA (Fig. 3A).

The *tert*-butyl ester (analog **7b**) obtained by using *tert*-butylacetate and $HClO_4$ per se induced an increase of the [cAMP]i similarly to methyl ester but without an additive effect if administered together with ABA (Fig. 3A). Also analogs **7** and **7b** were tested at the concentration of 10 μM and 1 μM and, at this concentration they lost the slight agonistic activity shown at 50 μM (data not shown).

Esters in general and methyl esters in particular may undergo hydrolysis by cellular esterases to produce the free acid. However, the very brief incubation time of granulocytes with the analog (1 min), and the apparently additive effect of analog **7** and ABA, make this possibility unlikely.

To obtain an alcoholic group at the C-1 position, the unstable acid chloride of ABA was reduced with $NaBH_4$ at low temperature.[28] This alcohol (analog **8**) induced a [cAMP]i increase similar to that observed with ABA (Fig. 3A) and for this reason no further experiments were performed at lower concentrations.

In plants, abscisyl alcohol can be converted to ABA;[39] it is possible that the same oxidation takes place also in granulocytes, although the brief incubation time of the cAMP assay (1 min) argues against a significant analog transformation.

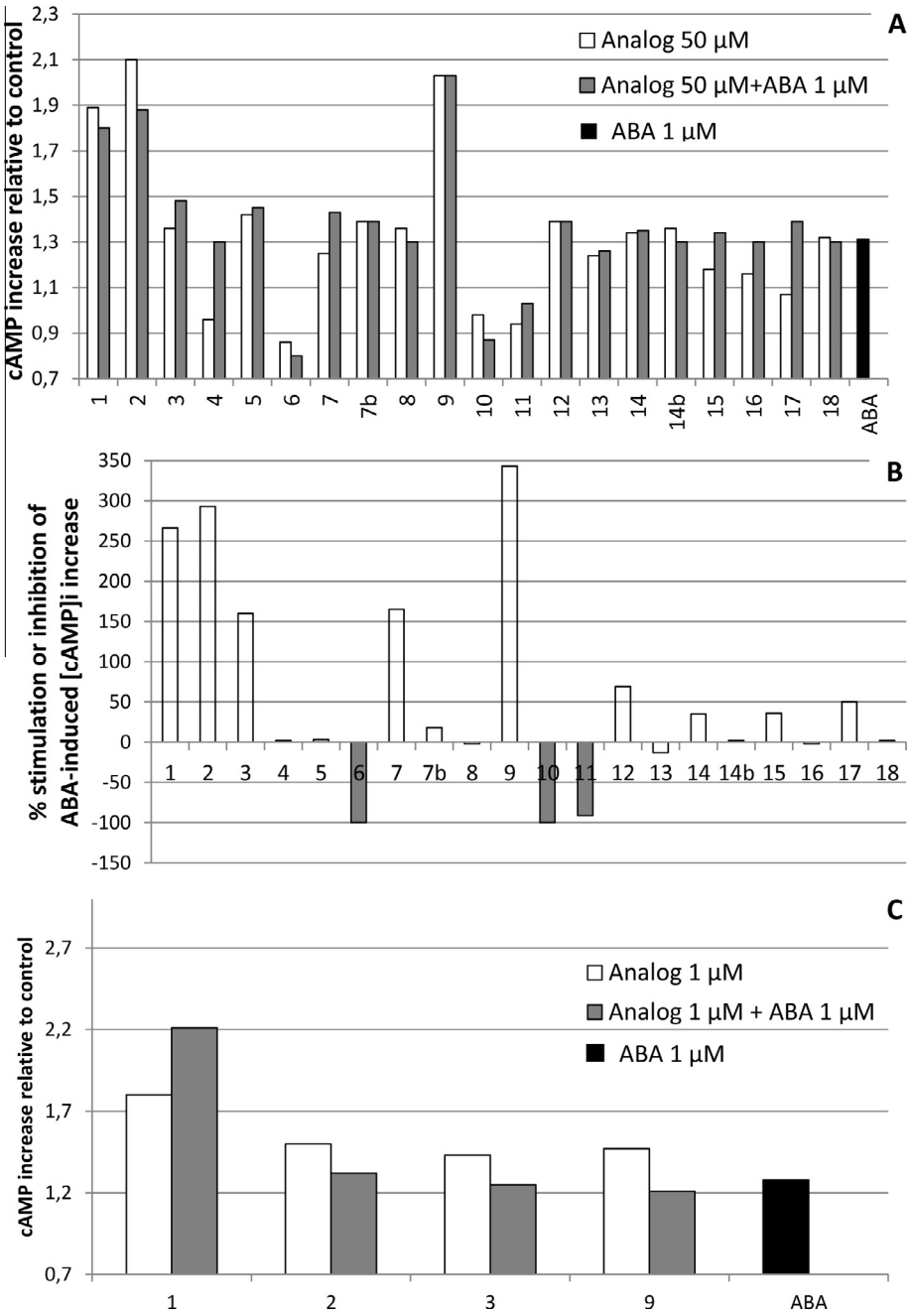

Figure 3. Screening of synthetic ABA analogs for their ability to inhibit the ABA-induced increase of the [cAMP]i in human granulocytes at 50 µM and 1 µM. Granulocytes were incubated for 1 min without (control) or with 1 µM ABA. 50 µM (panel A) or 1 µM (panel C) of the indicated analog was tested, in the presence or in the absence of 1 µM ABA. In panels A and C results are expressed as [cAMP]i increase relative to control, untreated cells are mean from triplicate incubations (SD <5% of the mean for all values). The black bar indicates the effect of ABA alone. Panel B. Results are expressed as percentage stimulation or inhibition of the [cAMP]i increase induced by ABA in the presence of the indicated analogs at 50 µM, relative to that induced by ABA alone. Analogs behaving as ABA-antagonists are highlighted in grey.

We also tested a derivative in which the carboxylic group was substituted with an aldehyde; its effect on the [cAMP]i was similar to that of ABA (data not shown).

To investigate whether a modification of the pentadienoic chain could confer ABA-antagonistic activity, two derivatives were synthesized (analogs **6** and **9**) in which this side chain was modified by lengthening of the backbone. In particular, a linker was introduced, containing carbon and oxygen atoms and ending with a carboxylic group. Analog **6**, which has a free carboxylic group, was obtained by reaction of ABA with bromoacetic acid. In the case of derivative **9**, which has a protected carboxylic group, a simple reaction with *tert*-butylbromoacetate in an anhydrous organic solvent allowed to obtain the desired compound with good yield.[29] Interestingly, analog **6** at 50 µM did not increase the [cAMP]i in granulocytes and it abrogated the [cAMP]i rise induced by 1 µM ABA (Fig. 3A); conversely, analog **9** per se induced a [cAMP]i increase higher than that elicited by ABA alone also at 10 µM (data not shown) and 1 µM (Fig. 3C).

Besides, when analog **9** was tested at the concentration of 1 µM in the presence of an equal concentration of ABA, the increase of the [cAMP]i was comparable to that observed with ABA alone (Fig. 3C), a behavior similar to that described above for analogs **2** and **3**.

The antagonistic activity of analog **6** on the [cAMP]i rise induced by 1 μM ABA was negligible at 10 μM analog and absent at 1 μM (data not shown).

Elongation of the ABA side-chain diminished the ability of the analog to increase the [cAMP]i, but only in the presence of a free acid in the same chain. Thus, it seems likely that one of the requirements for activity is the presence of a double bond conjugated to the carboxylic group. In analog **9**, the side chain is longer but the presence of *tert*-butyl group increases the hydrophobicity of the molecule. This feature seems to promote a significant [cAMP]i increase (Fig. 3A). This result also highlights the importance of the orientation of the ABA carboxyl group.

In analog **5**, the pentadienoic chain was substituted with a five ring cycle (Fig. 2). This analog does not have the –OH group in C1′ that is reported to be critical for biological activity in plants,[18,19] but contains a carbonyl group in C4′. This compound, derived from the reaction of ABA with pyrophosphoric acid,[34] had a similar effect on granulocyte [cAMP]i as ABA at all concentrations tested, even though the side chain is devoid of the carboxyl group and has a fairly hydrophobic lactone cycle.

2.3.2. Effect of the modification of the configuration at C-2′ and C-3′

With this group of analogs, we tried to determine how the degree of ABA ring unsaturation affects the biological activity of the hormone.

Analog **10** contains a 2′–3′epoxy bridge, which eliminates the unsaturation in the ring. Epoxidation of (±)-ABA with H_2O_2 in an alkaline solution produced a racemic mixture of one diastereoisomer, because addition of the hydrogen peroxide occurs only at the less hindered α-face, opposite to the side chain.[30] Analog **10** completely inhibited the ABA-induced [cAMP]i rise (Fig. 3A), in line with previously published results.[40] Analog **10** has been already shown to compete with ABA for binding to recombinant LANCL2, with an IC_{50} value of 6.56 nM, and to inhibit ABA-induced chemotaxis, phagocytosis, ROS and PGE2 production in human granulocytes.[40]

Given the unusual behavior of analog **10**, which appears to antagonize the pro-inflammatory effects of ABA on granulocytes, several other derivatives containing an epoxy bridge were synthesized and tested on granulocyte cAMP. Analogs **11** and **12** (Fig. 1) were obtained by the direct oxidation of alpha- and beta-ionone, respectively, with *m*-chloroperoxybenzoic acid,[35] and showed two different actions. Analog **11**, where the epoxy group is in the same position as in analog **10** (C2′–C3′), behaved as an ABA-antagonist at 1 μM too, inhibiting the ABA-induced [cAMP]i increase in granulocytes similarly to analog **10** (data not shown); conversely, analog **12**, in which the epoxy group is in a different position of the ring (C1′–C2′), did not antagonize the effect of ABA, and rather induced a [cAMP]i increase of its own at 50 μM (Fig. 3A). This activity was completely lost at 10 μM and 1 μM (not shown). The antagonistic activity of analog **11** on the [cAMP]i rise induced by 1 μM ABA was negligible at 10nM analog (not shown), unlike that of analog **10**, which had an EC_{50} of 0.7 ± 0.2 nM.[40] However, analog **11** lacks the keto group in C4′ and the pentadienoic side chain with the carboxylic group, which are present in analog **10**. In fact, the 1-carboxyl group seems to have a specific electronic interaction with the receptor and shortening of this side-chain of ABA seriously diminishes activity.

These results suggest that the ABA-antagonistic activity of analog **10** is due not only to the presence of the epoxy group in the ring, but also to presence of the C4′ keto group and of the pentadienoic side chain with a free carboxyl group. The antagonistic effect of analogs **10** and **11** may be due to steric reasons as the epoxy ring in both cases retains the methyl group at C2′, coplanar with the ring, but at the same time apparently creates a new point of interaction with the receptor.

2.3.3. Effect of the modification of the 4′-carbonyl oxygen

We previously reported that ABA biotinylated at the C4′ keto group binds to granulocyte membranes and that this effect is antagonized by excess of unmodified ABA.[3]

The 4′-carbonyl oxygen on the ABA ring was selectively reduced to a hydroxyl group with $NaBH_4$, generating the 1′,4′ diol (analog **13**). The final product was a racemic mixture of the *trans*- and of the *cis*-form derivatives.[32] The racemic mixture and the purified *cis* and *trans* isomers of 1′,4′-diol ABA all showed a similar effect on the granulocyte [cAMP]i as ABA (Fig. 3A).

The same results were obtained at 10 μM and 1 μM (not shown). This result was surprising, given that the keto-to-hydroxyl reduction and the consequent absence of the double bond significantly modify the conformation of the ABA cyclohexene ring. The similar effect of ABA and of its 1′,4′-diol-derivative on granulocyte [cAMP]i levels suggested that the C4′ carbonyl group is not essential for ABA activity. To further investigate this hypothesis, the *cis* and *trans* 1′,4′-diols were selectively acetylated with acetic anhydride in pyridine, obtaining the corresponding 4′-O-acetates (analog **14**).[26] The racemic mixture and the purified *cis*- and *trans*-isomers of the 4′-acetate analog all showed, as expected, a similar ABA-like activity on granulocyte [cAMP]i (Fig. 3A).

Other derivatives containing different groups at the C4′ position (e.g., ketimine or secondary amine groups) were also synthesized and tested, but none behaved as an ABA-antagonist or agonist on granulocyte [cAMP]i (data not shown). As an example, the compound obtained by the reaction of ABA and aniline followed by reductive amination with sodium cyanoborohydride[27] (analog **14b**) showed an ABA-like activity on granulocyte [cAMP]i (Fig. 3A).

Together, these results suggest that the 4′-carbonyl group of ABA is not fundamental for its effect on the [cAMP]i.

2.3.4. Effect of the modification of the ring saturation

Several ABA analogs containing a cyclohexane ring have been tested on plants, but none containing an aromatic ring.[18–22] In analog **15** (Fig. 2), the cyclohexene is substituted by a benzene moiety. Treatment of ABA methyl ester with acetic anhydride and toluene-*p*-sulfonic acid led to the simultaneous aromatization of the original cyclohexene ring and to the introduction of an acetate group at position 4′ with a previously described mechanism.[31] This ABA analog retains the 7′- and 8′-methyl groups, but lacks the 1′-hydroxyl and the 9′-methyl group of ABA that are believed to play a significant role in ABA activity in plants.[18–22] This derivative behaved as a very weak agonist on granulocyte [cAMP]i and did not alter the effect of ABA (Fig. 3A). For this reason no further experiments were performed with analog **15** at lower concentrations.

Thus, presence of the aromatic ring apparently prevents analog **15** from competing with ABA in human granulocytes: in fact, the geometry of the ring is profoundly modified, possibly preventing binding of this analog to the receptor.

2.3.5. Effect of the concomitant modification of different positions in the ABA structure

So far, we examined the activity of many compounds, with the exclusion of analog **15**, that harbored a single variation of the ABA molecule.

Next, we synthesized several analogs containing modifications in different positions of the ABA molecule. Firstly, functionalities at C1 and C4′ were concomitantly modified: in analogs **16** and **17** (Fig. 2) the ketone group at C4′ was substituted with a hydroxyl group and the carboxylic function at C1 was substituted with an ester. Neither compound had any effect on the [cAMP]i nor did it show any inhibitory activity on the ABA-induced [cAMP]i increase in granulocytes (Fig. 3A). Analog **18** (Fig. 2), obtained by the selective reduction of the ABA methyl ester in C1 and of the carbonyl group in C4′ with diisobutylaluminium hydride,[28] showed an

ABA-like activity on granulocyte [cAMP]i, similar to that of analog **7** (ABA methyl ester), that is perhaps explainable with the oxidation to acid or with the presence of the electrostatic characteristic of the alcohol. These slight activities were not confirmed using analogs **16**, **17**, **18** at 10 μM or 1 μM (data not shown).

As the presence of the epoxy group confers an antagonistic activity to analogs **10** and **11**, other derivatives containing this modification were synthesized. Due to the reactivity of the epoxy group, the only part of the ABA molecule that could be concomitantly modified, without altering the epoxy functionality, was the carboxyl group in C1.[41] We tested several derivatives containing the epoxy group in C2′ and C3′ as well as ester substitutions of the carboxyl group. Almost all derivatives tested indeed behaved as ABA antagonists on granulocyte [cAMP]i, although when tested at concentrations lower than 50 μM, they were all less active than analog **10** (data not shown). These results, however, confirm that the epoxy modification in C2′–C3′ is critical for ABA-antagonism.

3. Conclusion

We described the synthesis, structural characterization and biological activity 'in vitro' of a series of derivatives of ABA, in an attempt to identify the molecular determinants associated with ABA-agonistic or antagonistic effects on human granulocytes. Thus, the compound library was tested on human granulocytes, to find ABA analogs capable of inhibiting the [cAMP]i rise induced by ABA. The systematic modification of the various parts of the molecule aimed to clarify which functional groups of ABA are important in determining its biological effect.

Specifically, results obtained by modifying the ABA structure only at the C4′ suggest that presence of the carbonyl group is not essential for ABA-like activity on granulocyte [cAMP]i.

All the analogs in which the carbonyl group is present and conjugated with a 2′ 3′ double bond (analog **1**, **2**, **3**, **5**, **7**, **7b**, **8**, **9**) show ABA-like activity at 50 μM. This action was confirmed only for analog **1**, **2**, **3**, **9** also at 10 μM (not shown) and 1 μM (Fig. 3C). Four derivatives do not contain this keto group (**13**, **14**, **14b**, **18**) but have, as a common feature, a polar chain containing carboxylic acid or alcohol group that allow an increase of the [cAMP]i even if this activity is evident only at 50 μM.

Analog **14**, in which this carbonyl is substituted with an acetyl group, retains the ability to stimulate the production of [cAMP]i, as the acetyl group allows conjugation with the cyclohexenic double bond.

Particularly interesting is the presence of the alcohol group in position 4′. In fact, alcohol group in position 4′ differently affects the [cAMP]i, depending on the chemical–physical characteristics of the group in C1. Presence of a hydrophobic group in C1 (such as the methyl and *tert*butyl esters in analogs **16** and **17**, respectively) abrogates stimulation of [cAMP]i production, while the increase of the [cAMP]i is maintained if C1 is polar, carboxylic or alcoholic (analogs **13** and **18**) only at high analog concentration.

If the carbonyl group in 4′ is preserved, the side chain determines the activity of the analogs tested. Elongation of the side chain with hydrophobic esters or amides ensures an ABA-like activity, as demonstrated in particular for analogs **1**, **2**, **3** and **9**.

On the contrary, the presence of a free carboxylic group (analog **6**) or the introduction of the polar amino group as substituent in the amide (analog **4**) abrogate the effect on the [cAMP]i albeit only at 50 μM.

Among the analogs tested, only those where the configuration at C-2′ and C-3′ was modified by introduction of an epoxidic ring abrogated the [cAMP]i increase induced by ABA on granulocytes.

The introduction of an epoxidic group in the position C-2′ and C-3′ conferred the ABA-antagonistic effect regardless of the type and length of the side chain and of the presence of a keto group in 4′. For this reason, analog **11**, where the epoxy group is in the same position as in analog **10** (2′–3′), also behaved similarly as an ABA-antagonist, although it lacked the keto group and had a shortened side-chain. On the contrary, analog **12**, in which the epoxy group is in a different position of the ring, between C1′and C2′, but which shares a ionone-like backbone with analog **11**, does not antagonize the effect of ABA, instead inducing itself a [cAMP]i increase. At 1 μM, the antagonistic activity of analog **11** on the [cAMP]i rise induced by ABA was lower than that of analog **10** (data not shown). Thus, it appears that the ABA-antagonistic effect is more pronounced if the pentadienoic chain and the C-4′ketone group are left unchanged.

These results are in agreement with a recent paper where substitution in the 3′ position of abscisic acid's ring produced a potent ABA antagonist that was sufficiently active to block stress-induced ABA responses in vivo on plants.[42]

Once the tridimensional structure of ABA receptor(s) in mammals is defined, it will be possible to verify the results and consequent predictions reported above and to probe the binding pockets with structurally defined compounds and hence design new derivatives to fit different binding sites. Given the present state of knowledge, we propose that these analogs, in particular **10** and **11** could be useful for biochemical studies aimed at identifying novel putative compounds for the treatment of inflammatory diseases.

4. Experimental

4.1. General

The analysis of the intermediates and the raw products was performed by liquid chromatography-electrospray mass spectrometry (HPLC–ESI–MS) using an Agilent 1100 series LC/MSD ion trap instrument. The products were then purified by preparative reverse phase high performance liquid chromatography (RP-HPLC) on a Shimadzu LC-9A preparative HPLC equipped with a Phenomenex C18 Luna column (21.20 × 250 mm). The separation was performed in gradient starting with 15% solvent B for 5 min, linearly increasing to 70% solvent B in 30 min and up to 100% B in 10 min. The solvent used was 0.1% trifluoroacetic acid (TFA) in water (A) and 0.1% TFA in acetonitrile (B).

The molecular weight of the products were finally confirmed by electrospray mass spectrometry using an ion trap analyzer that allowed to perform MS and MS^2 analysis.

^{1}H NMR and ^{13}C NMR spectra were recorded on a Varian Mercury Plus at 300 MHz and 75 MHz, respectively. Chemicals shifts are reported as δ values (ppm).

4.2. Chemicals

Synthetic (±)-ABA and the natural (S)-(+)-ABA are available commercially and both the resolution and HPLC separation of the racemic mixture have been reported.[36]

However, because of the high cost, the use of (+)ABA as a starting material for analogue synthesis has been impractical and, until now, most ABA analogues have been prepared by total synthesis starting from of the racemic form as (±)-ABA.

4.3. Synthesis of abscisic acid analogs

4.3.1. (2*Z*,4*E*)-*N*,*N*-Diethyl-5-(1′-hydroxy-2′,6′,6′-trimethyl-4′-oxo-2′-cyclohexen-1′-yl)-3 methylpenta-2,4-dienamide (1)

(±)-Abscisic acid (10 mg, 0.038 mmol, 1 equiv) was dissolved in 1 ml of *N*,*N* dimethylformamide and O-(7-azabenzotriazol-1-yl)-

1,1,3,3 tetramethyluroniumhexafluorophosphate (HATU) (14.5 mg, 0.038 mmol, 1 equiv) and *N*,*N*-Diisopropylethylamine (DIPEA) (13 μl, 0.076 mmol, 2 equiv) were slowly added to the stirred mixture at room temperature in the dark. After few minutes a solution of diethylamine (8 μl, 0.076 mmol, 2 equiv) was added and the reaction mixture was mixed at room temperature, protected from light. When reaction was complete, about 24 h, the solvent was evaporated under vacuum and the residue was dissolved in deionized water. The pH was adjusted to 9 with addition of NaOH 0.1 N and the solution was extracted with diethyl ether (3 × 5 ml). The organic solution was finally evaporated under vacuum. The residue was purified by reverse phase high performance liquid chromatography (RP-HPLC) to give the title compound (8 mg, yield of 66%) as oil.

ESI–MS *m*/*z*: 319.3.

^{1}H NMR (DMSO-d_6, 300 MHz): T = 25 °C; δ 0.97 [s, 3H, **8′**]; 0.96 [s, 3H, **9′**]; 1.35 [m, 6H, **CH$_3$**]; 1.93 [s, 3H, **7′**]; 1.98 [d, 3H, **6**, J = 1.2 Hz]; 2.28 (d, 1H, **5′**, J = 15.4 Hz] 2.74 [d, 1H, **5′**, J = 15.5 Hz]; 3.75 [m, 4H, **N–CH$_2$**]; 3.64 [s, 1H, **OH**]; 5.91 [s, 1H, **5**]; 6.08 [d, 1H, **3′**, J = 16.2 Hz]; 6.48 [d, 1H, **4**, J = 16.2 Hz]; 6.93 [d, 1H, **2**, J = 16 Hz].

^{13}C NMR: (DMSO-d_6), T = 25 °C; δ 12.95[–**CH$_3$**]; 18.85 [**7′**]; 20.86 [**6**]; 23.19 [**8′**]; 24.16 [**9′**]; 41.31 [**6′**]; 42.45 [–**N–CH$_2$**]; 49.31[**5′**]; 50.87 [**O–CH$_3$**]; 78.41 [**1′**]; 116.63 [**2**]; 126.04 [**3′**]; 126.99 [**4**]; 138.45 [**5**]; 150.77 [**3**]; 163.05 [**2′**]; 165.69 [**1**]; 197.21 [**4′**].

4.3.2. (2*Z*,4*E*)-5-(1′-Hydroxy-2′,6′,6′-trimethyl-4′-oxo-2′-cyclohexen-1′-yl)-3-methyl-*N*-phenylpenta-2,4-dienamide (2)

(±)-Abscisic acid (10 mg, 0.038 mmol, 1 equiv) was dissolved in 1 ml of *N*,*N* dimethylformamide and HATU (14.5 mg, 0.038 mmol, 1 equiv) and DIPEA (13 μl, 0.076 mmol, 2 equiv) were slowly added to the stirred mixture in the dark at room temperature. After few minutes a solution of aniline (6.8 μl, 0.076 mmol, 2 equiv) was added and the reaction mixture was mixed at room temperature, protected from light, for about 48 h.

When reaction was complete, the solvent was evaporated under vacuum and the residue was dissolved in deionized water. The pH was adjusted to 9 with addition of NaOH 0.1 N and the solution was extracted with diethyl ether (3 × 5 ml). The organic solution was finally evaporated under vacuum. The residue was purified by RP-HPLC to give the title compound (7 mg, yield of 54%) as oil.

ESI–MS *m*/*z*: 339.2.

^{1}H NMR (DMSO-d_6, 300 MHz): T = 25 °C; δ 0.93 [s, 3H, **9′**]; 0.96 [s, 3H, **8′**]; 1.84 [d, 3H, **7′**, J = 1.4 Hz]; 1.98 [d, 3H, **6**, J = 1.0 Hz]; 2.09 [d, 1H, **5′**, J = 16.7 Hz], 2.53 [d, 1H, **5′**, J = 16.7 Hz]; 5.19 [s, 1H, **OH**]; 5.84–5.79 [m, 1H, **3′**]; 5.88 [m, 1H, **2**]; 6.16 [d, 1H, **5**, J = 16.0 Hz], 6.48 [d, 1H, **4**, J = 16.0 Hz;] 7.19 [m, 1H, **CH**]; 7.41–7.43 [m, 2H, **CH**]; 7.50–7.58 [m, 2H, **CH**]; 9.87 [s, 1H, –**NH**].

^{13}C NMR: DMSO-d_6, T = 25 °C; δ 18.96 [**7′**]; 21.08 [**6**]; 23.24 [**8′**]; 24.22 [**9′**]; 41.35 [**6′**]; 49.41 [**5′**]; 78.44 [**1′**]; 113.84[**CH phenyl**]; 120.57[**CH phenyl**]; 121.46 [**2**]; 125.84 [**3′**]; 127.78 [**4**]; 132.59 [**C–NH**]; 136.09 [**5**]; 145.39 [**3**]; 153.12 [**CH phenyl**]; 163.55 [**2′**]; 163.67 [**1**]; 197.35 [**4′**].

4.3.3. (2*Z*,4*E*)-5-(1′-Hydroxy-2′,6′,6′-trimethyl-4′-oxo-2′-cyclohexen-1′-yl)-*N*-(4 methoxyphenyl)-3-methylpenta-2,4-dienamide (3)

(±)-Abscisic acid (20 mg, 0.075 mmol, 1 equiv), was dissolved in 2 ml of *N*,*N* dimethylformamide and HATU (30 mg, 0.075 mmol, 1 equiv) and DIPEA (26 μl, 0.15 mmol, 2 equiv) were slowly added to the stirred mixture in the dark. After few minutes a solution of *p*-anisidine (19 mg, 0.151 mmol, 2 equiv) was added and the reaction mixture was mixed at room temperature, protected from light, for about 48 h monitoring the reaction with HPLC analysis. When the reaction was complete, the solvent was evaporated under vacuum and the residue was dissolved in deionized water. The pH was adjusted to 9 with addition of NaOH 0.1 N and the solution was extracted with diethyl ether (3 × 5 ml). The organic solution was finally evaporated under vacuum. The residue was purified by RP-HPLC to obtain the title compound (12.7 mg, yield of 46%) as brown oil.

ESI–MS *m*/*z*: 369.2.

^{1}H NMR (DMSO-d_6, 300 MHz): T = 25 °C; δ 0.93 [s, 3H, **9′**]; 0.96 [s, 3H, **8′**]; 1.84 [d, 3H, **7′**, J = 1.4 Hz]; 1.98 [d, 3H, **6**, J = 1.0 Hz]; 2.09 [d, 1H, **5′**, J = 16.7 Hz], 2.53 [d, 1H, **5′**, J = 16.7 Hz]; 3.71 [s, 3H, **OCH$_3$**] 5.19 [s, 1H, **OH**]; 5.84–5.79 [m, 1H, **3′**]; 5.88 [m, 1H, **2**]; 6.16 [d, 1H, **5**, J = 16.0 Hz], 6.48 [d, 1H, **4**, J = 16.0 Hz]; 6.91–6.83 [m, 2H, **CH**]; 7.58–7.50 [m, 2H, **CH**]; 9.87 [s, 1H, –**NH**].

^{13}C NMR: DMSO-d_6, T = 25 °C; δ 18.96 [**7′**]; 21.08 [**6**]; 23.24 [**8′**]; 24.22 [**9′**]; 41.35 [**6′**]; 49.41 [**5′**]; 55.17 [**O–CH$_3$**]; 78.44 [**1′**]; 113.84[**CH phenyl**]; 120.57[**CH phenyl**]; 121.46 [**2**]; 125.84 [**3′**]; 127.78 [**4**]; 132.59[**C–NH**]; 136.09 [**5**]; 145.39 [**3**]; 155.12[**C–O–CH$_3$**]; 163.55 [**2′**]; 163.67 [**1**]; 197.35 [**4′**].

4.3.4. (2*Z*,4*E*)-*N*-(4-aminophenyl)-5-(1′-hydroxy-2′,6′,6′-trimethyl-4′-oxo-2′-cyclohexen-1′-yl)-3-methylpenta-2,4-dienamide (4)

(±)-Abscisic acid (10 mg, 0.038 mmol, 1 equiv) was dissolved in 0.5 ml of *N*,*N* dimethylformamide and HATU (14 mg, 0.038 mmol, 1 equiv) and DIPEA (13 μl, 0.076 mmol, 2 equiv) were slowly added to the stirred mixture in the dark at room temperature. After a few minutes, a solution of 1,4 phenylendiamine (16 mg, 0.076 mmol, 2 equiv) in 300 μl of *N*,*N* dimethylformamide was added and the reaction mixture was mixed at room temperature, protected from light, overnight monitoring the reaction with thin layer chromatography (TLC) (ethanol/ethyl acetate/ammonia solution 25% 10:90:0.25).

When reaction was complete the solvent was evaporated under vacuum and the residue was dissolved in deionized water. The solution was extracted with diethyl ether (3 × 2 ml). The aqueous solution was finally extracted with ethyl acetate (EtOAc) (3 × 2 ml). The organic solution was then evaporated under vacuum. The residue was purified by RP-HPLC to obtain the title compound (7 mg, yield of 52%) as oil.

ESI–MS *m*/*z*: 354.2.

^{1}H NMR (DMSO-d_6, 300 MHz): T = 25 °C; δ 0.93 [s, 3H, **9′**]; 0.96 [s, 3H, **8′**]; 1.84 [d, 3H, **7′**, J = 1.4 Hz]; 1.98 [d, 3H, **6**, J = 1.0 Hz]; 2.09 [d, 1H, **5′**, J = 16.7 Hz], 2.53 [d, 1H, **5′**, J = 16.7 Hz]; 5.19 [s, 1H, **OH**]; 5.84–5.79 [m, 1H, **3′**]; 5.88 [m, 1H, **2**]; 6.16 [d, 1H, **5**, J = 16.0 Hz]; 6.48 [d, 1H, **4**, J = 16.0 Hz]; 6.50–6.58 [m, 2H, **CH**]; 6.29 [s, 2H, **NH2**]; 7.41–7.43 [m, 2H, **CH**]; 9.87 [s, 1H, –**NH**].

^{13}C NMR: DMSO-d_6, T = 25 °C; δ 18.96 [**7′**]; 21.08 [**6**]; 23.24 [**8′**]; 24.22 [**9′**]; 41.35 [**6′**]; 49.41 [**5′**]; 78.44 [**1′**]; 113.84 [**CH phenyl**]; 120.57 [**CH phenyl**] 121.46 [**2**]; 125.84 [**3′**]; 127.78 [**4**]; 132.48[**C–NH**]; 136.09 [**5**]; 145.39 [**3**]; 155.12 [**CH–NH$_2$**]; 163.55 [**2′**]; 163.67 [**1**]; 197.35 [**4′**].

4.3.5. (*Z*)-4-Methyl-5-((2′,6′,6′-trimethyl-4′-oxo-2′-cyclohexen-1′-yl) methylene)furan-2(5*H*)-one (5)

Pyrophosphoric acid (200 mg, 1.2 mmol, 10 equiv) was dissolved in 5 ml of methanol and the mixture was heated at 90° for 5 min. (±)-Abscisic acid was added to this solution (30 mg, 0.12 mmol, 1 equiv) and stirred at the same temperature until complete dissolution. When the reaction was complete, it was allowed to reach the ambient temperature and the solvent was evaporated under vacuum. The residue was then purified by RP-HPLC to obtain the title compound (23 mg, yield of 74%) as brown oil.

ESI–MS *m*/*z*: 246.1.

^{1}H NMR (DMSO-d_6, 300 MHz): T = 25 °C; 0.97 [s, 3H, **9′**]; δ 1.00 [s, 3H, **8′**]; 1.81 [d, 3H, **7′**, J = 1.4 Hz]; 2.21 [d, 3H, **6**, J = 1.5 Hz]; 2.42

[d, 1H, **5′**, *J* = 17 Hz]; 2.6 (d, 1H, **5′**, *J* = 16.9 Hz]; 3.2 [d, 1H, **1′**, *J* = 11 Hz]; 5.59 [d, 1H, **5**, *J* = 11 Hz]; 5.88 [s, 1H, **3′**]; 6.20 [m, 1H, **2**].

^{13}C NMR: (DMSO-d_6): *T* = 25 °C; δ 11.4 [**6**]; 22.9 [**7′**]; 27. 21 [**8′**]; 26.34 [**9′**]; 36.2 [**6′**]; 47.28 [**5′**]; 125.21 [**3′**]; 160.31 [**2′**]; 48.7 [**1′**]; 116.12 [**2**]; 152.9 [**4**]; 109.8 [**5**]; 155.71 [**3**]; 167.95 [**1**]; 197.10 [**4′**].

4.3.6. 2-(2-(((2*Z*,4*E*)-5-(1′-Hydroxy-2′,6′,6′-trimethyl-4′-oxo-2′-cyclohexen-1′-yl)-3-methylpenta-2,4-dienoyl)oxy)acetoxy) acetic acid (6)

2-bromoacetic acid (14 μl, 0.1 mmol, 2 equiv) and K_2CO_3 (24 mg, 0.35 mmol, 7 equiv) were added to a solution of (±)-ABA (13 mg, 0.05 mmol, 1 equiv) in 2 ml of dry *N*,*N* dimethylformamide. The reaction was mixed at room temperature, protected from light for 24 h, monitoring the reaction with TLC (ethanol/ethylacetate/acetic acid 10:90:0.25). When the reaction was complete, the solvent was evaporated under vacuum and the residue was dissolved in deionized water. The pH was adjusted to 7 and the solution was extracted with diethyl ether (3 × 3 mL). The organic extracts were dried with anhydrous sodium sulfate and filtered. The organic solution was evaporated under vacuum. The residue was purified by RP-HPLC to obtain the title compound (6 mg, yield of 32%) as white powder.

ESI–MS *m*/*z*: 380.1.

^{1}H NMR (DMSO-d_6, 300 MHz): *T* = 25 °C; δ 0.92 [s, 3H, **8′**]; 0.95 [s, 3H, **9′**]; 1.81 [d, 3H, **7′**, *J* = 1.3 Hz]; 2.04 [d, 3H, **6**, *J* = 1.2 Hz]; 2.10 [d, 1H, **5′**, *J* = 17.0 Hz]; 2.54 [d, 1H, **5′**, *J* = 17.0 Hz]; 4.94 [s, 2H, **O–CH$_2$–CO**]; 5.11[s, 2H, **O–CH$_2$–CO**]; 5.26 [s, 1H, **OH**]; 5.85–5.79 [m, 2H, **3′** e **2**]; 6.35 [d, 1H, **5**, *J* = 15.9 Hz]; 7.71 [d, 1H, **4**, *J* = 15.9, 0.6 Hz]. 11.00 [s, 1H, **OH**].

^{13}C NMR: (DMSO-d_6): *T* = 25 °C; δ 18.82 [**7′**]; 20.94 [**6**]; 24.19 [**8′**]; 27.68 [**9′**]; 41.32 [**6′**]; 49.31 [**5′**]; 62.60 [**O–CH$_2$–**]; 63.70 [**O–CH$_2$–**]; 78.40 [**1′**]; 115.82 [**2**]; 126.08 [**3′**]; 126.91 [**4**]; 139.07 [**5**]; 152.25 [**3**]; 162.89 [**2′**]; 164.52 [**1**]; 166.99 [**C═O**]; 171.99 [**C═O**]; 197.18 [**4′**].

4.3.7. (2*Z*,4*E*)-methyl5-(1′-hydroxy-2′,6′,6′-trimethyl-4′-oxo-2′-cyclohexen-1′-yl)-3-methylpenta-2,4-dienoate (7)

A stirred solution of (±)-ABA (50 mg, 0.189 mmol, 1 equiv) dimethylsulfate (60 mg, 0.473 mmol, 2.5 equiv) and K_2CO_3 (104 mg, 0.756 mmol, 4 equiv) in acetone (4 ml) was heated under reflux for 1 h. The reaction was allowed to warm to ambient temperature, extracted with ethyl acetate and the combined organic layer washed with a solution of ammoniumhydrogencarbonate 0.1 M. The organic phase was dried with anhydrous sodium sulfate, filtered, concentrated under vacuum and liophylized to afford a white solid.

The residue was purified by RP-HPLC to obtain the title compound (44 mg, yield of 85%) as powder.

ESI–MS *m*/*z*: 278.1.

^{1}H NMR (DMSO-d_6, 300 MHz): *T* = 25 °C; δ 0.92 [s, 3H, **9′**]; 0.96 [s, 3H, **8′**]; 1.82 [d, 3H, **7′**, *J* = 1.4 Hz]; 2.01[d, 3H, **6** *J* = 1.3 Hz]; 2.10 [d, 1H, **5′**, *J* = 16.6 Hz]; 2.53 [d, 1H, **5′**, *J* = 16.6 Hz]; 3.61 [s, 3H, **O–CH$_3$**]; 5.25 [s, 1H, **OH**]; 5.77–5.73 [m, 1H, **2**]; 5.81–5.85 [m, 1H, **3′**]; 6.30 [d, 1H, **5**, *J* = 15.9 Hz]; 7.70 [dd, 1H, **4**, *J* = 15.9, 0.8 Hz].

^{13}C NMR: (DMSO-d_6): *T* = 25 °C; δ 18.85 [**7′**]; 20.86 [**6**]; 23.19 [**8′**]; 24.16 [**9′**]; 41.31 [**6′**]; 49.31 [**5′**]; 50.87 [**O–CH$_3$**]; 78.41 [**1′**]; 116.63 [**2**]; 126.04 [**3′**]; 126.99 [**4**]; 138.45 [**5**]; 150.77 [**3**]; 163.05 [**2′**]; 165.69 [**1**]; 197.21 [**4′**].

4.3.8. (2*Z*,4*E*)-*tert*-butyl 5-(1′-hydroxy-2′,6′,6′-trimethyl-4′-oxo-2′-cyclohexen-1′-yl)-3-methylpenta-2,4-dienoate (7b)

$HClO_4$ (7 μl, 0.12 mmol, 1.5 equiv) was slowly added to a solution of (±)-ABA (20 mg, 0.076 mmol, 1 equiv) in *tert*-butyl acetate (600 μl, 3.8 mmol, 50 equiv) at *T* = 0 °C. The reaction mixture was stirred at room temperature for 12 h, then washed with 1 N hydrochloric acid in water. The resultant aqueous solution was adjusted to pH 9 by addition of 10% K_2CO_3, and then extracted with diethyl ether (3 × 1 mL). The combined organic phases were dried with anhydrous sodium sulfate, filtered and concentrated to give an oil. The residue was purified by RP-HPLC to obtain the title compound (5 mg, yield of 21%) as oil.

ESI–MS *m*/*z*: 320.4.

^{1}H NMR (DMSO-d_6, 300 MHz): *T* = 25 °C; δ 0.95 [s, 3H, **8′**]; 0.96 [s, 3H, **9′**]; 1.38 [m, 9H, (**CH$_3$**)$_3$]; 1.83 [s, 3H, **7′**]; 1.98 [d, 3H, **6**, *J* = 1.2 Hz]; 2.28 (d, 1H, **5′**, *J* = 15.4 Hz] 2.74 [d, 1H, **5′**, *J* = 15.5 Hz]; 3.64 [s, 1H, **OH**]; 5.63 [d, 1H, **2**, *J* = 16 Hz]; 5.91 [s, 1H, **5**]; 6.08 [d, 1H, **3′**, *J* = 16.2 Hz]; 6.48 [d, 1H, **4**, *J* = 16.2 Hz].

^{13}C NMR: (DMSO-d_6): *T* = 25 °C; δ 18.85 [**7′**]; 20.86 [**6**]; 23.19 [**8′**]; 24.16 [**9′**]; 28.7 [(**CH$_3$**)$_3$] 41.31 [**6′**]; 49.31[**5′**]; 78.41 [**1′**]; 80.98 [**O–C**]; 116.63 [**2**]; 126.04 [**3′**]; 126.99 [**4**]; 138.45 [**5**]; 150.77 [**3**]; 163.05 [**2′**]; 165.69 [**1**]; 197.21 [**4′**].

4.3.9. (2*Z*,4*E*)-5-(1′-Hydroxy-4′-oxo-2′,6′,6′-trimethyl-2′-cyclohexenyl)-3-methyl-pentadien-1-ol (8)

Oxalyl chloride (0.025 ml, 0.28 mmol, 2 equiv) was added dropwise to a solution of (±)-ABA (50 mg, 0.19 mmol, 1 equiv) in 2 ml of dry *N*,*N* dimethylformamide at *T* = 10 °C. After 30 min the reaction was stopped and the solvent was evaporated under vacuum. The residue was dissolved in 1 ml of dry tetrahydrofuran (THF) and $NaBH_4$ (55 mg, 1.4 mmol, 1 equiv) was added at *T* = −55 °C to the stirred solution. When the reaction was complete, 100 μl of 0.1 M acetic acid was added to the mixture. The reaction was poured into cold water (1 mL), and finally extracted with EtOAc (3 × 3 mL). The EtOAc extract was dried with anhydrous sodium sulfate, filtered and concentrated under vacuum to afford the final product.

The residue was purified by RP-HPLC to obtain the title compound (18 mg, yield of 37%) as yellow oil.

ESI–MS *m*/*z*: 250.2.

^{1}H NMR ($CDCl_3$, 300 MHz): *T* = 25 °C; δ 0.97 [s, 3H, **8′**]; 0.99 [s, 3H, **9′**]; 1.85 [3H, **s**, **7′**]; 1. 98 [s 3H, **6**]; 2.26 [d, 1H, **5′**, *J* = 17 Hz], 2.45 [d, 1H, **5′**, *J* = 17 Hz]; 4.29 [d, 2H, **–CH$_2$–OH**, *J* = 6.5 Hz]; 5.25 [s, 1H, **OH**]; 5.62 [t, 1H, **2**, *J* = 6.5 Hz] 5.80 [d, 1H, **5**, *J* = 15.5 Hz]; 5.85 [m, 1H, **3′**]; 5.90 [s, **–OH**, 1H,]; 6.76 [d, 1H, **4**, *J* = 15.5 Hz].

4.3.10. (2*Z*,4*E*)-2-(*tert*-butoxy)-2-oxoethyl-5-(1′-hydroxy-2′,6′,6′-trimethyl-4′-oxo-2′cyclohexen-1′-yl)-3-methylpenta-2,4-dienoate (9)

*Tert*butyl-2-bromoacetate (100 μl, 0.605 mmol, 4 equiv) and K_2CO_3 (48 mg, 0.35 mmol, 2.3 equiv) were added to a solution of (±)-ABA (40 mg, 0.151 mmol, 1 equiv) in 2 ml of dry *N*,*N* dimethylformamide. The reaction was mixed at room temperature, protected from light, up to completeness. The solvent was evaporated under vacuum and the residue was dissolved in about 3 ml of deionized water. The pH was adjusted to 9 with addition of NaOH 0.1 N and the solution was extracted with EtOAc (3 × 3 mL). The EtOAc extract was dried with anhydrous sodium sulfate and filtered. The organic solution was evaporated under vacuum. The residue was purified by RP-HPLC to obtain the title compound (35 mg, yield of 61%) as powder. ESI–MS *m*/*z*: 378.20.

^{1}H NMR (DMSO-d_6, 300 MHz): *T* = 25 °C; δ 0.92 [s, 3H, **8′**]; 0.95 [s, 3H, **9′**]; 1.41 [s, 9H, **$_3$($_3$HC)CO–**]; 1.82 [d, 3H, **7′**, *J* = 1.4 Hz]; 2.04 [d, 3H, **6**, *J* = 1.2 Hz]; 2.10 [d, 1H, **5′**, *J* = 17.0 Hz]; 2.54 [d, 1H, **5′**, *J* = 17.0 Hz]; 4.54 [s, 2H, **O–CH$_2$–CO**]; 5.26 [s, 1H, **OH**]; 5.85–5.79 [m, 2H, **3′** e **2**]; 6.35 [d, 1H, **5**, *J* = 15.9 Hz]; 7.71 [dd, 1H, **4**, *J* = 15.9, 0.6 Hz].

^{13}C NMR: (DMSO-d_6): *T* = 25 °C; δ 18.82 [**7′**]; 20.94 [**6**]; 24.19 [**8′**]; 27.68 [**9′**]; 41.32 [**6′**]; 49.31[**5′**]; 60.60 [**O–CH$_2$–**]; 78.40 [**1′**]; 81.48 [(**H$_3$C)C**]; 115.82 [**2**]; 126.08 [**3′**]; 126.91 [**4**]; 139.07 [**5**]; 152.25 [**3**]; 162.89 [**2′**]; 164.52 [**1**]; 166.99 [**C═O**]; 197.18 [**4′**].

4.3.11. (2*Z*,4*E*)-5-(2-hydroxy-1,3,3-trimethyl-5-oxo-7-oxabicyclo[4.1.0]heptan-2-yl)-3-methylpenta-2,4-dienoic acid (10)

30% H_2O_2 (250 μl, 8.09 mmol, 70 equiv) and 6 N NaOH (50 μl) were added at 0 °C to a stirred solution of (±)-ABA (30 mg, 0.113 mmol, 1 equiv) in methanol (MeOH) (1 ml). The mixture was stirred, protected from light, for 96 h at 0 °C and then diluted with H_2O.

After lowering the pH to 2 with 3 N HCI, the mixture was extracted with EtOAc (3 × 3 ml). The organic layer was concentrated under reduced pressure. The residue was purified by RP-HPLC to obtain the title compound (25 mg, yield of 76%) as yellow oil.

ESI–MS *m*/*z* 280.1.

^{1}H NMR (DMSO-d_6, 300 MHz): *T* = 25 °C; δ 0.79 [s, 3H, **8′**]; 0.96 [s, 3H, **9′**]; 1.27 [s, 3H, **7′**]; 1.93 (d, 1H, **5′**, *J* = 15.4 Hz]; 1.98 [d, 3H, **6**, *J* = 1.2 Hz]; 2.74 [d, 1H, **5′**, *J* = 15.5 Hz]; 3.22 [s, 1H, **3′**]; 5.25 [s, 1H, **OH**]; 5.66–5.68 [m, 1H, **2**]; 6.22 [dd, 1H, **5**, *J* = 15.8, 0.5 Hz]; 7.81 [dd, 1H, **4**, *J* = 15.8, 0.7 Hz] 11.00 [s, 1H, **OH**].

^{13}C NMR: (DMSO-d_6), *T* = 25 °C; δ 19.65 [**7′**]; 20.93 [**6**]; 24.92 [**8′**]; 26.02 [**9′**]; 41.55 [**6′**]; 47.28 [**5′**]; 62.11 [**3′**]; 66.71 [**2′**]; 76.15 [**1′**]; 118.12 [**2**]; 126.9 [**4**]; 138.18 [**5**]; 149.31 [**3**]; 166.95 [**1**]; 206.00 [**4′**].

4.3.12. 4-(2′,3′-Epoxy-2′,6′,6′-trimethylcyclohexyl)-3-buten-2-one (11)

m-Chloroperoxybenzoic acid (13.4 mg, 0.078 mmol, 1.5 equiv) was added to a solution of (+)-α-Ionone (11 μl, 0.052 mmol, 1 equiv) in 600 μl of methylene chloride, being cooled in an ice bath.

The reaction mixture was stirred at room temperature for 8 h and then filtered and washed with aqueous ammonium hydrogen carbonate 0.1 M. The organic phase was dried with anhydrous sodium sulfate, and evaporated under reduced pressure. The residue was purified by RP-HPLC to obtain a the title compound (8.7 mg, yield of 80%) as an yellow oil.

ESI–MS *m*/*z*: 208.2.

^{1}H NMR (DMSO-d_6, 300 MHz): *T* = 25 °C; δ 0.76 [s, 3H, **9′**]; 0.94 [s, 3H, **8′**]; 1.01 [m, 1H, **5′**]; 1.26 [s, 3H, **7′**]; 1.40 [m, 1H, **5′**]; 1.80–2.00 [m, 2H, **4′**]; 2.09 [d, 1H, **1′**, *J* = 10 Hz]; 2.30 [s, 3H, **CH_3CO**]; 3.10 [m, 1H, **3′**]; 6.10 [d, 1H, **3**, *J* = 16 Hz]; 6.73 [dd, 1H, **4**, *J* = 16 Hz].

4.3.13. 4-(1′,2′-Epoxy-2′,6′,6′-trimethylcyclohexyl)-3-buten-2-one (12)

m-Chloroperoxybenzoic acid (13.4 mg, 0.078 mmol, 1.5 equiv) was added to a solution of (+)-β-ionone (11 μl, 0.052 mmol, 1 equiv) in 600 μl of methylene chloride, being cooled in an ice bath.

The reaction mixture was stirred at room temperature for about 12 h and then filtered and washed with aqueous ammonium hydrogen carbonate 0.1 M. The organic phase was dried with anhydrous sodium sulfate, and evaporated under reduced pressure. The residue was purified by RP-HPLC to obtain the title compound (7.4 mg, yield of 68%) as an yellow oil.

ESI–MS *m*/*z*: 208.2.

^{1}H NMR (DMSO-d_6, 300 MHz): *T* = 25 °C; 0.94 [s, 3H, **8′**]; 1.14[s, 6H, **7′e 9′**]; 1.40 [m, 1H, **5′**]; 1.30–1.9 [m, 6H, **4′**, **3′**, **5′**]; 2.30 [s, 3H, **CH_3CO**]; 6.10 [d, 1H, **3**, *J* = 16 Hz]; 7.01 [dd, 1H, **4**, *J* = 16 Hz].

4.3.14. (2*Z*,4*E*)-5-(1′,4′-Dihydroxy-2′,6′,6′-trimethyl-4′-oxo-2′-cyclohexen-1′-yl)-3-methylpenta-2,4-dienoic acid (13)

(±)-ABA (10 mg, 0.038 mmol, 1 equiv) in 800 μl of methanol and 100 μl of deionized water was cooled to 0 °C. Sodium borohydride (7 mg, 0.18 mmol, 4.5 equiv) was added to the resulting solution and the mixture was stirred at *T* = 0 °C for about 6 h, monitoring the reaction by TLC (ethanol/ethylacetate/acetic acid 10/90/0.25). The reaction was poured into water (5 mL), acidified to pH 2 with 1 N HCI and finally extracted with EtOAc (3 × 1 mL). The EtOAc extract was dried with anhydrous sodium sulfate, filtered, and concentrated under vacuum to give a colorless solid.

The residue was purified by RP-HPLC to obtain two different compounds (*trans*-diol ABA 4 mg, yield of 40%; *cis*-diol ABA 4.8 mg, yield of 47%) as oils.

ESI–MS *m*/*z*: 266.2.

^{1}H NMR (DMSO-d_6, 300 MHz): *T* = 25 °C; δ 0.92 [s, 3H, **9′**]; 0.98 [s, 3H, **8′**]; 1.65 [d, 3H, **7′**, *J* = 1.4 Hz]; 1.73 [d, 1H, **5′**, *J* = 16.6 Hz] 1.81 [d, 1H, **5′**, *J* = 16.6 Hz] 2.01 [d, 3H, **6** *J* = 1.3 Hz]; 2.80 [s, 1H, **OH**]; 4.25 [m, 1H, **4′**]; 5.25 [s, 1H, **OH**]; 5.63 [m, 1H, **3′**]; 5.69 [m, 1H, **2**]; 6.05 [d, 1H, **5**, *J* = 15.9 Hz]; 7.70 [dd, 1H, **4**, *J* = 15.9 Hz], 11.00 [s, 1H, **OH**].

^{1}H NMR: (DMSO-d_6, 300 MHz): *T* = 25 °C; δ 0.90 [s, 3H, **9′**]; 1.00 [s, 3H, **8′**]; 1.63 [d, 3H, **7′**, *J* = 1.4 Hz]; 1.73 [d, 1H, **5′**, *J* = 16.6 Hz] 1.78 [d, 1H, **5′**, *J* = 16.6 Hz] 2.02[d, 3H, **6** *J* = 1.3 Hz]; 2.80 [s, 1H, **OH**]; 4.22 [m, 1H, **4′**]; 5.25 [s, 1H, **OH**]; 5.67 [m, 1H, **3′**]; 5.69 [m, 1H, **2**]; 6.11 [d, 1H, **5**, *J* = 15.9 Hz]; 7.25 [dd, 1H, **4**, *J* = 15.9 Hz]. 11.00 [s, 1H, **OH**].

4.3.15. (2*Z*,4*E*)-5-(4′-acetoxy-1′-hydroxy-2′,6′,6′-trimethyl 2′-cyclohexen-1′-yl)-3-methylpenta-2,4-dienoic acid (14)

Trans ABA diol (27 mg, 0.096 mmol, 1 equiv) was dissolved in a 1:2 (v:v) mixture of acetic anhydride and pyridine (1 mL), and the solution was stirred at *T* = 40 °C for about 3 h, monitoring the reaction with TLC (ethanol/ethylacetate/acetic acid 10:90:0.25). The reaction was poured into water, finally extracted with EtOAc (3 × 1 mL). The EtOAc extract was dried with anhydrous sodium sulfate, filtered, and concentrated under vacuum to give a colorless solid. The residue was purified by RP-HPLC to obtain the title compound (23 mg, yield of 82%) as colorless solid.

ESI–MS *m*/*z*: 308.2.

^{1}H NMR (DMSO-d_6, 300 MHz): *T* = 25 °C; δ 0.90 [s, 3H, **9′**]; 0.94 [s, 3H, **8′**]; 1.56 [d, 3H, **7** *J* = 1.4 Hz]; 1.71 [d, 1H, **5′**, *J* = 16 Hz] 1.80 [d, 1H, **5′**, *J* = 16 Hz]; 1.91[d, 3H, **6** *J* = 1.1 Hz]; 1.97 [s, 3H, **O–$COCH_3$**]; 5.20 [m, 1H, **4′**]; 5.25 [s, 1H, **OH**]; 5.33 [m, 1H, **3′**]; 5.55 [m, 1H, **2**]; 6.02 [d, 1H, **5**, *J* = 15.9 Hz]; 7.61 [dd, 1H, **4**, *J* = 15.9, 0.8 Hz]. 11.00 [s, 1H, **OH**].

The *cis* diol (20 mg, 0.075 mmol, 1 equiv) was treated as above and the mixture was stirred at *T* = 40° for about 2 h monitoring the reaction with TLC (ethanol/ethylacetate/acetic acid 10:90:0.25). The reaction was poured into water, finally extracted with EtOAc (3 × 1 mL). The EtOAc extract was dried with anhydrous sodium sulfate, filtered and concentrated under vacuum to give a colorless solid. The residue was purified by RP-HPLC to obtain the title compound (18 mg, yield of 77%) as solid.

ESI–MS *m*/*z*: 308.2.

^{1}H NMR (DMSO-d_6, 300 MHz): *T* = 25 °C; δ 0.94 [s, 3H, **9′**]; 0.97 [s, 3H, **8′**]; 1.62 [d, 3H, **7** *J* = 1.4 Hz]; 1.72 [d, 1H, **5′**, *J* = 16 Hz] 1.81 [d, 1H, **5′**, *J* = 16 Hz]; 1.93[d, 3H, **6** *J* = 1.1 Hz]; 2.01 [s, 3H, **O–$COCH_3$**]; 5.25 [m, 1H, **4′**]; 5.50 [m, 1H, **2**]; 5.68 [m, 1H, **3′**]; 6.21 [d, 1H, **5**, *J* = 15.9 Hz]; 7.61 [dd,1H, **4**, *J* = 15.9, 0.8 Hz].

4.3.16. (2*Z*,4*E*)-5-(1′-hydroxy-2′,6′,6′-trimethyl-4′-(phenylamino) 2′-cyclohexen-1′-yl)-3-methylpenta-2,4-dienoic acid (14b)

(±)-ABA (30 mg, 0.113 mmol, 1 equiv) was dissolved in 5 ml of methanol at room temperature for few minutes. Aniline (16 μl, 0.170 mmol, 1.5 equiv) and acetic acid (100 μl, 1.748 mmol, 10 equiv) were added. The mixture was refluxed for about 4 h. The reaction was allowed to warm to ambient temperature, then, sodium cyanoborohydride (20 mg, 1.917 mmol, 11 equiv) was added in several aliquots over a period of 30 min. The reaction mixture was stirred at room temperature, protected from light, for 24 h, monitoring the reaction with TLC (hexane/ethylacetate/acetic

acid 50:35:1). The reaction was poured into cold water (5 mL), the pH was adjusted to 8 with addition of aqueous ammonium hydrogen carbonate 0.1 M and finally extracted with EtOAc (3 × 10 mL). The EtOAc extract was dried with anhydrous sodium sulfate, filtered and concentrated under vacuum to afford a mixture of the two different products.

The residue was purified by RP-HPLC to obtain the title compound (30 mg, 73%) as yellow oil.

ESI–MS *m*/*z*: 341.4.

^{1}H NMR (DMSO-d_6, 300 MHz): T = 25 °C; δ 0.90 [s, 3H, **9′**]; 0.94 [s, 3H, **8′**]; 1.56 [d, 3H, **7** J = 1.4 Hz]; 1.71 [d, 1H, **5′**, J = 16 Hz] 1.80 [d, 1H, **5′**, J = 16 Hz]; 1.91[d, 3H, **6** J = 1.1 Hz]; 3.20 [m, 1H, **4′**]; 4.05[s, 1H, **NH**] 5.25 [s, 1H, **OH**]; 5.33 [m, 1H, **3′**]; 5.55 [m, 1H, **2**]; 6.02 [d, 1H, **5**, J = 15.9 Hz]; 7.19 [m, 1H, **CH phenyl**]; 7.41–7.43 [m, 2H, **CH phenyl**]; 7.50–7.58 [m, 2H, **CH phenyl**]; 7.61 [dd, 1H, **4**, J = 15.9, 0.8 Hz]. 11.00 [s, 1H, **OH**].

^{13}C NMR: (DMSO-d_6): T = 25 °C; δ 18.85 [**7′**]; 20.86 [**6**]; 23.19 [**8′**]; 24.16 [**9′**]; 41.31 [**6′**]; 43.34 [**5′**]; 50.87 [**O–CH$_3$**]; 54.7 [**4′**]; 78.41 [**1′**]; 116.63 [**2**]; 118.04 [**3′**]; 120.11 [**C phenyl**]; 122.32 [**C phenyl**]; 126.99 [**4**]; 129.50 [**C phenyl**]; 137.45 [**5**]; 139.05 [**2′**]; 146.85 [**C phenyl**]; 150.77 [**3**]; 169.69 [**1**].

4.3.17. (2*Z*,4*E*)-methyl-5-(4′-acetoxy-2′,5′,6′-trimethylphenyl)-3-methylpenta-2,4-dienoate (15)

Analog **7** (10 mg, 0.036 mmol, 1 equiv) was stirred with acetic anhydride (500 μl, 5.23 mmol) and p-toluene-p-sulfonic acid (25 mg, 0.145 mmol, 3.8 equiv) for 24 h at room temperature, monitoring the reaction with TLC (hexane/ethylacetate/acetic acid 50:35:1). About 1 ml of water was added and the final solution was extracted with EtOAc (3 × 1 mL). The organic phase was dried with anhydrous sodium sulfate, filtered, and concentrated under vacuum. The residue was purified by RP-HPLC to obtain the title compound (8 mg, yield of 73%) as a gum.

ESI–MS *m*/*z*: 302.1.

^{1}H NMR (DMSO-d_6, 300 MHz): T = 25 °C; δ 2.02 [s, 3H, **6**]; 2.15 [s, 3H, **5′**]; 2.24 [s, 3H, **6′**]; 2.25 [d, 3H, **7′**, J = 1.4 Hz]; 2.31 [s, 1H, **4′**], 5.79 [s, 1H, **2**]; 6.78 [s, 1H, **3′**]; 6.93 [d, 1H, **4**, J = 16.0 Hz], 7.73 [d, 1H, **5**, J = 16.0 Hz] 11.00 [s, 1H, **OH**].

4.3.18. (2*Z*,4*E*)-methyl-5-(1′,4′-dihydroxy-2′,6′,6′-trimethyl 2′-cyclohexen-1′-yl)-3-methylpenta-2,4-dienoate (16)

Analog **7** (10 mg, 0.036 mmol, 1 equiv) in 800 μl of methanol and 100 μl of deionized water was cooled to 0 °C. Sodium borohydride (7 mg, 0.18 mmol, 5 equiv) was added to the resulting solution and the mixture was stirred at T = 0° for about 24 h, monitoring the reaction with TLC (ethanol/ethylacetate/acetic acid 10:90:0.25). The reaction was poured into water (5 mL), acidified to pH 2 with 1 N HCI and finally extracted with EtOAc (3 × 1 mL). The EtOAc extract was dried with anhydrous sodium sulfate, filtered, and concentrated under vacuum to give a colorless solid.

The residue was purified by RP-HPLC to obtain two different products (a *trans*-diol methylester derivative, 3 mg, yield of 40% and a *cis*-diol methylester derivative, 4 mg, yield of 47%) as oils.

ESI–MS *m*/*z* 280.2.

^{1}H NMR (DMSO-d_6, 300 MHz): T = 25 °C; δ 0.94 [s, 3H, **9′**]; 0.99 [s, 3H, **8′**]; 1.68 [d, 3H, **7′** J = 1.4 Hz]; 1.69 [d, 1H, **5′**, J = 16.6 Hz] 1.81 [d, 1H, **5′**, J = 16.6 Hz]; 2.00[d, 3H, **6** J = 1.1 Hz]; 2.81 [s, 1H, **OH**]; 3.71 [s, 3H, **O–CH$_3$**]; 4.25 [m, 1H, **4′**]; 5.25 [s, 1H, **OH**]; 5.70 [m, 1H, **2**]; 5.81 [m, 1H, **3′**]; 6.05 [d, 1H, **5**, J = 15.9 Hz]; 7.72 [dd, 1H, **4**, J = 15.9, 0.8 Hz].

^{1}H NMR (DMSO-d_6, 300 MHz), T = 25 °C; δ 0.92 [s, 3H, **9′**]; 1.01 [s, 3H, **8′**]; 1.67 [d, 3H, **7′** J = 1.4 Hz]; 1.69 [d, 1H, **5′**, J = 16.6 Hz] 1.83 [d, 1H, **5′**, J = 16.6 Hz]; 2.02[d, 3H, **6** J = 1.1 Hz]; 2.81 [s, 1H, **OH**]; 3.69 [s, 3H, **O–CH$_3$**]; 4.22 [m, 1H, **4′**]; 5.25 [s, 1H, **OH**]; 5.70 [m, 1H, **2**]; 5.75 [m, 1H, **3′**]; 6.10 [d, 1H, **5**, J = 15.9 Hz]; 7.69 [dd, 1H, **4**, J = 15.9, 0.8 Hz].

^{13}C NMR: (DMSO-d_6): T = 25 °C; δ 18.85 [**7′**]; 20.86 [**6**]; 23.19 [**8′**]; 24.16 [**9′**]; 41.31 [**6′**]; 43.31 [**5′**]; 50.87 [**O–CH$_3$**]; 67.25 [**4′**]; 78.41 [**1′**]; 116.63 [**2**]; 126.04 [**2′**]; 126.99 [**4**]; 138.45 [**5**]; 141.05 [**3′**]; 150.77 [**3**]; 165.69 [**1**].

4.3.19. (2*Z*,4*E*)-2-(*tert*-Butoxy)-2-oxoethyl 5-(1′,4′-dihydroxy-2′,6′,6′ trimethyl 2′-cyclohexen-1′-yl)-3-methylpenta-2,4-dienoate (17)

A solution of analog **9** (5 mg, 0.013 mmol, 1 equiv) in 800 μl of methanol and 100 μl of deionized water was cooled to T = 0 °C. Sodium borohydride (5 mg, 0.13 mmol, 10 equiv) was added to the resulting solution and the mixture was stirred at T = 0 °C for about 20 h, monitoring the reaction with TLC (ethanol/ethylacetate/acetic acid 10:90:0.25). The reaction was poured into water acidified to pH 2 with 1 N HCI and finally extracted with EtOAc (3 × 1 mL). The EtOAc extract was dried with anhydrous sodium sulfate, filtered, and concentrated under vacuum to give a colorless solid. The residue was purified by RP-HPLC to obtain the title compound, containing a mixture of two different isomers (4.5 mg, yield of 90%) as oils.

ESI–MS *m*/*z*: 380.2.

^{1}H NMR (DMSO-d_6, 300 MHz): T = 25 °C; δ 0.92 [s, 3H, **8′**]; 0.95 [s, 3H, **9′**]; 1.41 [s, 9H, **$_3$($_3$HC)CO**–]; 1.67 [d, 1H, **5′**, J = 17.0 Hz]; 1.81 [d, 3H, **7′**, J = 1.4 Hz]; 1.85 [d, 1H, **5′**, J = 17.0 Hz]; 2.04 [d, 3H, **6**, J = 1.2 Hz]; 2.85 [s, 1H, **OH**]; 4.22 [m, 1H, **4′**], 4.54 [s, 2H, **O–CH$_2$–CO**]; 5.26 [s, 1H, **OH**]; 5.55–5.74 [m, 2H, **3′** e **2**]; 6.35 [d, 1H, **5**, J = 15.9 Hz]; 7.71 [dd, 1H, **4**, J = 15.9, 0.6 Hz].

^{13}C NMR: (DMSO-d_6): T = 25 °C; δ 18.82 [**7′**]; 20.94 [**6**]; 24.19 [**8′**]; 27.68 [**9′**]; 41.32 [**6′**]; 49.31[**5′**]; 60.60 [**O–CH$_2$**–]; 78.40 [**1′**]; 81.48 [(**H$_3$C**)**C**]; 115.82 [**2**]; 126.08 [**3′**]; 126.91 [**4**]; 139.07 [**5**]; 152.25 [**3**]; 162.89 [**2′**]; 164.52 [**1**]; 166.99 [**C═O**]; 197.18 [**4′**].

4.3.20. (2*Z*,4*E*)-5-(1′,4′-dihydroxy-2′,6′,6′-trimethyl-2′-cyclohexen-1-yl)-3-methyl-2,4-pentadien-1-ol (18)

Diisobutylaluminium hydride (1.5 M in toluene, 253 μl, 0.38 mmol, 4 equiv) was carefully added with a syringe to a stirred solution of analog **7** (25 mg, 0.089 mmol, 1 equiv) in 1.5 ml diethylether at T = −20° under N_2 atmosphere. The solution was stirred at the same temperature for about 2 h, monitoring the reaction with HPLC analysis. Then, 100 μl of methanol was added to the mixture to stop the reaction. The mixture was stirred at room temperature for 30 min and finally the solvent was evaporated under vacuum and the residue was dissolved in about 1 ml of deionized water. The solution was extracted with EtOAc (3 × 1 mL). The EtOAc extract was dried with anhydrous sodium sulfate, filtered, and concentrated under vacuum to give a colorless solid.

The residue was purified by RP-HPLC to obtain the title compound containing two different isomers (a *cis* analog 4.5 mg, yield 20% and a *trans* analog, 6.5 mg, yield 30%) as oils.

ESI–MS *m*/*z*: 252.2.

^{1}H NMR ($CDCl_3$, 300 MHz): T = 25 °C; δ 0.90 [s, 3H, **8′**]; 0.94 [s, 3H, **9′**]; 1.64 [3H, **s**, **7′**]; 1.73 [2H, **d**, **5′**]; 1.85 [3H, **s**, **6**]; 2.59 [3H, br s, 3**OH**]; 4.28 [m, 3H, –**CH$_2$OH e 4′**]; 5.57 [m, 2H, **3′e 2**] 5.67 [d, 1H, **5**, J = 15.5 Hz]; 6.73 [d, 1H, **4**, J = 15.5 Hz].

^{1}H NMR ($CDCl_3$, 300 MHz): T = 25 °C; δ 0.87 [s, 3H, **8′**]; 0.98 [s, 3H, **9′**]; 1.51 [dd, 1H, J = 10.13 Hz, **5′**]; 1.62 [3H, **s**, **7′**]; 1.70 [dd, 1H, **5′**, J = 6.13 Hz]; 1.85 [**s**, 3H, **6**]; 2.75 [3H, br s, **3OH**]; 4.20 [m, 3H, –**CH$_2$OH e 4′**]; 5.56[m, 2H, **3′e 2**] 5.74 [d, 1H, **5**, J = 15.5 Hz]; 6.56 [d, 1H, **4**, J = 15.5 Hz].

4.3.21. Determination of intracellular cAMP in human granulocytes

Human granulocytes were isolated by density gradient centrifugation of buffy coats,[3] prepared from freshly drawn blood of

healthy human volunteers. Granulocytes were then incubated overnight in DMEM/F12 supplemented with 10% of fetal bovine serum (FBS), penicillin (50 units/ml) and streptomycin (50 μg/ml) (DMEM/F12 complete). The physical stimulation of the cells during their purification results in an artificial elevation of basal [cAMP]i values, compromising the ability of the cells to respond to ABA. After overnight rest, cells were centrifuged at 200×*g* for 5 min and resuspended at 1.5×10^7/ml in Hanks' Balanced Salt Solution (HBSS) with calcium and magnesium. Granulocytes were preincubated in the presence of 10 μM cAMP phosphodiesterase inhibitor 4-[(3-butoxy-4-methoxyphenyl)-methyl]-2-imidazolidinone (Ro-20-1724) for 5 min at 25 °C, duplicate 200 μl-aliquots of cells were incubated without (control) or with 1 μM (±)-ABA, in the absence or in the presence of different analogs at 50 μM final concentration, for 1 min at 25 °C. The reaction was stopped by the addition of 13 μl of 0.6 M perchloric acid (PCA) at 4 °C. The cell lysates were centrifuged for 5 min at 17,000×*g* at 4 °C and the supernatant was adjusted to pH 7.5 with 3 M potassium carbonate (K_2CO_3). The reaction led to the development of CO_2 gas and the formation of a white precipitate of potassium perchlorate ($KClO_4$), which was removed by a 5-min centrifugation at 17,000×*g* at 4 °C.

The intracellular cAMP concentration was determined with a competitive radioimmunological test [^{3}H]-cAMP assay system (Amersham, GE Healthcare), following the manufacturer's instructions.

Author disclosure statement

The authors declare no competing financial interest.

Acknowledgements

The authors would like to thank Dr. Paola Fossa for helping in revision manuscript. This work was supported in part by the Italian Ministry of Education University and Scientific Research, by the University of Genova, by the Fondazione CARIGE, by the Compagnia di S. Paolo, and by Regione Liguria.

References and notes

1. Nambara, E.; Marion, P. *Annu. Rev. Plant Biol.* **2005**, *56*, 165.
2. Xue-Xuan, X.; Hong-Bo, S.; Yuan-Yuan, M.; Jun-Na, S.; Dong-Gang, G.; Cheng-Jiang, R. *Crit. Rev. Biotechnol.* **2010**, *30*, 222.
3. Bruzzone, S.; Moreschi, I.; Usai, C.; Guida, L.; Damonte, G.; Salis, A.; Scarfì, S.; Millo, E.; De Flora, A.; Zocchi, E. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 5759.
4. Bruzzone, S.; Bodrato, N.; Usai, C.; Guida, L.; Moreschi, I.; Nano, R.; Antonioli, B.; Fruscione, F.; Magnone, M.; Scarfì, S.; De Flora, A.; Zocchi, E. *J. Biol. Chem.* **2008**, *283*, 32188.
5. Bodrato, N.; Franco, L.; Fresia, C.; Guida, L.; Usai, C.; Salis, A.; Moreschi, I.; Ferraris, C.; Verderio, C.; Basile, G.; Bruzzone, S.; Scarfì, S.; De Flora, A.; Zocchi, E. *J. Biol. Chem.* **2009**, *284*, 14777.
6. Magnone, M.; Bruzzone, S.; Guida, L.; Damonte, G.; Millo, E.; Scarfì, S.; Usai, C.; Sturla, L.; Palombo, D.; De Flora, A.; Zocchi, E. *J. Biol. Chem.* **2009**, *284*, 17808.
7. Magnone, M.; Sturla, L.; Jacchetti, E.; Scarfì, S.; Bruzzone, S.; Usai, C.; Guida, L.; Salis, A.; Damonte, G.; De Flora, A.; Zocchi, E. *FASEB J.* **2012**, *26*, 1261.
8. Bruzzone, S.; Ameri, P.; Briatore, L.; Mannino, E.; Basile, G.; Andraghetti, G.; Grozio, A.; Magnone, M.; Guida, L.; Scarfì, S.; Salis, A.; Damonte, G.; Sturla, L.; Nencioni, A.; Fenoglio, D.; Fiory, F.; Miele, C.; Beguinot, F.; Ruvolo, V.; Bormioli, M.; Colombo, G.; Maggi, D.; Murialdo, G.; Cordera, R.; De Flora, A.; Zocchi, E. *FASEB J.* **2012**, *26*, 1251.
9. Gusta, L. V.; Reaney, M. J. T.; Abrams, S. R.; Robertson, A. J.; Abrams, G. D. *Comments Agric. Food Chem.* **1990**, *2*, 143.
10. Walker-Simmons, M. K.; Anderberg, R. J.; Rose, P. A.; Abrams, S. R. *Plant Physiol.* **1992**, *99*, 501.
11. Blake, T. J.; Tan, W.; Abrams, S. R. *Physiol. Plant.* **1990**, *80*, 365.
12. Flores, A.; Dorffling, K. *J. Plant Growth Regul.* **1990**, *9*, 133.
13. Flores, A.; Grau, A.; Laurich, F.; Dorffling, K. *J. Plant Physiol.* **1988**, *132*, 362.
14. Reaney, M. J. T.; Gusta, L. V.; Abrams, S. R.; Shaw, A. C.; Ewan, B. *Plant Physiol.* **1990**, *93*, S-7.
15. Schubert, J.; Roser, K.; Grossmann, K.; Sauter, H.; Jung, J. *J. Plant Growth Regul.* **1991**, *10*, 27.
16. Walton, D. C. In *Abscisic Acid*; Addicott, F. T., Ed.; Praeger: New York, 1983; p 113.
17. Milborrow, B. V. In *Abscisic acid*; Letham, D. S., Goodwin, P. B., Higgins, T. J. V., Eds.; Elsevier/North Holland Biomedical Press: New York, 1978; p 295. Vol. 1.
18. Churchill, G. C.; Ewan, B.; Reaney, M. J. T.; Abrams, S. R.; Gusta, L. W. *Plant Physiol.* **1992**, *100*, 2024.
19. Churchill, G. C.; Ewan, B.; Reaney, M. J. T.; Abrams, S. R.; Gusta, L. W. *Plant Growth Regul.* **1998**, *25*, 35.
20. Walker-Simmons, M. K.; Reaney, M. J. T.; Quarrie, S. A.; Perata, P.; Vernieri, P.; Abrams, S. R. *Plant Physiol.* **1991**, *95*, 46.
21. Walker-Simmons, M. K.; Rose, P. A.; Shaw, A. C.; Abrams, S. R. *Plant Physiol.* **1994**, *106*, 1279.
22. Perras, M.; Rose, P. A.; Pass, E. W.; Chatson, K. B.; Balsevich, J. J.; Abrams, S. R. *Phytochemistry* **1997**, *46*, 215.
23. Ueno, K.; Araki, Y.; Hirai, N.; Saito, S.; Mizutani, M.; Sakata, K.; Todoroki, Y. *Bioorg. Med. Chem.* **2005**, *13*, 3359.
24. McCourt, P. a.; Creelman, R. b. *Curr. Opin. Plant Biol.* **2008**, *11*, 474.
25. Sturla, L.; Fresia, C.; Guida, L.; Grozio, A.; Vigliarolo, T.; Mannino, E.; Millo, E.; Bagnasco, L.; Bruzzone, S.; De Flora, A.; Zocchi, E. *Biochem. Biophys. Res. Commun.* **2011**, *415*, 390.
26. Belsevich, J.; Bishop, G.; Jacques, S. L.; Hogge, L. R.; Olson, D. J. H.; Laganiere, N. *Can. J. Chem.* **1996**, *74*, 112.
27. Liu, X.; Ma, L.; Lin, Y.; Tang, Lu. Y. *J. Chromatogr. A* **2003**, *1021*, 209.
28. Ward, D. E.; Yuanzhu, G. *Synth. Commun.* **1997**, *27*, 2133.
29. Kim, K. H.; Fan, X. J.; Nielsen, P. E. *Bioconjug. Chem.* **2007**, *18*, 567.
30. Todoroki, Y.; Hirai, N. *Tetrahedron* **1995**, *25*, 6911.
31. Beale, M. H.; Chen, A.; Harrison, P. A.; Willis, C. L. *J. Chem. Soc. Perkin. Trans. 1* **1993**, 3061.
32. Hirai, N.; Yoshida, R.; Todoroki, Y.; Ohigashi, N. *Biosci. Biotechnol. Biochem.* **2000**, *64*, 1448.
33. Kim, B. T.; Min, Y. K.; Asami, T.; Park, N. K.; Kwon, O. Y.; Cho, K. Y.; Yoshida, S. *J. Agric. Food Chem.* **1999**, *47*, 313.
34. Mallaby, R.; Ryback, G. *J. Chem. Soc. Perkin. Trans. 2* **1972**, *2*, 919.
35. Aleu, J.; Brenna, E.; Fuganti, C.; Serra, S. *J. Chem. Soc. Perkin Trans. 1* **1999**, 271.
36. Zaharia, L. I.; Walker-Simmon, M. K.; Rodriguez, C. N.; Abrams, S. R. *J. Plant Growth Regul.* **2005**, *24*, 274.
37. Lin, B. L.; Wang, H. J.; Wang, J. S.; Zaharia, L. I.; Abrams, S. R. *J. Exp. Bot.* **2005**, *56*, 2935.
38. Ueno, K.; Yoneyama, H.; Mizutani, M.; Hirai, N.; Todoroki, Y. *Bioorg. Med. Chem.* **2007**, *15*, 6311.
39. Rock, C. D.; Heath, T. G.; Gage, D. A.; Zeevaart, J. A. *Plant Physiol.* **1991**, *97*, 670.
40. Grozio, A.; Millo, E.; Guida, L.; Vigliarolo, T.; Bellotti, M.; Salis, A.; Fresia, C.; Sturla, L.; Magnone, M.; Galatini, A.; Damonte, G.; De Flora, A.; Bruzzone, S.; Bagnasco, L.; Zocchi, E. *Biochem. Biophys. Res. Commun.* **2011**, *415*, 696.
41. Zocchi, E.; De Flora, A.; Millo, E.; Bruzzone, S.; Guida, L.; Grozio, A.; Salis, A.; Magnone, M. European Patent 2511272A1, 2012.
42. Takeuchi, J.; Okamoto, M.; Akiyama, T.; Muto, T.; Yajima, S.; Sue, M.; Seo, M.; Kanno, Y.; Kamo, T.; Endo, A.; Nambara, E.; Hirai, N.; Ohnishi, T.; Cutler, S. R.; Todoroki, Y. *Nat. Chem. Biol.* **2014**, *10*, 477.