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Phosphonamide pyrabactin analogues as abscisic acid agonists†

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A four step synthesis towards novel phosphonic pyrabactin analogues is presented. Via a stomatal closure and germination assay, the ability of the analogues to selectively induce the ABA-signaling pathway was demonstrated.

The phytohormone abscisic acid (ABA, Figure 1) **1** is a crucial factor in the adaptive response of plants to abiotic environmental stresses. ABA **1** is known to induce stomatal closure, thereby reducing transpiration during periods of heat, drought or salt stress.[1] In view of the ongoing climate change, causing an intensification of earth's hydrological cycle and thus entailing more droughts, manipulation of the ABA-content and -signaling is a promising method to improve the plant's tolerance to water scarcity and the crop yield in suboptimal growth conditions.

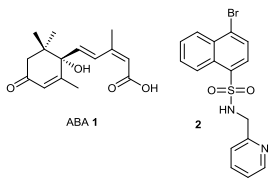
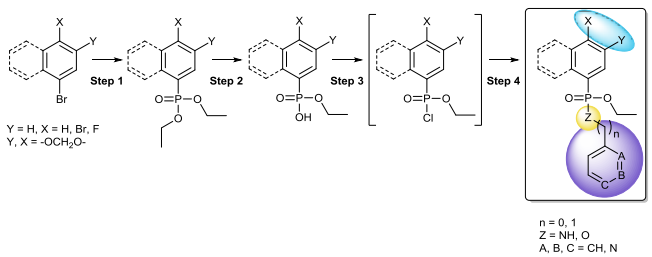


Figure 1. Plant growth regulators (*S*)-(+)-abscisic acid ((+)-ABA) **1** and pyrabactin **2**.

While treatment of crops with exogenous ABA **1** resulted in an increased drought resistance,[2] the potential for its application in agricultural settings is limited. In addition to the unwanted hormonal effect on seed germination and plant growth, ABA **1** is light sensitive and degrades rapidly when in contact with plants.[3] Recently, a synthetic, more stable, ABA-agonist named pyrabactin **2** (Figure 1) was identified through chemical genetic analysis.[4] Although there is no apparent structural resemblance to ABA **1**, pyrabactin **2** is able to elicit ABA-responses, such as the promotion of guard cell closure and the inhibition of seed germination, but fails to trigger substantial vegetative responses.[4-5] The fact that not all ABA-pathways are affected made pyrabactin **2** an indispensable tool for genetic studies and the identification of the PYR1/PYL/RCAR START-domain receptor family.[6] Furthermore, because of its inherent specificity and high stability, pyrabactin **2** can be used as a lead compound to synthesize agricultural applicable ABA-agonists that act as anti-transpirant without side effects on plant growth and development.[7]

This work presents the synthesis and biological evaluation of a small library of pyrabactin analogues, containing phosphonate and phosphonamide linkers instead of the originally reported sulfonamide bridge (**2**). This may result in an enhanced biological compatibility as sulfonamides tend to have a negative effect on plant growth and are regularly used as herbicides.[8] Phosphate, on the other hand, is an essential plant nutrient to sustain optimal growth and the plant enzymatic system is well equipped to handle phosphates, phosphonates and phosphonic acids. Furthermore, phosphorus and sulfur have a similar geometry and atomic weight. As such, similar receptor binding can be expected. The proposed synthetic pathway is in analogy with the first reported pyrabactin synthesis route[9] and comprises the phosphonylation of aryl halides (step 1), activation of the phosphonate function (step 2-3), and coupling with an amine or alcohol (step 4, Scheme 1).

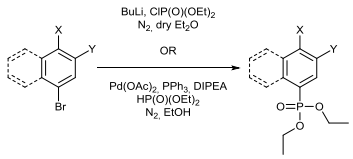


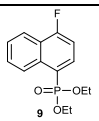
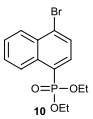
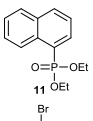
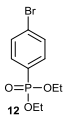
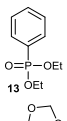
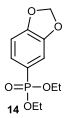
Scheme 1. Proposed synthetic route towards phosphorus containing pyrabactin analogues.

The first step in the synthetic sequence, the coupling reaction of aryl halides with diethyl phosphate or phosphite, was performed in two distinct ways: lithium-halogen exchange and transition metal catalysis (Table 1).

All three halogen containing phosphonates **9**, **10**, and **12** were prepared by treating bromoarenes **3**, **4**, and **6** with butyl lithium and diethyl chlorophosphate, using the lithium-halogen exchange approach. The synthesis of diethyl 4-fluoronaphth-1-ylphosphonate **9** proceeded without difficulties as expected, since lithium exchange selectively occurs with the bromide (Table 1, Entry 1).

Table 1. Synthesis of diethyl arylphosphonates.



Entry	Aryl bromide	Aryl phosphonate	Reagents (equivalents)	Reaction conditions	Yield (%)[a]
1	 **3**	 **9**	BuLi (1.5) $ClP(O)(OEt)_2$ (1.5)	-78 °C, 1 h -78 °C (add phosphate) 20 °C, 1 h	88
2	 **4**[b]	 **10**	BuLi (1.1) $ClP(O)(OEt)_2$ (10)	-78 °C, 0.5 h -40 °C, 0.5 h -78 °C (add to phosphate) 20 °C, 1 h	74
3	 **5**	 **11**	$Pd(OAc)_2$ (0.02) PPh_3 (0.06) DIPEA (1.5) $HP(O)(OEt)_2$ (1.2)	78 °C, 16 h	96
4	 **6**	 **12**	BuLi (1.1) $ClP(O)(OEt)_2$ (10)	-78 °C, 0.5 h -40 °C, 0.5 h -78 °C (add to phosphate) 20 °C, 1 h	64
5	 **7**	 **13**	$Pd(OAc)_2$ (0.02) PPh_3 (0.06) DIPEA (1.5) $HP(O)(OEt)_2$ (1.2)	78 °C, 16 h	82
6	 **8**	 **14**	$Pd(OAc)_2$ (0.02) PPh_3 (0.06) DIPEA (1.5) $HP(O)(OEt)_2$ (1.2)	78 °C, 16 h	85

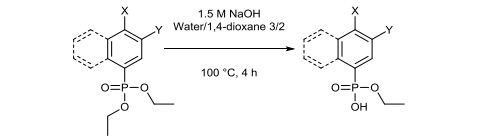
[a] After purification via column chromatography with a mixture of ethyl acetate and hexanes as eluent and silica as adsorbent. [b] 1,4-Dibromonaphthalene **4** was purchased from TCI Europe or synthesized from naphthalene (Y = 87%) based on a modified procedure of Cakmak et al.[10]

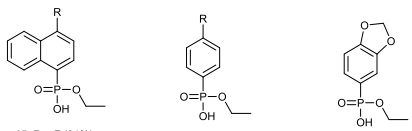
The only observed by-product was 4-fluoronaphthalene, due to early quenching of the reactive species. In order to avoid this side reaction, the amounts of both reagents were elevated from 1.1 to 1.5 equivalents and the reaction time after chlorophosphate addition was extended from 30 minutes to 1 h. The synthesis of the corresponding 4-bromonaphth-1-ylphosphonate **10** entailed more complications as both bromine atoms are susceptible to lithium-halogen exchange. Indeed, the reaction of 1,4-dibromonaphthalene **4** with butyl lithium and diethyl chlorophosphate, using the optimized procedure for the synthesis of diethyl 4-fluoronaphth-1-ylphosphonate **9**, generated multiple side products. The most common by-products were either formed by double lithiation of the starting material or via reaction of lithiated naphthalene with the desired end product. Several adjustments were made to improve the procedure and minimize inefficient side product formation. First of all, as the solubility of 1,4-dibromonaphthalene **4** in Et_2O appeared to be poor at -78 °C, lithiation was performed for an additional 30 minutes at -40 °C in order to obtain complete dissolution and lithiation of the starting material. To avoid double lithiation at the elevated temperature, the amount of added butyl lithium was reduced to 1.1 equivalents. Self-condensation was circumvented by ensuring a constant excess of diethyl chlorophosphate via increasing the amount to 10 equivalents. Moreover, lithiated 4-bromonaphthalene was added to the excess of diethyl chlorophosphate in Et_2O. Prior to this inversed addition, both solutions were cooled to -78 °C to minimize temperature rise during transfer via the cannula. As such, acceptable conversion and yield were obtained (Table 1, Entry 2). The same procedure was applied for the synthesis of diethyl 4-bromophen-1-ylphosphonate **12** (Table 1, Entry 4).

Diethylphosphonates **11**, **13**, and **14** were prepared via the Hirao cross-coupling reaction, an efficient method for organo-phosphorus syntheses developed in the early 1980's.[11] Several Hirao-based procedures were evaluated, including the use of microwave technology and acetate additives.[12] Despite the good yields reported in these papers, we only managed to achieve a limited conversion and/or poor purity. However, the method developed by Gooβen and Dezfuli, which employs an alcoholic solvent and a sterically demanding tertiary amine as base, generated satisfactory results and all products were obtained in very good yields (Table 1, Entry 3, 5, 6).[13] Although the yields using this method were generally higher than the yields obtained via the lithium-halogen exchange method, the procedure could not be applied to improve the phosphonylation of dibromoarenes as the Hirao-coupling reaction is not selective towards one bromine atom.

Prior to the coupling reaction of the synthesized phosphonates (**9**-**14**) with an amine or alcohol, activation of the phosphonate group for nucleophilic addition is required. The activation was carried out in two steps: hydrolysis with NaOH to obtain the monoethyl arylphosphonate and subsequent conversion to the corresponding phosphonyl chloride. The monohydrolysis reaction proceeded efficiently in a mixture of 1,4-dioxane and water containing 1.5 M NaOH. Both solvents were proven to be required because reactions in 2.5 M NaOH as well as in 1,4-dioxane with six equivalents NaOH, both at reflux, yielded only trace amounts of hydrolyzed product. The optimized reaction conditions are a 1:1 to 2:3 ratio dioxane:water and a 4 h reflux period. All products (**15**-**20**) were obtained in good to excellent yield and purity after acid-base extraction (Table 2).

Table 2. Monohydrolysis of diethyl arylphosphonates.





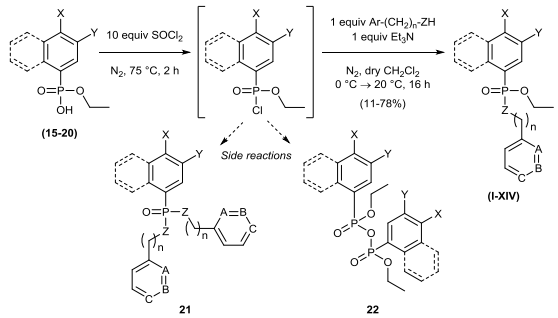
15: R = F (91%)
16: R = Br (93%)
17: R = H (94%)

18: R = Br (90%)
19: R = H (74%)

20 (86%)

A first approach to the synthesis of activated phosphonyl chlorides involves the use of thionyl chloride as chlorinating agent (Scheme 2).[14] $SOCl_2$ also served as solvent as dilution of the reaction mixture with dry Et_2O entailed a severe drop in yield. The monoethyl arylphosphonates (**15**-**20**) were dissolved in 10 equivalents $SOCl_2$ and heated to reflux. After completion of the reaction, $SOCl_2$ was evaporated and the unpurified residue was redissolved in dry CH_2Cl_2. One equivalent of the appropriate amine or alcohol and triethyl amine were added and the mixture was stirred for 16 h at 20 °C. Extractive work up was generally followed by purification via column chromatography. In some cases, additional purification steps

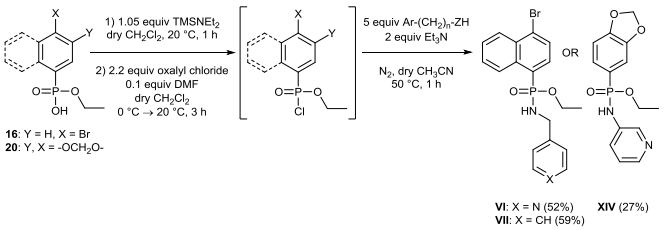
were required (see ESI†, Table S-1). The isolated yields range from 11% to 78%.



Scheme 2. Activation and coupling reaction of monoethyl arylphosphonates **15-20** with an amine or alcohol using thionyl chloride.

Two types of by-products, both formed during the activation step, were frequently detected (see ESI†, Scheme S-2, Figure S-1 and S-2, Table S-2). Most commonly, self-condensation of the activated phosphonate with the inactivated equivalent took place. The resulting dimeric anhydride (**22**, Scheme 2), however, can react during the coupling step, which results in at most 50% of the desired analogue and 50% of starting product. The second occurring phenomenon was overactivation of the phosphonic acid and hence, double addition-elimination of the nucleophile (**21**, Scheme 2). The dual activated phosphonic dichloride is formed via a $SOCl_2$-driven dealkylation mechanism, similar to the cleavage of dialkylphosphonates with trimethylsilyl halide.[15]

In attempt to avoid these side reactions, a second chlorination method, using $TMSNEt_2$ and oxalyl chloride, was evaluated (Scheme 3).[16] This alternative activation method proved to be successful for the synthesis of pyrabactin analogues **VI** and **VII** starting from ethyl 4-bromonaphth-1-ylphosphonic acid **16**, and enhanced the yield of the activation and coupling step from 11-13% to 52-59%. In contrast, the synthesis of analogue **XIV** using oxalyl chloride and DMF resulted in only slightly improved yield compared to the thionyl chloride method, as formation of dimeric anhydride remained significant.



Scheme 3. Activation and coupling reaction of monoethyl arylphosphonates **16** and **20** with an amine using oxalyl chloride.

Despite these difficulties in the final synthetic step, a total of 14 phosphonic pyrabactin analogues were synthesized in acceptable overall yields. A summary of their structures is given in Figure 2.

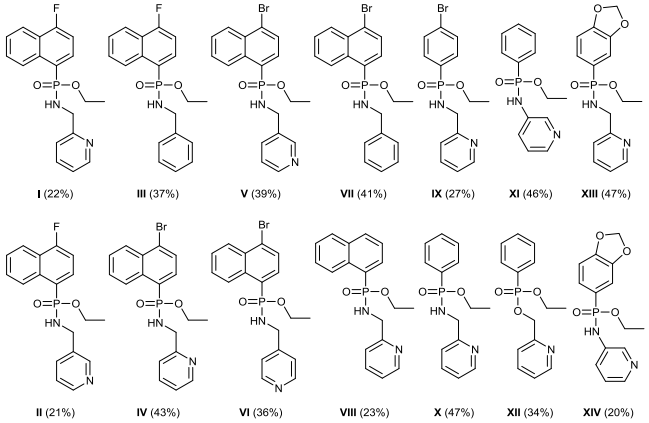


Figure 2. Overview of the prepared phosphonamide and phosphonate pyrabactin analogues and their overall yield (4 steps).

The ability of the novel phosphonamide and phosphonate pyrabactin analogues to selectively elicit certain ABA-related responses in plants was assessed via a stomatal closure and germination assay. In case of abiotic environmental stress, such as decreased water availability or increased salinity, ABA **1** accumulates in the leaves where it mediates stomatal closure by changing the osmotic potential of the guard cells through the regulation of Ca^{2+} levels.[17] As a measure for drought response, ABA-induced stomatal closure has been extensively studied in for example *Vicia faba*, *Nicotiana tabacum* and *Arabidopsis thaliana*.[18] In this biological assay, the effect of the phosphonic pyrabactin analogues on the stomatal aperture of detached abaxial tobacco (*N. tabacum*) leaf epidermal strips was measured and compared to pyrabactin **2** and ABA **1** (Figure 3). As both isomers of abscisic acid show different biological activity, the optically pure natural enantiomer ((+)-ABA) was used as reference.[19] More detailed graphs for each treatment, specifying the standard error of the mean and the significance of the difference between the aperture averages, can be found in Figure S-5 of the ESI†.

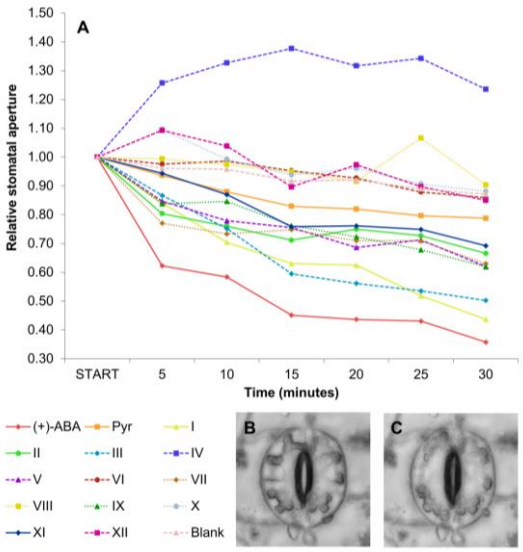


Figure 3. A. Average stomatal aperture in time, relative to the aperture measured at the start of the experiment, following 10 µM treatment with ABA **1**, pyrabactin **2** and pyrabactin analogues **I–XII** (stock solutions in DMSO). **B.** Untreated epidermal stoma. **C.** Epidermal stoma 30 minutes after treatment with 10 µM ABA in DMSO.

In the absence of the test compounds, a reduction of the stomatal aperture of approximately 10% was observed after 15

minutes, with no further change up to 30 minutes (see ESI†, Figure S-4). Therefore, 10% stomatal closure was considered a result of the incubation conditions.

ABA **1** is highly effective in reducing the stomatal opening and functions within 5 minutes. A relative closure of 64% was reached 30 minutes after incubating the epidermal peel in 10 µM ABA **1** solution. Pyrabactin **2** also induced stomatal closure rapidly, but to a much lower degree (21%) than ABA **1**. Several compounds of the small library of phosphonic pyrabactin analogues (**I**, **II**, **III**, **V**, **VII**, **IX** and **XI**) were found to reduce the stomatal aperture to a greater extent than pyrabactin **2** (Figure 3). While compounds **II**, **V**, **VII**, **IX** and **XI** are moderate inducers of stomatal closure (31-38% reduction in pore opening area), analogues **I** and **III** strongly affected the stomatal aperture, reducing the opening with more than 50% after 30 minutes. The speed at which the stomata close, indicated by the incline of the graph (Figure 3), is lower than for ABA **1**. A few analogues (**VI**, **VIII**, **X** and **XII**) did not significantly affect the size of the stomatal aperture.

Remarkably, unlike the other phosphonamide pyrabactin analogues described, compound **IV** did not cause closure of the stomata, but induced opening when the intrinsic closure of 10% in untreated stomata is taken into account. This observation is intriguing, as a high structural resemblance is present between analogue **IV** and the newly identified ABA-agonists. Moreover, analogue **IV** is not a true ABA-antagonist,‡ suggesting that this molecule does not induce the typical ABA-signaling process in the stomata.

A second well known hormonal effect of ABA **1** is the inhibition of seed germination.[20] Also pyrabactin **2** was proven to be an effective germination inhibitor.[4] The inhibitory effect on seed germination of the novel phosphonic pyrabactin analogues was investigated via an *Arabidopsis thaliana* germination assay. The seeds were incubated on medium containing 10 µM of (+)-ABA **1**, pyrabactin **2** or the different pyrabactin analogues and the germination ratio was documented 10 days post growth at 25 °C (see ESI†, Figure S-6). The assay revealed that the analogues were poor inhibitors of germination (less than 14% inhibition). Only one compound (**II**) markedly suppressed seed germination (60% inhibition) compared to ABA **1** and pyrabactin **2** (38% and 34% inhibition respectively). These results indicate enhanced specificity of the novel pyrabactin analogues towards vegetative responses and confirm their selectivity in inducing certain ABA-signaling pathways.

In conclusion, 14 novel phosphonamide and phosphonate pyrabactin analogues were prepared in 4 steps. In general, the pyrabactin analogues induce ABA-like stomatal closure responses, indicating that they likely act as ABA-agonists. In addition, the analogues do not exert a general influence on the ABA-signaling pathway, as was confirmed by the absence of the typical ABA germination inhibition capacity. The ability to protect leaves from excess evaporation via the stomatal pores, without interfering with germination, makes these analogues very promising leads for agro-pharmaceuticals. Indeed, drought is one of the main factors limiting crop productivity, causing more than 50% yield loss worldwide.[21] Furthermore, the synthesized pyrabactin analogues may be helpful to unravel ABA-perception and –signaling involved in stomatal regulation. Complementary studies regarding compound stability and the effect on rooting processes of different species are ongoing to assess their applicability in agriculture.

Acknowledgements

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Notes and references

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† Electronic Supplementary Information (ESI) available. See DOI: 10.1039/b000000x/

‡ The absence of an antagonistic action of pyrabactin analogue **IV** was proven by measuring the pore area of 100 random stomata before and after treatment with 10 µM ABA **1** and a combination of 10 µM ABA **1** and 10 µM analogue **IV**. Upon combinational treatment, a pore area reduction of 39% was observed after 60 minutes, indicating that the effect of ABA **1** (pore area reduction of 31%) was not counteracted.

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