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Discovery of a potent and selective inhibitor for human carbonyl reductase 1 from propionate scanning applied to the macrolide zearalenone

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ABSTRACT

In order to extend the chemical diversity available for organic polyketide synthesis, the concept of propionate scanning was developed. We observed that naturally occurring polyketides frequently comprise not only acetate, but also some propionate as building blocks. Therefore our approach consists of a systematic replacement of some of the acetate building blocks during synthesis by propionate moieties, resulting in additional methyl groups that may give rise to different properties of the polyketides. Here we present the results of a first 'proof of concept' study where a novel zearalenone analogue **5** was prepared that comprises an additional methyl group at C5′. Key steps in the synthesis of **5** include a Marshall–Tamaru reaction, a Suzuki cross-coupling reaction, and a Mitsunobu lactonization. Compared to the parent zearalenone (**1**), analogue **5** showed reduced binding to a panel of human protein kinases and no binding to human Hsp90. On the other hand, however, **5** turned out to be a potent (IC_{50} = 210 nM) inhibitor of human carbonyl reductase 1 (CBR1).

1. Introduction

Many therapeutically useful compounds are based on natural products,[1] and natural products are often considered validated starting points for drug design. It has been argued that a relationship may exist between the enzymes that catalyze the biosynthesis of natural products and the targets of these compounds:[2] enzymes binding a compound during its biosynthesis may comprise similar protein fold topologies, that is, a similar arrangement of secondary structure elements around the active site, as the proteins targeted by the drug. It is obvious that natural products 'encode' the structural properties required for binding to their target proteins. Therefore, the concept of biology-oriented synthesis (BIOS) was developed that employs core structures delineated from natural products as scaffolds of compound collections.[3] For example, Waldmann and co-workers were successful in the development of biologically active molecules using BIOS.[4] In addition, many other groups have reported on the synthesis of libraries from natural product-like scaffolds.[5]

For any given natural product–receptor pair the question arises which functional group(s) and/or substituent(s) represent key elements for the binding affinity and selectivity. To elucidate this, scanning methods can provide valuable information. A common example is alanine scanning, where the replacement of individual amino acids within a peptide by L-alanine often reveals the relative importance of these side chain groups.[6,7] A fluorine scan applied to an inhibitor of thrombin showed the F···C=O interaction to be of special importance for the efficacy.[8] In order to map a binding site, affinity labelling and related approaches have been used.[9]

In natural products, such as terpenes or polyketides methyl groups represent common substituents in addition to hydroxyl- or keto groups. A somewhat surprising finding with many polyketide-based macrolactones is the exclusive use of acetate as building block. A prototypical example is zearalenone (**1**) (Fig. 1) which belongs to the wide-spread resorcylic lactones (RAL).[10] The biosynthesis of benzolactones of the zearalenone type is based on the Claisen-condensation of nine acetates.[11] The intermediate ***A*** undergoes an intramolecular aldol condensation to form the benzoic acid ***B***.

Other macrolactones such as dictyostatin (**2**), on the other hand, are made from acetate and propionate (Fig. 2), whilst the macrolactone part of erythromycin, erythronolide B (**3**), is entirely made from propionate entities.

One obvious effect of methyl substituents on a macrolactone core is the resulting conformational control.[12] In addition, they

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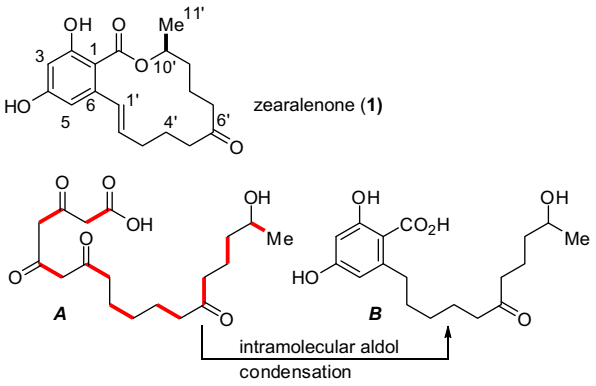


Figure 1. Structure and intermediates in the biosynthesis of of zearalenone (**1**). The acetate building blocks in ***A*** are indicated by red bonds.

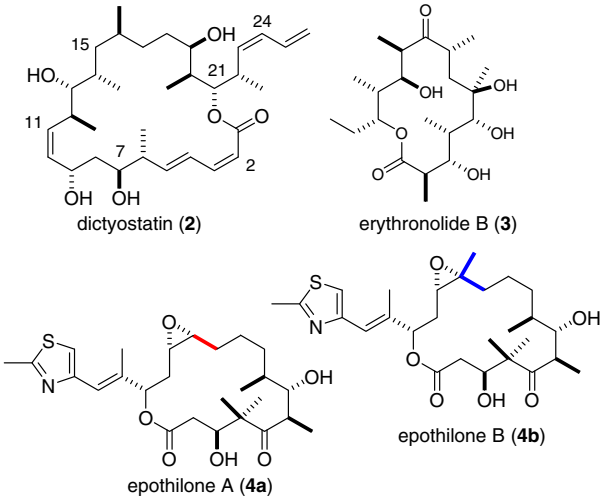


Figure 2. Structures of important polyketides; red = acetate, blue = propionate.

may allow the molecule to take advantage of hydrophobic pockets in the binding site. The compounds epothilone A (**4a**) and epothilone B (**4b**) that show anti-cancer activity represent an example for a natural acetate/propionate pair. Due to the additional methyl group epothilone B is significantly (approx. 10 times) more active than epothilone A.[13] The significance of the methyl group is also apparent from the fact that all epothilone derivatives which are in clinical studies or use are analogues of epothilone B.

We devised the concept of *propionate scanning* based on the biosynthesis of polyketides.[14] In general, it comprises the systematic replacement of all acetates in reasonable positions in polyacetate-based natural products with propionate such that one acetate is replaced in each of the products. A more extended version could be called methyl-scanning where a methyl group is attached to the available methylene functions in a macrolactone. However, we feel the non-propionate analogues would be less validated. We chose RAL zearalenone as the first compound to apply propionate scanning to. Zearalenone has a relatively simple structure,[15] but belongs to a very interesting family of natural products of which many are inhibitors of kinases, ATPases, and chaperones.[10] Despite the lack of obvious similarity with ATP, these compounds have been shown to bind to the ATP-binding pocket of ATPases including kinases. Applied to zearalenone, eight possible analogues can be conceived from propionate scanning (Fig. 3).

The classical benzolactone, the fungal metabolite zearalenone (**1**) was first isolated in 1962 from the fungus *Gibberella zeae* and

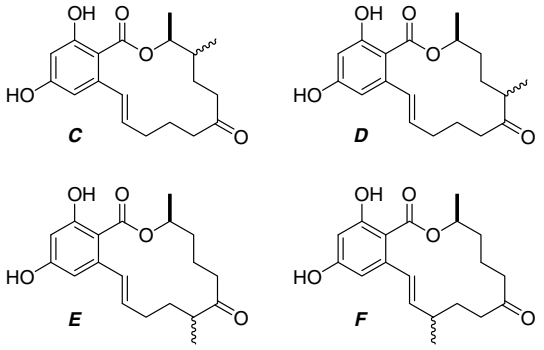


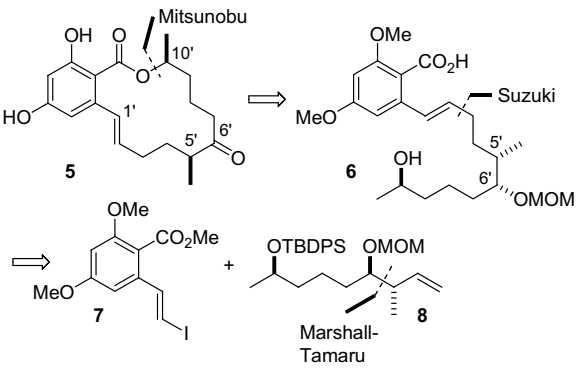
Figure 3. Reasonable mono-propionate analogues of zearalenone.

reported to comprise high toxicity because of anabolic and oestrogenic properties. It is also an antibacterial acting chemical.[16] It mimics 17-estradiol, the principal hormone produced by the human ovary, in binding to oestrogen receptors in mammalian target cells. Zearalenone is found worldwide as contaminant in cereal crops and is recognized as endocrine disruptor by having chronic oestrogenic effects on mammals. A dietary concentration of zearalenone as low as 1.0 ppm may lead to hyperestrogenic syndromes in pigs; higher concentrations can have adverse effects on reproduction such as disrupted conception and abortion.[17]

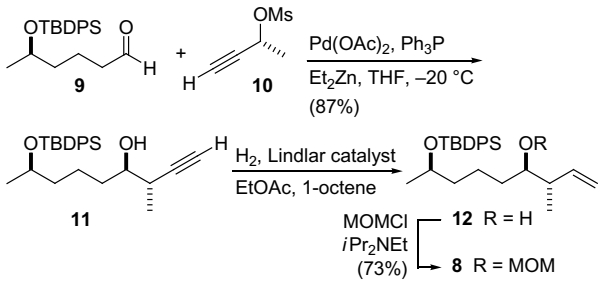
2. Chemistry

We used Mitsunobu macrolactonization to form the macrolactone ring of **5** (Scheme 1). The corresponding *seco*-acid **6** was cut further at the vinylic position, leading to iodovinyl benzoate **7** and alkene **8**. The alkene may be prepared by addition of a crotylmetal species or an equivalent thereof to an aldehyde. In order to establish the vicinal *anti* OH/Me pattern at position C6′/C5′ (zearalenone numbering) we used the Marshall–Tamaru reaction,[18–20] which relates to the reaction of an aldehyde with a chiral allenyl zinc reagent.

Aldehyde **9** which can be easily prepared from (*R*)-propylene oxide,[21] was made to react with propargylic mesylate[22] **10** in presence of palladium(II) acetate, diethylzinc, and triphenylphosphine (Scheme 2). The intermediate allenylzinc species then underwent a S_E2' addition to the aldehyde, yielding mainly the *anti* homopropargylic alcohol **11**. Stirring of the reaction mixture for 72 h at −20 °C gave the best yield and diastereomeric ratio (9:1, as shown by NMR, see Supplementary data). The alkyne **11** was hydrogenated to the alkene **12** in the presence of Lindlar catalyst (5% Pd on $CaCO_3$, poisoned with lead). To prevent over-reduction to the single bond, the Lindlar reduction of alkyne **11** was performed in



Scheme 1. Retrosynthetic analysis for zearalenone analogue **5**.

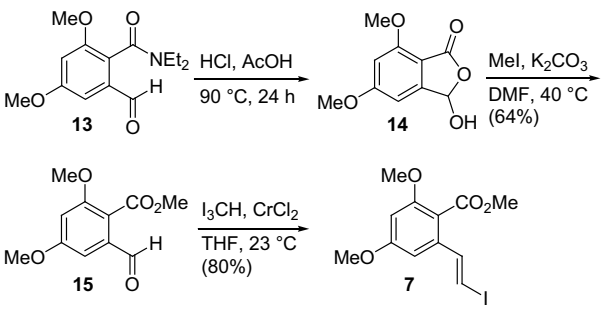


Scheme 2. Synthesis of protected homoallyl alcohol **8** via Marshall–Tamaru reaction.

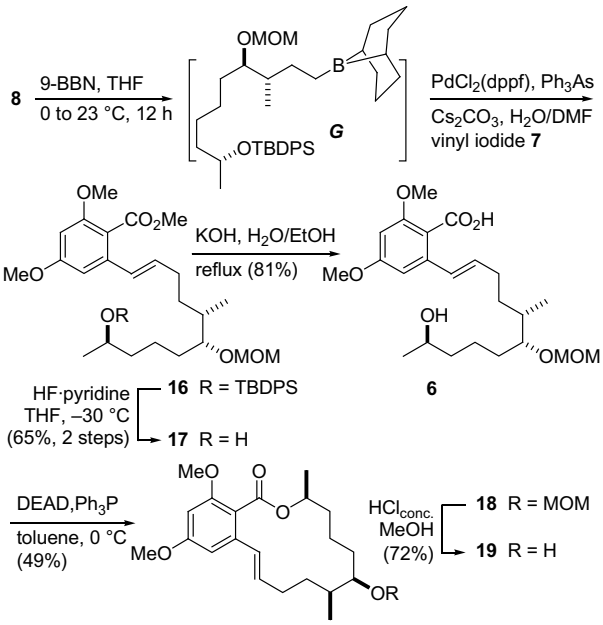
a solvent mixture containing 1-octene. The hydroxyl function of the product **12** was protected with MOMCl[23] under basic conditions, thus, providing building block **8**. The overall yield for the three step sequence was 65%.

The synthesis of the vinyl iodide **7**, required for the Suzuki cross-coupling reaction, started with the known *N*,*N*-diethyl-2-formyl-benzamide[24] **13** (Scheme 3). The amide **13** was treated with HCl (3 N) at 90 °C, resulting in hydrolysis and hemiacetal formation to provide 3-hydroxy-5,7-dimethoxyphthalide (**14**). Refluxing the mixture gave inferior yields. The in situ prepared phthalide was subsequently methylated under alkylating conditions. Best conditions for the methylation reaction were 40 °C in DMF, giving methyl 2-formylbenzoate **15** resulting in a good overall yield. The vinyl iodide **7** could be obtained by Takai olefination[25] of aldehyde **15**. NMR analysis showed that the desired *E* compound was obtained in a 5:1:1 ratio over the unwanted *Z* compound and the corresponding vinylbenzoate (See Supplementary data). Both by-products did not interfere in the following Suzuki coupling reaction.

Building blocks **7** and **8** were combined via a Suzuki cross-coupling reaction (Scheme 4).[26,21] Following hydroboration of alkene **8** with 9-BBN (0–23 °C, overnight), the intermediate borane ***G*** was subjected to a palladium-catalyzed coupling reaction with iodostyrene **7**. Application of a slight excess (1.5 equiv) of **7** in the Suzuki coupling reaction resulted in a reasonable yield of the alkene **16**. To reach the *seco*-acid **6**, the silyl ether of **16** was cleaved with the HF·pyridine complex in THF, leading to hydroxy ester **17**. Saponification of the methyl ester with KOH in EtOH/H_2O (95:05) under reflux resulted in *seco*-acid **6**. For the macrolactone formation standard Mitsunobu conditions (DEAD, Ph_3P, toluene, 0 °C) were used.[27] Thus, a diethyl azodicarboxylate (DEAD) solution was added slowly to a mixture of *seco*-acid **6** and triphenylphosphine in toluene at 0 °C. In order to favour cyclization to **18**, the reaction was performed at relatively high dilution. Cleavage of the MOM protecting group was accomplished under acidic conditions, thus providing hydroxy lactone **19**.



Scheme 3. Synthesis of vinyl iodide **7**.

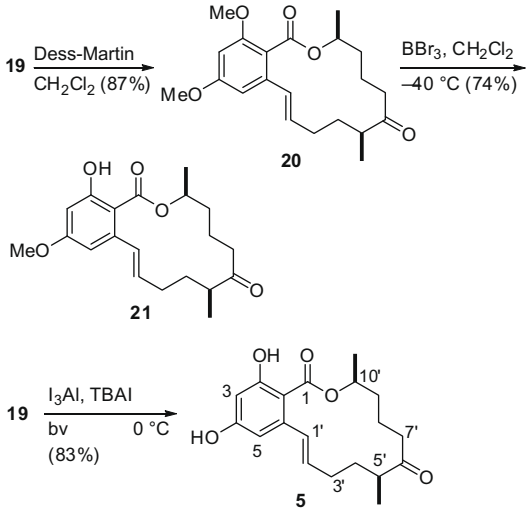


Scheme 4. Synthesis of the lactone **19** via Suzuki cross-coupling and Mitsunobu macrolactonization as key steps.

Lactone **19** served as the precursor for a few additional zearalenone derivatives. Thus, oxidation of **19** with Dess–Martin periodinane provided macrocyclic ketone **20** (Scheme 5). Treatment of **20** with BBr_3 was expected to cleave both methyl ethers.[28] However, under these conditions only the methyl ether *ortho* to the carboxyl function was cleaved, thus leading to analogue **21**. In order to cleave both methyl ether functions we found I_3Al, prepared in situ, to be suitable.[29] Under these conditions, the zearalenone analogue **5** was obtained in 83% yield.

3. Biology

A family of natural products with high attractivity for the creation of analogues using propionate scanning are the benzolactones. Members of this family have a vast spectrum of biological activities including, for example, protein kinase inhibitors, inhibitors of heat



Scheme 5. Synthesis of various zearalenone analogues from key intermediate **19** via cleavage of the methyl ether functions.

shock protein 90 (Hsp90), oestrogen receptor agonists, and antibiotics.[10,30] As described above, the prototypic benzolactone, zearalenone, mimics oestrogen in binding to its receptor, and uses modes of binding to ATPases similar to that of ATP. Intensive research programs are ongoing to identify specific kinase inhibitors despite a high structural similarity within the ATP binding site of these proteins. With an estimated 30% of all cellular proteins becoming transiently phosphorylated the essential role of the protein kinases, comprising approx. 3% of all expressed genes,[31] in cellular signalling pathways is obvious. Their connection to numerous disease mechanisms including cancer, diabetes and inflammation was shown in many studies, thus the development of small-molecular-weight compounds to target inhibition of protein kinases is recognized as a promising strategy to address various forms of cancer.[32] Hsp90 is an ATP-dependent chaperone that is one of the most abundant proteins in eukaryotic cells (1–2%). In assisting non-covalent folding/unfolding of a large number of proteins it plays a central role for a multitude of functions such as cell cycle regulation and signal transduction. Pharmacological inhibition of Hsp90 has been shown to be beneficial in the treatment of multistep carcinogenesis.[33]

Interestingly, binding of inhibitors targeted at protein kinases to non-ATP binding proteins was observed in a number of cases. For example, human carbonyl reductase 1 (CBR1) is inhibited by a hydroxyl-substituted analogue of the well-known Src-family kinase inhibitor.[34] CBR1 is a member of a large class of oxidoreductases, namely short-chain dehydrogenases/reductases (SDR). CBR1 is one of the major xenobiotic carbonyl reducing enzymes in humans.[35] The outcome of these reactions is compound and tissue-specific, that is, could lead to more potent or less active compounds. For example, CBR1 catalyzes the reduction of doxorubicin, a widely used antineoplastic drug, to the highly cardiotoxic metabolite doxorubicinol. Therefore, inhibition of CBR1 by small-molecular-weight compounds may proof beneficial in combination therapy during doxorubicin-based chemotherapy.[35]

Within our integrated structural and functional genomics platform directed against human medicinal target classes[36] we evaluated the biological potential of the collection of zearalenone analogues. Binding of compounds to proteins is commonly detected using differential scanning fluorimetry (DSF), a generic thermal-shift (ΔT_m) detection assay, as described.[37–39] Firstly, we tested the zearalenone collection (**1**, **5**, **19**, **20**, **21,** including also geldanamycin and radicicol) against a panel of catalytic domains of human protein kinases from diverse subfamilies (for the distribution of the selected proteins over the phylogenetic tree of the human protein kinase family, see Supplementary data, Fig. S1). As the interaction chart (Table 1) shows, only relatively small effects were observed. Within this group of compounds, the natural product zearalenone (**1**) stabilized lymphocyte-originating kinase (LOK) and STE20-like kinase (SLK) by little more than 4 °C, commonly corresponding to low micromolar inhibition.[37] These two kinases are closely related both structurally and with respect to promiscuity for kinase inhibitors that are currently either pursued in clinical trials or approved as drugs.[40] In addition to LOK and SLK, zearalenone also showed stabilization above the threshold (2 °C) for PIM1, PIM2, PAK5 and GAK. In contrast to these effects, the additional methyl group, present on the analogue **5** results in a strong decrease in binding affinity: For all but SLK ($\Delta\Delta T_m \sim$ 1 °C), Pim1 (ΔT_m near threshold) and GAK (ΔT_m similar to that with zearalenone) the stabilization was lost entirely, that is, the ΔT_m fell below the threshold of 2 °C. In addition to the effects observed upon addition of the methyl group, the screens also showed the importance of the aromatic hydroxyl groups for binding to kinases: both dimethoxy analogues (**19** and **20**) did not bind to any of the tested proteins.

It is important to note that the extent of stabilization depends on the individual properties of the protein; it is dependent on the entropic and enthalpic components, respectively of the binding.[41] For protein kinases a linear correlation between ΔT_m and IC_{50} is apparent, most likely because of the two-lobe architecture where the nucleotide (or an inhibitor in its place) exerts most of its stabilizing effect through 'locking' of the two domains together, which in effect is dominant over the mode of binding of the inhibitor. For almost all other protein families, however, the effect of a ligand on the stability of the protein it binds to is not predictable. On the other hand, however, there is evidence that for a certain protein the comparison of T_m shifts caused by different ligands correlate with their affinities. We observed that the known nanomolar inhibitors of Hsp90, radicicol and geldanamycin, caused only a small stabilization of the protein, of 5.1 and 3.2 °C, respectively. In comparison, zearalenone seems to bind with less affinity to Hsp90. No stabilization was observed with the analogue **5.**

In thermal shift assays CBR1 and other human SDRs such as CBR3 were screened both with the oxidized and reduced cofactor ($NADP^+$, NADPH) as well as in the absence of cofactor (Table 2). In general, consistent with previous finding on a large set of human oxidoreductases, thermal shifts for SDR proteins are much lower than for kinases. To the collection of zearalenone analogues, a couple of structurally related compounds were added to the screening experiments. Most hits were obtained for CBR1 in the presence of the cofactor $NADP^+$. Very promising was the obvious selectivity shown by zearalenone analogue **5** that gave only a distinct T_m shift with CBR1/$NADP^+$. Taking into account the impaired kinase activity in comparison to the natural product, this hit seemed auspicious for further investigations.

3.1. Substrate Test for CBR1

Stabilization by all of the tested compounds was dependent on the presence of $NADP^+$. Neither in the presence of the reduced cofactor, NADPH, nor without cofactor we observed any significant T_m shift. A likely reason for this effect is that binding of the ligand requires polar interactions with the positively charged nicotinamide ring. However, it could not be ruled out that in presence of NADPH the compounds would be reduced to a product with lower affinity, that is, producing a lower stabilizing effect. To test for the latter possibility, all compounds that caused a T_m shift (exception: triclosan, which does not comprise any reducible group) were tested in an activity assay at concentrations of 200 μM, respectively.

However, no CBR1 activity was observed towards the zearalenone analogue **5**. Furthermore, the activity towards the natural product **1** was below 10% of that towards isatin (Table 3).

3.2. Inhibition of the CBR1-Isatin reaction

We then used the activity of CBR1 towards isatin as an orthogonal assay to the DSF results, which allows analysis of potential inhibitory properties. In order to study CBR1 inhibition, we have chosen the CBR1-catalyzed reduction of isatin as reference reaction (Fig. 4). Isatin has been shown to exhibit various pharmacological actions and is reduced by CBR1 to 3-hydroxy-2-oxoindole.

Reductase activity of purified CBR1 was assayed by monitoring the oxidation of NADPH to $NADP^+$. The fluorescence intensity for NADPH at 460 nm (after excitation at 355 nm) decreases as the cofactor is oxidized (Table 4). The reaction rate of the isatin reduction was determined from linear regression to the data after normalization to the fluorescence increase upon injection (correction for compound-dependent quenching).

Table 1
Inhibition array of the screened kinases and Hsp90

Inhibitor	PIM1	PIM2	PIM3	GAK	MAPK6 (ERK3)	MAPK11 (P38b)	DYRK1	FES	MAP3K5	STK3 (MST2)	SLK	LOK	MAP2K6	CSNK1G1 (CK1γ1)	PLK4	PAK6	Hsp90
Geldanamycin																	3.2
Radicicol																	5.1
Dimethoxy ZEA Analogue **19**																	
Dimethoxy ZOL Analogue **20**																	
Mono OMe ZEA Analogue **21**											2.5						
Zearalenone (ZEA) **1**	3.0	2.6		2.4							4.4	4.6					2.3
ZEA Analogue **5**	2.2			2.9							3.5						

Yellow fields indicate a T_m shift >2 °C and orange fields of >4 °C, respectively. Blank fields indicate a T_m shift below 2 °C.

Table 2
Stabilization array for human carbonyl reductase 1 (CBR1) and 3 (CBR3)

Compound	CBR1 apo	CBR1 $NADP^+$	CBR1 NADPH	CBR3 apo	CBR3 $NADP^+$	CBR3 NADPH
Quercetin						
Genistein						
Wedelolactone	2.0	5.1				
Geldanamycin,						
Radicicol		4.6				
Triclosan		3.4				
Chrysin		4.6				
Biochanin A		2.7				
Naringenin		2.3				
Dimethoxy ZEA Analogue **19**						
Dimethoxy ZOL Analogue **20**						
Mono OMe ZEA Analogue **21**						
Zearalenone (ZEA) (**1**)		3.6				
ZEA Analogue **5**		3.9				

The threshold for weak binding was set to 2 °C, stabilization effects above the threshold are colour-coded (T_m shift above, respectively: 2 °C, yellow; 3 °C, ochre; 4 °C, orange; 5 °C, red).

During the present study, following up on the results from the DSF screen led to the identification of low micromolar and, in the case of ZEA analogue **5**, even nanomolar CBR1 inhibitors. Interestingly however, not all compounds that stabilized did inhibit the enzyme strongly. The natural product zearalenone (**1**) did not lead to inhibition higher than 50% (see Supplementary data, Fig. S2). Therefore, no IC_{50} value could be determined. The binding affinity of zearalenone to the substrate binding site of CBR1 seems to be much lower than that of the analogue **5**.

Table 3
Activity test for compounds with T_m shift >2 °C and additional zearalenone analogues

Compound	Activity compared to isatin (%)	T_m shift (°C)
Wedelolactone	5.8	5.1
Radicicol	–	4.6
Chrysin	16.3	4.6
ZEA Analogue **5**	–	3.9
Zearalenone (ZEA) (**1**)	5.7	3.6
Biochanin A	9.3	2.7
Naringenin	4.7	2.3
ZEA analogue **20**	31.3	<1
ZOL analogue **19**	–	<1
ZEA analogue **21**	1.5	<1

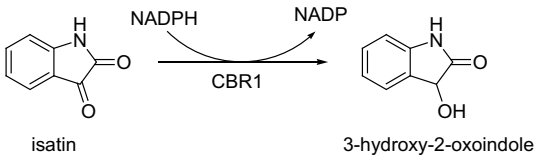


Figure 4. Reduction of isatin by CBR1.

Table 4
IC_{50} values for tested compounds, as well as their K_i values calculated including the assay concentration of isatin (50 μM)

Compound	IC_{50} (μM)	K_i (μM)
Wedelolactone	3.78 ± 0.9	0.60 ± 0.14
Radicicol	3.27 ± 2.5	0.52 ± 0.40
Triclosan	0.40 ± 0.1	0.06 ± 0.02
Chrysin	0.67 ± 0.3	0.11 ± 0.05
Biochanin A	12.71 ± 8.8	2.03 ± 1.40
Naringenin	21.48 ± 7.3	3.43 ± 1.17
ZEA analogue **5**	0.21 ± 0.03	0.04 ± 0.01
Zearalenone (ZEA) (**1**)	–	–

4. Conclusion

Zearalenone (**1**) and the analogues **5**, **19**, **20**, and **21** were screened versus a diverse set of therapeutically relevant human

kinases and the chaperone Hsp90. The screening results show that the zearalenone scaffold is likely to serve as a platform for kinase, Hsp90 or SDR enzyme inhibitor development. Furthermore, the modification of the underlying framework of the natural product zearalenone by the synthetic concept called *propionate scanning* changed the binding affinity of the compound dramatically for different targets. The incorporation of a propionate building block in the fourth position with a (*S*)-methyl group on C5′ turned the resulting zearalenone analogue **5** into a potent (IC_{50} = 210 nM) and very specific inhibitor for the carbonyl reductase CBR1. In comparison to zearalenone the side effect on inhibition of Hsp90 or kinases is greatly reduced.

5. Materials and methods

5.1. Protein expression and purification

All proteins were expressed in *Escherichia coli*. Detailed protocols for expression and purification of each protein can be downloaded from the Structural Genomics Consortium web site (www.sgc.ox.ac.uk). Either a His6 tag (N- or C-terminal) or a GST fusion system was used to aid purification. Recombinant proteins were >95% pure as judged by SDS/PAGE, and protein identity was confirmed by mass spectrometry.

5.2. Compounds

Compounds **5** and **19**–**21** were synthesized as described in the Supplementary data. Quercetin, Genistein, Wedelolactone, Geldanamycin and Radicicol were purchased from Calbiochem (EMD Bioscience), and all other compounds from Sigma Aldrich.

5.3. Thermal shift assays

Differential scanning fluorimetry (DSF) was carried out by using a Real-Time PCR Mx3005p machine (Stratagene) according to the procedure described by Niesen et al.[38] The protein solutions used for all fluorescence measurements employed a final protein concentration of 2 μM in a buffer consisting of 10 mM HEPES, pH 7.5 and 150 mM NaCl.

5.4. Kinetics of NADPH oxidation

The assay was performed in wells of a 384-well low-volume microtiter plate (Corning); the final reaction volume was 30 μL. Oxidation of NADPH to NADP was monitored from the decrease in fluorescence at 460 nm (excitation: 355 nm) after addition of 200 nM protein and 200 μM NADPH to the compound(s) dissolved in 50 mM sodium phosphate, pH 6.8, 1 mM $MgCl_2$ (injector integrated into plate reader: Polarstar Omega, BMG Labtech). The reaction rate was determined from linear regression to the data after normalization to the fluorescence increase upon injection (correction for compound-dependent quenching). Ability of compounds to function as CBR1 substrates was tested in triplicates at concentrations of 200 μM, respectively. Inhibition was determined by titration in presence of two concentrations of isatin (50 μM, 200 μM). Background activities (in absence of protein) at each substrate concentration were subtracted, and data fitted to a sigmoidal dose-response model to determine the IC_{50} (Graphpad Prism 5). Inhibition constants, K_i, were calculated according to the equation:

$$K_i = \frac{IC_{50}}{1 + \frac{s}{K_m}},$$

where *s*, concentration of substrate isatin; K_m, Michaelis constant for isatin (45.9 ± 10.2 μM).

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Supplementary data

Procedures for the syntheses described in Schemes 2–5. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2008.11.076.

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