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ARTICLE TYPE

Exploring a cascade Heck-Suzuki reaction based route to kinase inhibitors using Design of Experiments

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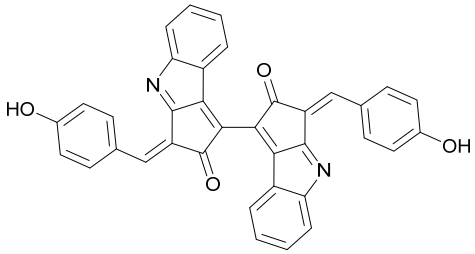
Design of Experiments (DoE) has been used to optimize a diversity oriented palladium catalyzed cascade Heck-Suzuki reaction for the construction of 3-alkenyl substituted cyclopenta[*b*]indole compounds. The obtained DoE model revealed a reaction highly dependent on the ligand. Guided by the model, an optimal ligand was chosen that selectively delivered the desired products in high yields. The conditions were applicable with a variety of boronic acids and were used to synthesize a library of 3-alkenyl derivatized compounds. Focusing on inhibition of kinases relevant for combating melanoma, the library was used in an initial structure-activity survey. In line with the observed kinase inhibition, cellular studies revealed one of the more promising derivatives to inhibit cell proliferation via an apoptotic mechanism.

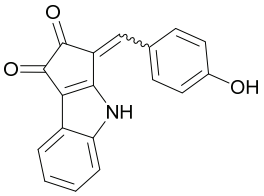
Introduction

While many tumour malignancies show a decrease in incidence, melanoma has continuously increased over the last 30 years.[1] Early diagnosed melanoma is associated with a high survivability. In contrast, patients with advanced melanoma (stage IV) only have a median survival of 6-10 month, with less than 5% surviving for more than 5 years.[2, 3] Recently, advances have been made in the treatment of metastatic melanoma by targeting the mutated kinase BRAF V600E, expressed in about half of all melanomas, with a selective inhibitor.[4, 5] The success has unfortunately been dampened by tumour adaptation, often giving only temporary therapeutic effects. Several possible solutions to avoid the development of resistance have been proposed, such as developing BRAF inhibitors based on new scaffolds or using a combination of inhibitors targeting different kinases.[6, 7]

We have previously reported the total syntheses of the biologically active cyanobacterial metabolites scytonemin (**1**) and nostodione A (**2**), both containing an unusual 3-benzylidene substituted cyclopenta[*b*]indole-2-one skeleton (Fig.1).[8, 9] In a recent study, nostodione A was synthesized together with a number of derivatives and were reported to display anti-*Toxoplasma gondii* activity.[10] Nostodione A has also been demonstrated to inhibit mitosis of sea urchin eggs by acting on the mitotic spindle.[11] Scytonemin was in 2002 reported to inhibit growth of Jurkat T-cells, which was explained by its ability to inhibit a number of cell cycle regulatory kinases.[12] Scytonemin has since then been demonstrated to inhibit a number of different cancer cell types.[13-15] In a recently initiated project, we are investigating the possibility to elaborate the 3-benzylidene substituted cyclopenta[*b*]indole-2-one skeleton into kinase inhibitor(s) relevant for combating melanoma. For this purpose, we need easily accessible derivatives of the skeleton to ultimately generate a library of compounds for the use in structure-activity modelling.



Scytonemin (**1**)

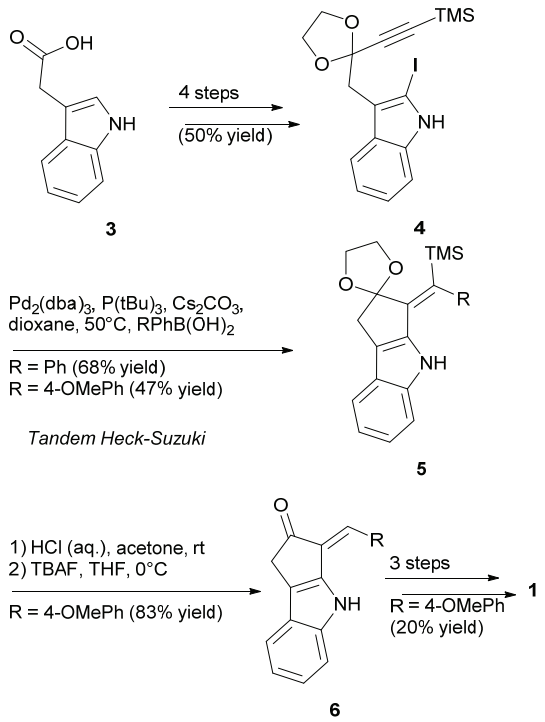


Nostodione A (**2**)

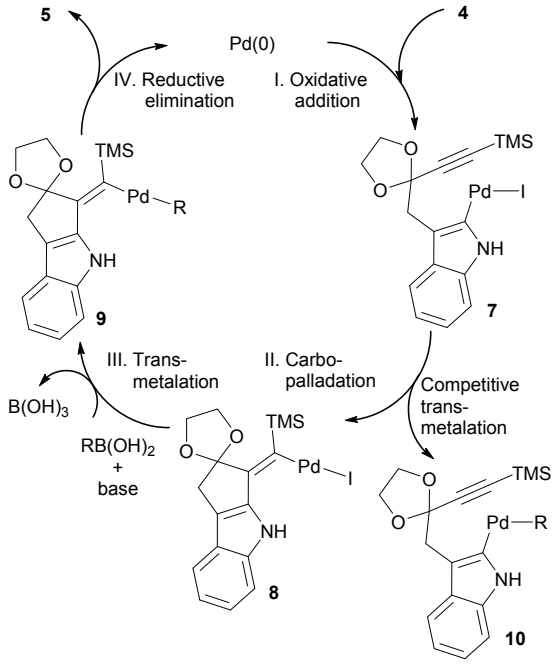
Fig. 1 Structures of scytonemin and nostodione A.

The synthesis of scytonemin is based on a palladium catalyzed tandem Heck-Suzuki reaction which has the potential to introduce different substituents at the exocyclic double-bond in the 3-position (Scheme 1). Here, oxidative addition of palladium to indole **4** forms intermediate **7**, which is annulated through carbopalladation to form **8** (Scheme 2).[16] The trapped vinylic palladium specie **8** reacts with an aryl boronic acid in a transmetallation step, forming **9** which after reductive elimination produce the desired product **5**. A number of examples of this type of cascade reaction can be found in the literature.[17-22]

Considering the vast number of commercially available boronic acid derivatives,[23] the reaction provides an excellent



Scheme 1 Synthetic route to scytonemin based on a diversity oriented tandem Heck-Suzuki coupling.



Scheme 2 Proposed catalytic cycle of the tandem Heck-Suzuki reaction.

opportunity for diversity oriented synthesis. However, with the previously used reaction conditions (Scheme 1),[9] only moderate yields were obtained. The yield was also affected by the electronic properties of the boronic acid coupling partners, which in part could be traced to the generation of a direct coupled byproduct obtained via intermediate **10** (Scheme 2) when electron rich substrates were used. Other studies on similar systems have also observed direct couplings,[19, 22] but also problems with hydride reduction of intermediate **8**, instead of transmetallation, to ultimately give a hydrogen bearing exocyclic double bond.[21] Another reported problem regards the incomplete control over the stereoselectivity in the formation of the exocyclic double bond.[17] The problems are usually solved by reaction screening, often guided by other studies and/or chemical perception, until satisfactory yields are obtained. This can be an excellent method for finding good reaction conditions with only a few attempts. However, by performing non-comparable experiments, a set of promising reaction conditions cannot be improved further by comparing them to other experiments in the screening process. It is also unlikely that a trial and error process will give any information to which reaction variables are important and what implications they have on the reaction mechanism. On the other hand, changing one variable at a time will give valuable, easily interpreted chemical information. However, only a small portion of the reaction space will be investigated, giving a local maximum of the yield to a high experimental prize.

As an alternative, Design of Experiments (DoE) is a methodical approach to investigate and optimize reaction variables (factors) by their simultaneous variation.[24] Each factor is usually investigated at two levels (+ and -) which defines the reaction space under study. By using statistical regression analysis of a measured reaction outcome, a precise quantitative data model is obtained, consisting of the significantly influencing reaction variables and also their interaction with each other. The model can be used to find optimal reaction conditions within the investigated reaction space, or to reveal in which direction outside the confinement to look for optimal conditions. However, for efficient use in DoE, the variables are required to be continuous, such as temperature and concentration. A clear limitation as many variables in a typical catalytic reaction are discrete, such as catalyst and ligand. To enable the use of discrete variables in DoE, another data analysis technique, principal component analysis (PCA), can be used in combination.[25, 26] When the discrete variables are chemical entities (e.g. ligands or solvents), these can be described by a large number of chemical properties, either physical attributes (e.g. melting point, density, HPLC retention times) or theoretical calculated (e.g. HOMO, LUMO, bond angles). PCA reduce the dimensions of the data set by introducing new latent variables, which capture as much of the variance (information) in the data set as possible.[27] The new latent variables (also called principal properties or principal components) can be used to describe the discrete variables in a continuous manner, ultimately enabling their use in DoE. The technique of combining DoE and PCA was pioneered by Carlson[25] and has recently been described in an instructive manner by Moseley.[26]

Here, we report the use of Design of Experiments (DoE) to optimize a diversity oriented tandem Heck-Suzuki reaction for the synthesis of cyclopenta[*b*]indole-2-one skeleton based compounds derivatized in the 3-position, with the purpose of engineering a library of compounds to be used in biological structure-activity studies. Scope and limitation of the reaction is investigated and an initial bioactivity study of synthesized compounds is presented.

Results and discussion

Design of Experiment

Table 1 Limits of investigated factors and constant values on non-investigated factors.

Factor	+	-	Constant values
t1[a]	-5.08	4.74	n/a
t2[a]	-6.00	4.11	n/a
Ham[b]	-0.27	0.54	n/a
Bor[c]	1 equiv.	3 equiv.	n/a
C[d]	0.04 M	0.08 M	n/a
H_2O[e]	1 equiv.	3 equiv.	n/a
Solvent	n/a	n/a	1,4-dioxane
Temperature	n/a	n/a	50°C
Precatalyst	n/a	n/a	$Pd_2(dba)_3$•$CHCl_3$
Catalytic loading	n/a	n/a	0.05 equiv.
Ligand loading	n/a	n/a	0.20 equiv.
Base	n/a	n/a	Cs_2CO_3
Base loading	n/a	n/a	2.5 equiv.
Time	n/a	n/a	24 h
Experiment scale	n/a	n/a	50 mg
Stirring speed	n/a	n/a	600 RPM
Reaction vessel	n/a	n/a	Tube (ø 15mm)

[a] The two first latent variables, *t1* and *t2*, for a P-donor ligand dataset according to Jover et al.[28] [b] Hammett constants of investigated *para*-substituted phenylboronic acids.[29] [c] Loading of boronic acids. [d] Reaction concentration. [e] Loading of water.

To resolve the previously discussed problems of the tandem Heck-Suzuki coupling, and thereby facilitating the synthesis of a diverse set of derivatives, an optimization study using design of experiments (DoE) was pursued. The number of experiments that need to be conducted increases rapidly with an increasing number of investigated factors; 3 factors give 2^3 experiments while 6 factors give 2^6 experiments in a full factorial design. With limited resources, it is therefore important to restrict the variables to those assumed to be most relevant. The variables investigated in the current study are: the ligand (*t1* and *t2*), the electronic property of the boronic acid (*Ham*), the loading of boronic acid (*Bor*), the overall concentration (*C*) and the loading of water (H_2O) (Table 1). The variable names are reported in *italic* throughout this article.

Ligands are well known to affect metal catalyzed reactions and have in other investigated cascade Heck-Suzuki reactions been shown to affect both the stereochemical outcome and the amount of direct coupled byproduct.[17, 19] Principal component analysis for extensive datasets of monodentate P-donor ligands, chelating P,P-donor ligands and N-heterocyclic carbene ligands using calculated chemical properties have recently been published.[28, 30, 31] In the present study, monodentate P-donor ligands were used exclusively due to commercial availability and documented applicability in both Heck and Suzuki reactions.[16] The published dataset consists of 348 ligands explained by 28 quantum mechanically calculated properties. The two first latent variables (*t1* and *t2*), which explain 65% of the variance in the dataset, were selected in this study to describe the ligands. The variables *t1* and *t2* have been described to roughly capture the electronic properties (low *t1* correspond to electron poor and high *t1* with electron rich ligands) and the size (low *t2* correspond to large and high *t2* with small ligands). Four ligands, covering a large part of the ligand space, were initially chosen for the investigation (Fig. 2). These were later complemented with four additional ligands to investigate non-linear relationships, *vide infra*. Triphenylphosphine was used as the center point.

As previously mentioned, the electronic properties of the

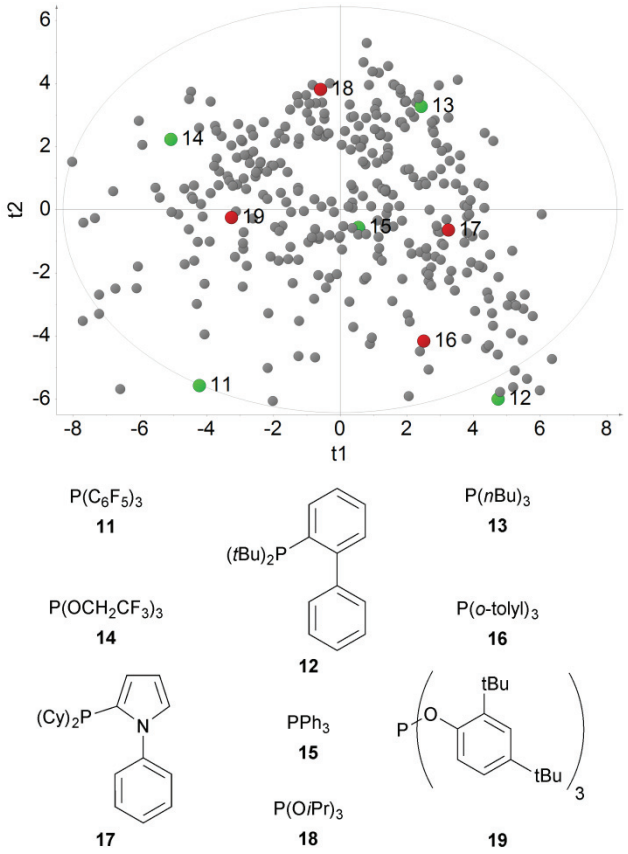


Fig. 2 Top: P-donor ligand space defined by the two first latent variables as described by Jover et al.[28] Initially chosen ligands in green and ligands complemented for investigating non-linear relationships in red. Bottom: structures of investigated ligands.

boronic acid coupling partner affects the reaction outcome, partly by affecting the rate of the transmetallation step.[22] Having a broad reaction scope is essential in this diversity oriented reaction. To model the electronic properties, and thereby finding reaction conditions applicable to a wide range of boronic acid derivatives, the Hammett constant was introduced as a reaction variable (*Ham*). 4-methoxyphenylboronic acid and 4-trifluoromethylphenylboronic acid were used as the low respective high limit. 4-chlorophenylboronic acid, with a Hammett constant of 0.23, was used as the center point.[29]

The byproduct obtained from direct coupling via intermediate **10** (Scheme 2) has previously been discussed. It is formed when the intramolecular carbopalladation (step II) is of similar rate to the undesired intermolecular transmetallation. To fine tune the relative intramolecular/intermolecular reaction rate, the global reaction concentration (*C*) and the amount of boronic acid (*Bor*) were introduced as reaction variables. Water is crucial in Suzuki couplings and has recently been shown to, via deprotonation by the base, form a palladium hydroxide specie essential for the transmetallation step.[32, 33] The amount of water (H_2O) will thereby affect the reaction and was therefore investigated as well.

Initially, solvents were also investigated by using the two first latent variables from a PCA of 113 solvents using nine descriptors.[25, 34] The solvents tested were methanol, N-methylpyrrolidone (NMP), 1,4-dioxane, *tert*-butyl methyl ether (TBME) and *p*-xylene. Unfortunately, these solvents produced too much variance in the reaction outcome and could therefore

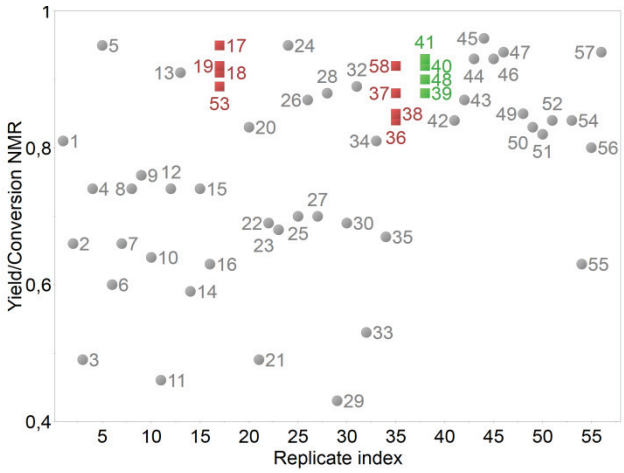


Fig. 3 Replicate plot of performed experiments. Replicates of the center point in green and replicates of the *Ham* axial points in red.

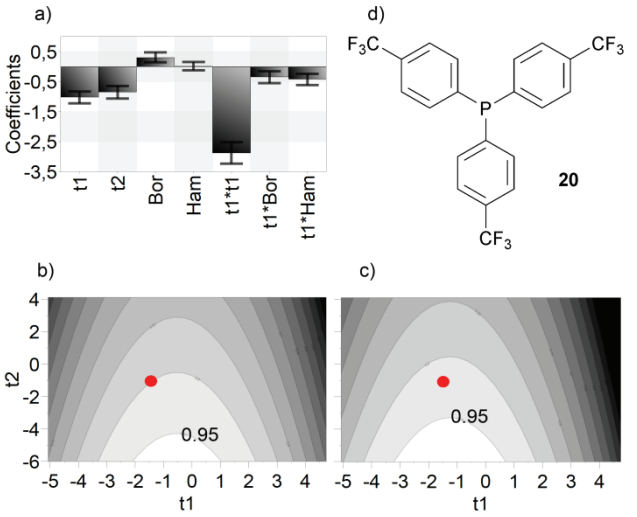


Fig. 4 a) Significant coefficients (parameters) at a 95% confidence level for yield/conversion (y/c) transformed according to $e^{2(y/c)}$. The ratio y/c was measured by NMR against benzyl benzoate as an internal standard. Multiple linear regression was used for data fitting after excluding outlying experiment No 31. b) Response surface map of the ligand space with *Ham* = -0.27 and *Bor* = 1.5 equivalent. y/c is presented on the contour lines with an decline of 0.05. The position of ligand **20** is shown as a red dot. c) Same as in b) but with *Ham* = 0.54. d) Structure of ligand **20**.

not be modelled. NMP degraded the substrate and only trace amount of product could be observed. Methanol gave varying results depending on the reaction conditions while TBME and xylene reacted sluggishly under all tested conditions. Consequently, the best solvent, 1,4-dioxane, was chosen for the remainder of the study. The rest of the factors were kept constant (Table 1). Cesium carbonate is a common choice as base in palladium catalyzed cross-couplings. Pd(0) precatalyst was chosen to avoid an activation step, which may be influenced by the choice of ligand and thereby complicating the model. A rather low temperature was chosen to maximize the stereoselectivity of the reaction.

The reactions were analyzed both by HPLC (using an external calibration curve) and NMR (using an internal standard) to minimize biasing of respective method. The analyzed and optimized response variable was the ratio between yield and conversion (y/c), which represents how much starting material that is converted to product. The ratio y/c is a more informative response variable than yield or conversion alone, as reactions with low yields are not automatically discarded as poor and reactions with high conversions are not automatically regarded as good.

To reduce the number of initial experiments, while still maintaining the possibility to model two factor interactions, a fractional factorial design of resolution V was chosen (Table S1, ESI†). The number of experiments were thereby reduced to 41 from 73 necessary in a full factorial design. The number of experiments can be reduced further by choosing fractional factorial designs of lower resolution, but at the cost of severe confounding (aliasing) of effects, i.e. the coefficients obtained after analysis are the sum of two or more "real" effects. In a resolution V design, main effects and interaction effects are only confounded with higher order interaction effects, which are unlikely to contribute.[24]

After the initial 41 experiments, we were pleased with the high degree of experimental reproducibility established by triplicate runs of each boronic acid (Fig. 3). We have previously observed problems with certain boronic acids, which prompted us to perform triplicate runs for each one of them. The triplicate runs were performed with intermediate values on all other variables, which in terms of factorial design correspond to the center point for 4-Cl-phenylboronic acid and *Ham* axial points for 4-OMe- and 4-CF_3-phenylboronic acid.

The HPLC and NMR analyses were in good agreement, indicating that the measured outcome was reliable. Further, the reaction did also display an almost complete stereoselectivity favouring the predicted isomer. Unfortunately, no linear interaction model could be obtained by multiple linear regression within the investigated reaction space. It is clear from Fig. 3 that the center point had among the highest responses. The center point has intermediate values on all variables and should consequently have an intermediate response (y/c) to fit a linear model. Thus, it appeared necessary to introduce quadratic term(s) to model the experimental data. Accordingly, axial points were introduced, thereby evolving the original fractional factorial design into a central composite face design.

Introduction of axial points for the variables *t1* and *t2* required four additional ligands to be investigated (Fig. 2). Additional experiments corresponding to the center point and axial points for *Ham* were also included to verify that the new set of experiments were comparable with the original experiments. Totally, another 17 experiments were performed (42 to 58, Fig. 3). Gratifyingly, introducing a quadratic *t1* term and transforming the response according to $e^{2(y/c)}$, produced a model which explained 86% of the observed variance and displayed a good predictive power (Fig. 4, Table 2). The model error was also small, i.e. the model showed no lack of fit. Introducing other quadratic terms (e.g. *t2*×*t2* or *Bor*×*Bor*) produced inferior models in terms of e.g. explained variance, predictive power and model error. One outlying experiment, no 31, was excluded after being rerun three times without improving its fitness to the model.

Of the investigated reaction variables, the ligand factors *t1* and *t2* were clearly the most important (Fig. 4). In fact, the ligand variables and their interactions with other variables are

Table 2 ANOVA and statistics for the quadratic model displayed in Fig. 4.[a]

	Degrees of freedom	Sum of squares	Mean squares	F	p
Total	57	1505.42	26.41		
Constant	1	1415.92	1415.92		
Total corr	56	89.50	1.60		
Regression	7	78.38	11.20	49.34	<0.0005
Residual	49	11.12	0.23		
Lack of fit	40	10.07	0.25	2.17	0.108
Pure error	9	1.05	0.12		

[a] Q2 = 0.827, R2 = 0.876, adj R2 = 0.858, Cond. no. = 3.637, RSD = 0.476

accountable for the five largest effects on the reaction. The combination of negative coefficients for *t1*×*t1* and *t1* can be interpreted as a reaction preference for ligands of an electron neutral to slightly electron deficient character, which is easiest visualized in a response surface map (Fig. 4). The negative coefficient of *t2* indicates a preference for larger ligands over smaller (Fig. 4). Further, the two interaction effects, *t1*×*Bor* and *t1*×*Ham* gave unusual insight to this reaction. The combination of the *t1*×*Bor* and *Bor* coefficients indicate a beneficial effect from high boronic acid loadings when using electron deficient ligands, but as the ligands become more electron rich the reaction benefits from lower boronic acid loadings. This can possibly be attributed to an enhancement in transmetallation rate when using high boronic acid loadings, thus producing more byproduct via intermediate **10** (Scheme 2). The interaction effect predicts electron poor ligands to have an antagonistic effect on the increased rate, and thereby suppressing the byproduct forming step. The negative *t1*×*Ham* effect shows that electron poor ligands are beneficial for electron poor boronic acids while the opposite relationship is observed for electron rich boronic acids. The electronic properties of boronic acids are known to affect the rate of transmetallation.[35] A recent computational study points toward a negative correlation between transmetallation rate and the electron density of boronic acids, i.e. electron poor boronic acids react faster than electron rich.[36] The interaction effect predicts that electron deficient ligands will counter the increased transmetallation rate, which is in line with the previously discussed interaction between boronic acid loading and ligand. Interestingly, the observed phenomenon contradicts the known literature. Although studies on ligand-transmetallation effects are scarce, a computational study predicts the opposite ligand behaviour, i.e. electron deficient ligands *increase* the rate of transmetallation.[37] Throughout this discussion, the latent variable *t1* has been used to account for pure electronic effects. It is important to remember that *t1* to some degree explains steric properties of the ligand as well, and the physical properties behind the interaction effects are therefore slightly more intricate than discussed here. It is also important to point out that the independent ligand effects to a large extent overrides these more subtle interaction effects, which is clear from the small differences between the ligand response contour maps for the two extremes of the *Ham* variable (Fig. 4). Finally, neither the reaction concentration nor the loading of water significantly affected the reaction within the investigated interval.

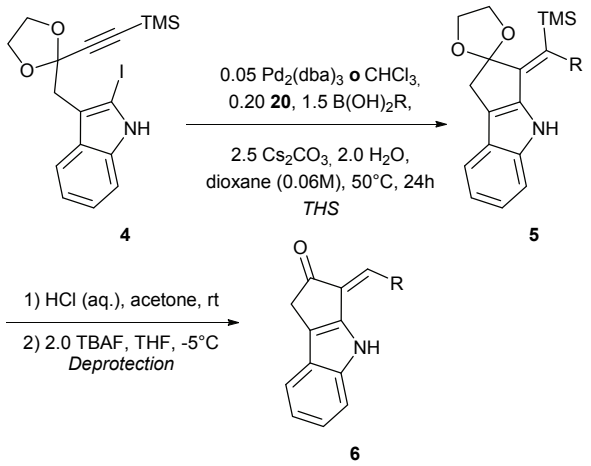
Taken together, the choice of ligand seemed imperative for optimizing the reaction within the examined reaction space. To this end, the response surface maps were used as a guiding hand in selecting a ligand applicable to both electron rich and electron poor boronic acids. Unfortunately, there are no commercially available ligands at the hotspots, but ligand **20** (Fig. 4) is quite close in both cases. Together with ligand **20**, 1.5 equivalents of the boronic acid and intermediate values on the non influential factors, concentration and water, were chosen as optimal reaction conditions. Larger excess of boronic acid is predicted to give slightly better results, but not enough to justify its use.

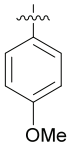
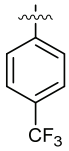
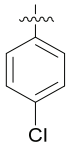
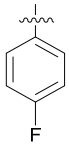
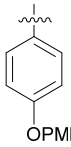
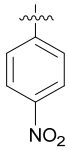
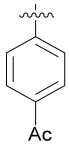
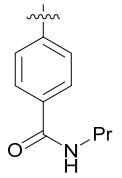
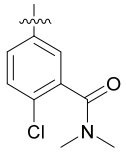
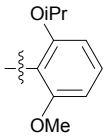
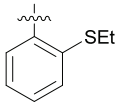
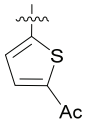
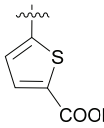
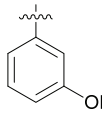
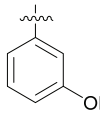
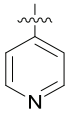
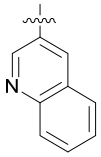
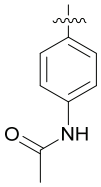
Scope and limitation

The applicability of the optimized tandem Heck-Suzuki reaction was investigated using 20 boronic acids derivatives, including the three used in the optimization study (Table 3). Good results were obtained for arylboronic acids with electron donating and inductively electron withdrawing substituents (entry 1-5). However, when conjugated electron withdrawing substituents were attached in the *para*-position, the results were less satisfactory, even with a four time increase in catalytic loading (entry 6-8). Electron withdrawing substituents in the *meta*-position, which do show any mesomeric effect, did on the other hand not affect the reaction negatively (entry 9). *Ortho*-substituted arylboronic acids were incompatible with this reaction (entry 10-11). Considering the sterically congested palladium intermediate **8** to which the aryl group need to be transferred, the observed incompatibility was not surprising. In contrast, the reaction had good compatibility with thienylboronic acids (entry 12-15). In analogy with the substituted phenylboronic acids, a conjugated electron withdrawing substituent in the pseudo *para*-position of the 2-thienyl derivative caused a significant drop in conversion (entry 14). However, increasing the catalytic loading produced sufficient material to be isolated. Noteworthy, the acid substituted thienylboronic acid did not participate in the reaction (entry 15). The lack of reactivity can most likely be attributed to a palladium-carboxylate ion coordination rather than to a change in electronic properties. This hypothesis was strengthened by the reactions with 3-phenolboronic acid (entry 16) and 3-benzyloxyphenylboronic acid (entry 17). While the protected phenol undergoes a smooth conversion to product, the phenol itself reacts sluggishly, suggesting a hampering phenoxy-palladium coordination at the basic conditions used. Pyridines were also challenging substrates (entry 18-19). The poor reaction of 4-pyridineboronic acid could be ascribed to the mesomeric electron-withdrawing property of the nitrogen. This cannot be the case for the 3-quinolinboronic acid, suggesting that another effect is in play. However, by increasing the catalytic loading, good results could be obtained with both pyridine derivatives. Finally, the electron neutral 4-acetamidphenylboronic acid reacted smoothly at a higher catalytic loading (entry 20).

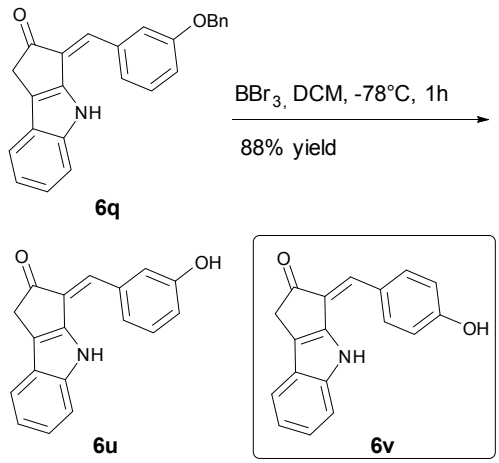
The products successfully obtained from the cascade reaction could be deprotected with moderate to good yields in a two step sequence (Table 3) to give precursors of both scytonemin and nostodione A derivatives. After TMS-excision, the exocyclic double bond isomerized spontaneously to produce a set of isomers. The thermodynamic equilibrium depended on the electronic characteristics of the aryl substituent. Most compounds had an equilibrium well shifted toward the E-isomer (>98%), which is the isomer depicted throughout this report. Compounds

Table 3 Scope and limitation of the tandem Heck-Suzuki reaction and yield of subsequent deprotections



Entry	Molecule	R	Yield THS[a] (%)	Yield deprot.[b] (%)
1	a		89	83[9]
2	b		89	75
3	c		92	87
4	d		86	70
5	e		93	80
6	f		35, 64[c]	60
7	g		13, 24[c]	n/a
8	h		8 7[c]	n/a
9	i		84	40%
10	j		12 20[c]	n/a
11	k		1	n/a
12	l		84	78
13	m		88	n/a
14	n		35 54[d]	86
15	o		0	n/a
16	p		15 26[c]	n/a
17	q		94	81
18	r		16 78[c]	66
19	s		16 73[c]	54
20	t		21 87[c]	47

[a] NMR yield using benzyl benzoate as internal standard. [b] Combined isolated yield over ketal hydrolysis and TMS-excision. n/a: precursor was not isolated from the tandem Heck-Suzuki reaction. [c] 0.20 $Pd_2(dba)_3$•$CHCl_3$, 0.8 **20**. [d] 0.15 $Pd_2(dba)_3$•$CHCl_3$, 0.6 **20**.



Scheme 3 Synthesis of *meta*-substituted scytonemin monomer **6u**. In the box: the scytonemin monomer **6v** obtained during synthesis of nostodione A.

with a strongly electron withdrawing substituent conjugated to the exo cyclic double bond did on the other hand demonstrate a more evenly distributed isomeric mixture, e.g. the E/Z ratios of **6f** and **6n** are close to 1:1.

The benzyl-group of **6q** could further be removed with borontribromide to give a *meta*-substituted monomeric structure of scytonemin (**6u**, Scheme 3). The *para*-substituted counterpart **6v**, which is interesting due to its similarity to both scytonemin and nostodione A, had already been obtained as an intermediate during the synthesis of nostodione A,[8] but could very well be synthesized here from a benzyl-protected substance in analogy with the *meta*-derivative.

Biological evaluation

Initially, the ability of scytonemin (**1**), nostodione A (**2**) and the scytonemin monomer (**6v**) to interact with kinases, either relevant to cancer in general or melanoma in particular, were investigated in a small screening study (Table S3, ESI†). Scytonemin, which has demonstrated kinase inhibitory properties in previous studies, did surprisingly not exhibit the most interesting bioactivity. Instead, the previously unreported monomeric compound **6v** demonstrated inhibition of BRAF V600E as well as IRAK4, Aurora A and Aurora B, all of which have been suggested as potential targets for melanoma treatment.[4, 5, 38, 39] Interestingly, we also found **6v** to be fluorescent, which allows drug properties such as membrane permeability and cellular localization to be imaged in live cells.[40, 41] Based on these results, cell studies were done to determine the viability of **6v** as a starting-point for developing kinase inhibitors. The *in vitro* toxicity was investigated in peripheral blood mononucleated cells (PBMC) by measuring the DNA-synthesis via incorporation of ^{3}H-thymidine after treatment with **6v**. The IC_{50} value was measured to ~20µM (Fig. 5a), which can be compared to IC_{50} values in the low nM range typical for cytotoxic compounds used in chemotherapy.[42-44] The low toxicity was a rewarding observation, as a highly toxic compound indicates that unknown, unwanted mechanisms are in

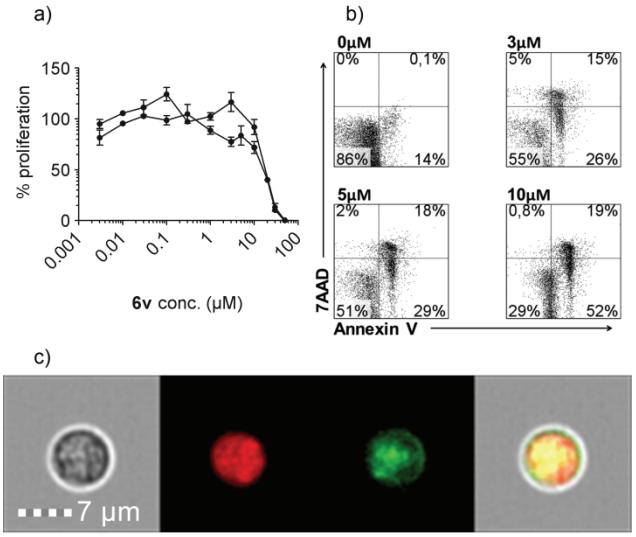


Fig. 5 a) Proliferation study of CD3/CD28 stimulated PBMC treated with a concentration range of **6v** for 2 days. Results from two separate experiments. b) Proliferating PBMC treated with 4 different concentrations of **6v** for 2 days were stained with AnnexinV/PE and 7AAD for the analysis of apoptosis. c) Representative fluorescent image of PBMC treated with 10 µM **6v,** showing from left to right: Bright field, nuclear staining with DRAQ5, fluorescence from **6v** and an overlay image of bright field, DRAQ5 and **6v**. Additional fluorescent images can be found in the electronic supplementary information (Fig. S5, ESI†).

effect, beside the kinase interactions looked for. Additionally, flow-cytometry analysis demonstrated that the cells undergo apoptosis upon treatment with **6v** (Fig. 5b). In line with these results, other studies have shown that selective inhibition of Aurora A, B and BRAF, all targets of **6v**, induce apoptosis in cells.[38, 45] To show that the substance can reach the desired kinases, which are located in the cytosol and nucleus, the cell permeability of **6v** was investigated by fluorescent microscopy. The molecule could to our delight be detected inside the cells, where it was distributed both in the cytosol and nucleus while leaving no observable damage to the cell membrane (Fig. 5c). This early examination of the cellular behaviour, showing **6v** to have limited toxicity, inducing apoptosis and being cell permeable was promising and the study proceeded with evaluating the kinase inhibiting properties of the compounds obtained from the scope and limitation survey.

The derivatives were tested against the four targets of the parent compound **6v**: Aurora A, Aurora B, BRAF V600E and IRAK4 (Fig. 6). Scytonemin (**1**) and nostodione A (**2**) were also included for comparison. Variation of the substituent at the exocyclic double bond had a large effect on the inhibition of BRAF and IRAK4, while the effect on the Aurora kinases was less significant. A striking example is the difference between the parent compound **6v** and compound **6a**, having a *para*-methoxy substituted phenyl ring instead of a *para*-hydroxy. While **6a** retained its inhibitory effects against Aurora A and B, the inhibition of both BRAF and IRAK4 was diminished completely. Most of the other derivatives did also display activity toward Aurora A and B, albeit less efficient compared to **6a** and **6v**. The derivatives inhibiting BRAF was on the other hand limited to **6i**, **6r**, **6t** and **6u**. Intriguingly, changing position of the hydroxyl group from *para* (parent compound **6v**) to *meta* (**6u**) did not alter the activity, while substitution to a methoxy (**6a**) completely

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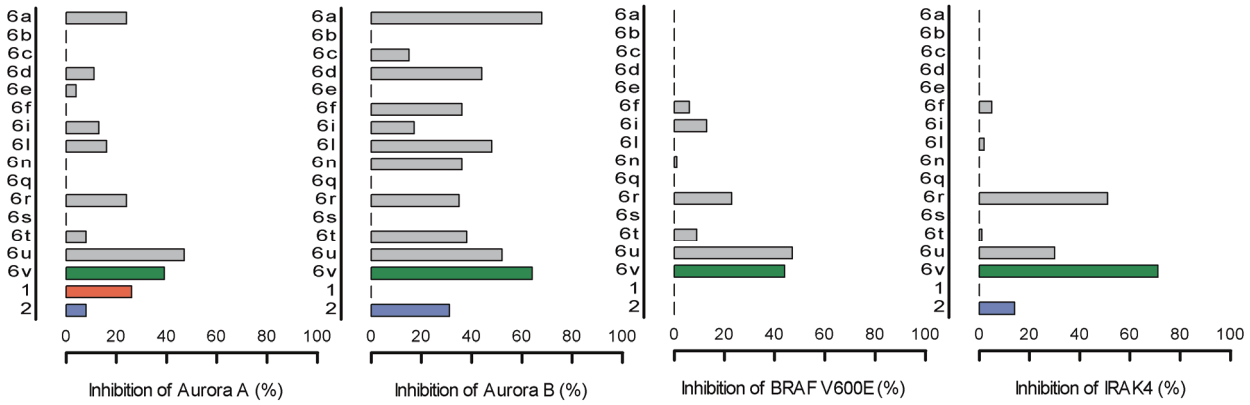

Fig. 6 Kinase inhibitory properties of investigated derivatives, parent derivative **6v** in green, scytonemin (**1**) in red and nostodione A (**2**) in blue. Reported inhibition of kinase activity after treatment with 10 µM compound and [ATP]≈Km. Results are reported as an average of two replicate experiments. Inhibition of Aurora B by scytonemin was not investigated.

erased it. Thus, a hydrogen bond donor seems to be necessary for good activity, albeit the optimal position is still unknown. The similar activities displayed by *para*- and *meta*-hydroxyl derivatives might also suggest that the BRAF binding pocket harbouring the derivatives is slightly flexible. Further, compound **6t** having a hydrogen bond donor in the same position as the parent compound demonstrated a weaker interaction with BRAF, implying either that the acetyl moiety sterically prevents a favourable hydrogen bond interaction or that a hydrogen bond acceptor is needed as well. The oxygen of the hydroxyl group can act as a hydrogen bond acceptor while the nitrogen of the amide cannot. The observed BRAF inhibition by **6r** supports the necessity of a hydrogen bond acceptor. IRAK4 displayed favourable interaction with only two derivatives, **6r** and **6u**. Here, changing position of the hydroxyl group from *para* (**6v**) to *meta* (**6u**) did change the activity, suggesting a more rigid kinase-inhibitor interaction. Further, inhibition by derivative **6r** signifies a constructive effect by the presence of a small hydrogen bond acceptor moiety. Another noteworthy trend is the inability of the more lipophilic compounds to inhibit kinases (i.e. **6b**, **6e**, **6q** and **6s**), which indicate that water solubility may be a limiting factor, a common phenomenon in medicinal chemistry. Finally, it is also interesting that the introduction of a second carbonyl in the 1-position (nostodione A, **2**) had a dramatic effect on the inhibiting properties of this scaffold. It reduced the activity against all kinases and totally diminished the inhibition of BRAF, compare **2** and **6v**.

Photophysical characterization of 6v

The molecule exhibits an absorption maximum at 410nm and emission maximum at 530nm in acetonitrile (Fig. S5, ESI†). It is as such suitable to excite with a 405nm laser.

Conclusions

We here report on the optimization of a cascade Heck-Suzuki reaction for the synthesis of various 3-alkenyl substituted cyclopenta[*b*]indole-2-one compounds. By using DoE, a predictive reaction model could be obtained. This was used to find optimal reaction conditions within the investigated reaction space. Of the investigated variables, the choice of ligand had the largest effect on the reaction, while variables of stoichiometric character such as concentration and reactant equivalents had little or no effect. This demonstrates the significance of modelling traditionally non-quantitative variables, such as ligands, in DoE. The choice of reagents (ligands, bases, catalysts, solvents etc.) often influence reactions more than the typical quantitative reaction variables (temperature, reaction stoichiometry etc.). By describing reagents with comparable quantitative data, which can be achieved by PCA, these can be included in continuous DoE modelling. Quantitative description of reagents is also a method for rationally choosing reagents for a screening process, where choices are often based on chemical intuition. The obtained reaction model also demonstrates how DoE can be used to acquire information otherwise hard to obtain. Specifically, it reveals that the optimal choice of ligand depends on the electronic properties of the boronic acid as well as the loading of boronic acid.

The applicability of the optimal reaction conditions described by the reaction model was investigated in a scope and limitation survey. This study revealed that *ortho*-substituted arylboronic acids were too bulky and that some arylboronic acids with conjugated electron withdrawing substituents were too unreactive to participate in the reaction. Apart from these limitations, the reaction conditions worked well and were used to synthesize 13 derivatives that could be transformed into precursors of scytonemin and nostodione A derivatives.

In bioactivity studies, we demonstrated that compound **6v** display anti-melanoma properties, inhibiting Aurora A, Aurora B, BRAF V600E and IRAK4. **6v** also inhibits proliferating PBMC. The PBMC were additionally shown to undergo apoptosis upon treatment with **6v**, in agreement with inhibition of Aurora A, B

and BRAF. Compound **6v** also display fluorescent properties, enabling its intracellular localization to be visualized. It exhibited cell permeably properties and was localized in both the cell cytosol and nucleus. The other synthesized derivatives were screened against the kinase targets of **6v** and interesting structural features affecting the bioactivity could be observed. The biological activity of the inhibitors displayed water solubility as a possible limitation, and future studies should investigate the possibility to introduce water solubilising substituents that do not interfere with the kinase inhibiting properties. Taken together, **6v** displays biological properties promising for further development into a melanoma relevant kinase inhibitor.

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Notes and references

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† Electronic Supplementary Information (ESI) available: Full experimental details, ^{1}H-NMR, ^{13}C-NMR and IR-spectra for all compounds. Detailed outline for DoE. See DOI: 10.1039/b000000x/

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