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Highly enantioselective synthesis and cellular evaluation of spirooxindoles inspired by natural products

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In biology-oriented synthesis the underlying scaffold classes of natural products selected in evolution are used to define biologically relevant starting points in chemical structure space for the synthesis of compound collections with focused structural diversity. Here we describe a highly enantioselective synthesis of natural-product-inspired 3,3′-pyrrolidinyl spirooxindoles—which contain an all-carbon quaternary centre and three tertiary stereocentres. This synthesis takes place by means of an asymmetric Lewis acid-catalysed 1,3-dipolar cycloaddition of an azomethine ylide to a substituted 3-methylene-2-oxindole using 1–3 mol% of a chiral catalyst formed from a N,P-ferrocenyl ligand and $CuPF_6(CH_3CN)_4$. Cellular evaluation has identified a molecule that arrests mitosis, induces multiple microtubule organizing centres and multipolar spindles, causes chromosome congression defects during mitosis and inhibits tubulin regrowth in cells. Our findings support the concept that compound collections based on natural-product-inspired scaffolds constructed with complex stereochemistry will be a rich source of compounds with diverse bioactivity.

Relevance to nature and biological prevalidation are among the most important criteria to be met by compound classes for chemical biology and medicinal chemistry research. Bioactive natural products are a proven and rich source of disease-modulating drugs and of efficient tools and reagents for the study of biological phenomena[1–5]. Their pronounced bioactivity has been rationalized by the fact that during biosynthesis and when exerting their biological functions they need to interact with multiple proteins as substrates and targets[6]. Because the number of structural motifs of proteins and natural products is limited[6], the underlying scaffold classes of natural products selected in evolution and of compound classes derived from or inspired by them can be regarded as biologically prevalidated starting points in chemical structure space for compound collection development. Based on this rationale we introduced biology-oriented synthesis (BIOS) as a hypothesis-generating approach for the design and synthesis of natural-product-inspired compound collections enriched by molecules with diverse bioactivity[7,8]. In BIOS, biological relevance and prevalidation are used to select the underlying scaffolds for the synthesis of compound collections with chemical diversity focused around a biologically selected starting point in chemical space. Given the structural complexity and richness in stereogenic centres of natural products, and consequently of the compound libraries inspired by their structure, the development of efficient enantioselective synthesis methods is at the heart of BIOS and related approaches such as diversity-oriented synthesis[2,8].

The 3,3′-pyrrolidinyl-spirooxindole scaffold defines the characteristic structural core of a large family of alkaloids with pronounced and diverse bioactivity profiles[9,10]. For instance, spirotryprostatin B **1** (Fig. 1) arrests the cell cycle at the G2/M phase and is an inhibitor of tubulin polymerization. Notably, non-natural spirooxindoles of this class, such as **2** (Fig. 1), inhibit the cell cycle and represent a novel type of potent non-peptidic inhibitor of the p53-MDM2 protein–protein interaction that is crucial for the regulation of the tumour-suppressing activity of the p53 protein[11–13].

The synthesis of spirooxindoles in general remains a significant challenge in organic chemistry[14,15]. 3,3′-pyrrolidinyl-spirooxindoles have been the subject of elegant asymmetric synthesis approaches, in particular multistep diastereoselective transformations using chiral auxiliaries[9,10]; however, only recently was the first enantioselective access to this heterocycle class reported through an organocatalysed three-component 1,3-dipolar cycloaddition[16]. Here we describe the first Lewis acid-catalysed highly enantioselective synthesis of 3,3′-pyrrolidinyl-spirooxindoles by means of a 1,3-dipolar cycloaddition of an azomethine ylide to a 3-arylidene- or alkylideneoxindole to generate an all-carbon quaternary spirocentre and three tertiary centres in a single reaction step. Initial cellular evaluation of a small compound collection prepared in this way identified a library member that arrests the cell cycle at the G2/M phase, induces multiple microtubule organizing centres and multipolar spindles, causes chromosome congression defects during mitosis and inhibits tubulin regrowth in cells.

In order to establish the enantioselectively catalysed 1,3-dipolar cycloaddition, (*E*)-configured benzylidene oxindole **(*E*)-3a** and glycine ester imine **4a** (Fig. 2a and Table 1) were treated with a variety of copper catalysts or silver acetate[17–20] and chiral ligands in different solvents in the presence of catalytic amounts of triethylamine (Supplementary Table S1 and Supplementary Fig. S1). From this initial series of experiments the use of 3 mol% of both N,P-ferrocenyl ligand **7a** and $CuPF_6(CH_3CN)_4$ in tetrahydrofuran (THF)

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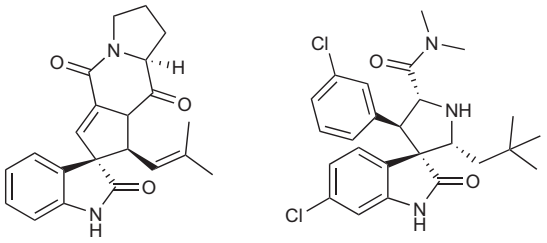


Figure 1 | Structures of a representative bioactive natural product structure with 3,3′-pyrrolidinyl-spirooxindole and a non-natural bioactive compound. Spirotryprostatin B **1** arrests the cell cycle at the G2/M transition and is an inhibitor of tubulin polymerization, and spirooxindole **2** is an inhibitor of the p53-MDM2 protein–protein interaction (see Supplementary Information).

emerged as most advantageous with respect to yield, enantioselectivity and diastereoselectivity (Table 1, entry 1). Chiral catalysts comprising a ferrocene ligand and a Cu(I) precursor have been used previously in enantioselective azomethine ylide cycloadditions with α,β-unsaturated esters[20–22], maleimides[19], nitro-olefines[23], vinyl sulfones[18] or fullerene[17] as dipolarophiles. Notably, if instead of ligand **7a**, which contains an NH_2 group, ligands **7b** or **7c** were used, in which the NH_2 group is replaced by an NR_2-group, the direction of the enantioselection is reversed (Supplementary Table S1 and Supplementary Fig. S1). Further variation of the reaction conditions to improve the method surprisingly reveal a non-linear dependence of the enantioselectivity on the ligand/Cu^+ ratio. Moderate increase of the ligand/Cu^+ ratio from 1:1 to 1.1:1 led to reduced diastereoselectivity and enantioselectivity (Table 1, entries 1 and 2); a further increase up to a ratio of 2:1 restored the diastereoselectivity and resulted in a significant increase in enantiomeric excess (e.e.) of up to 98%, which was gratifyingly combined with a higher yield (Table 1, entries 3–5). The non-linear effect may arise because of the formation of an additional complex; at a 1:1 ratio, intermediate **8** is generated as well as catalytically active Cu^+ with no chiral ligands coordinated, but in the presence of a small excess of ligand the equilibrium between Cu^+ ions and the chiral ligands is changed, and a complex with two ligand molecules coordinated to one Cu^+ may also be generated. This leads to a high concentration of catalytically active Cu^+ ions without chiral ligands and hence a decrease in the enantioselectivity of the product is observed. From the complex with two ligands, intermediate **8** may be formed by exchange of one ligand molecule for the amino acid ester imine and subsequent deprotonation. Increasing the ligand/Cu^+ ratio to 2:1 would ensure that all the Cu^+ ions were coordinated by two ligands, one of which would then be exchanged for the imine to subsequently form **8**.

To the best of our knowledge such a non-linear effect of the ligand/Cu^+ ratio on the enantioselectivity of 1,3-dipolar cycloadditions has not been described before. Furthermore, identification of this optimized ligand/Cu^+ ratio allowed us to reduce the catalyst loading to 1% without a negative impact on diastereoselectivity, enantioselectivity or yield (Table 1, entries 5–7). Even at 0.1% catalyst loading, product **5a** is formed with an e.e. of 91% (Table 1, entries 7–10). The absolute configuration of the major stereoisomer was unambiguously determined by crystal structure analysis (Supplementary Fig. S2).

In order to rationalize the steric course of the reaction we propose the formation of intermediate complex **8** in which (1) the metal atom is simultaneously coordinated by the ligand **7a** and the glycine ester imine **4a** (Fig. 2b); (2) unfavourable steric interactions are minimized; and (3) the dipolarophile attacks from the least hindered face. Deprotonation of the chiral complex **8** leads to the azomethine ylide, which undergoes the 1,3-dipolar cycloaddition. The benzylidene oxindole **3a** attacks the azomethine

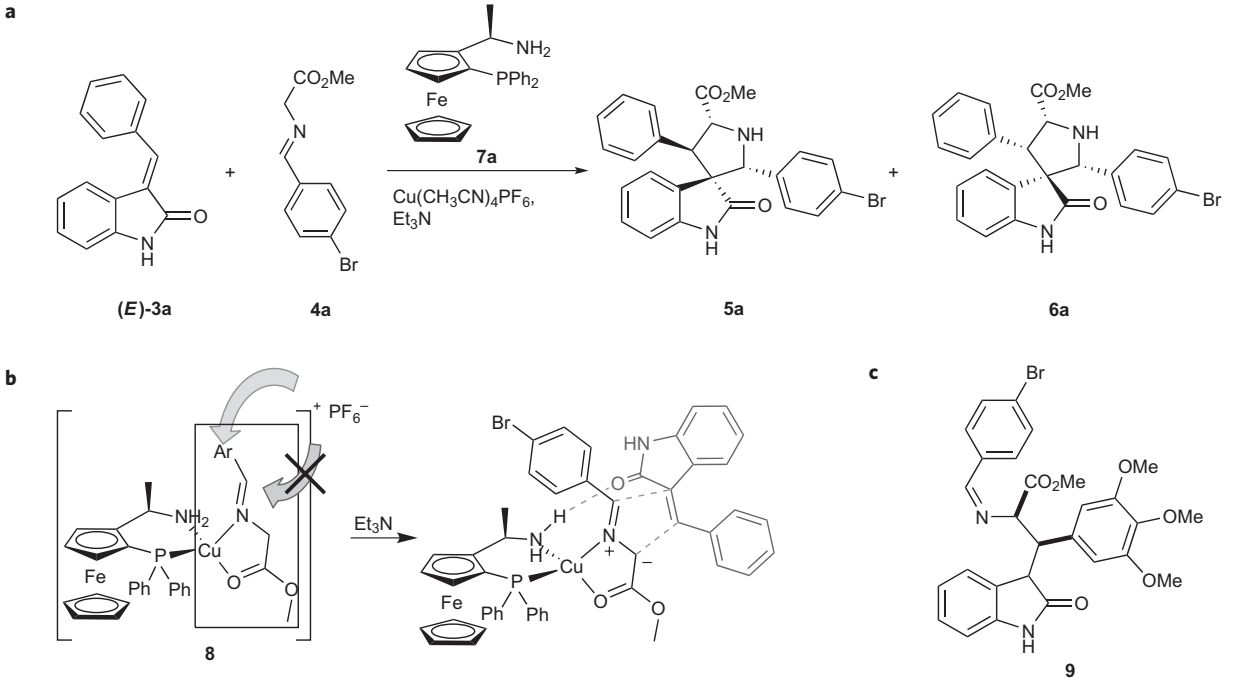


Figure 2 | Representative enantioselective 1,3-dipolar cycloaddition between an arylidene oxindole and an amino acid ester imine in the presence of a chiral catalyst, and proposed rational for the observed stereocontrol. a, Enantioselective 1,3-dipolar cycloaddition between dipolarophile (*E*)-**3a** and the 1,3-dipole formed from Schiff base **4a** in the presence of a chiral Lewis acid catalyst formed *in situ* from $Cu(CH_3CN)_4PF_6$ and a ferrocenyl P-N ligand **7a**. **b**, Proposed structure of the tetrahedral complex **8** formed by coordination of Cu^+ with the ligand **7a** and imine **4. c**, The Michael adduct **9** most likely formed in the reaction of (*Z*)-3-(3,4,5-trimethoxybenzylidene)indolin-2-one with **4a**.

Table 1 | Evaluation of reaction conditions for the catalytic 1,3-dipolar cycloaddition.

Entry*	[Cu^+] (mol%)	[7a] (mol%)	Time (h)	d.r.† 5a/5b	Yield (%)‡	e.e. of 5a (%)§
1	3	3	1	15/1	86	90
2	3	3.3	1	10/1	85	72
3	3	4	1	10/1	77	93
4	3	5	1	10/1	84	98
5	3	6	1	15/1	92	98
6	2	4	1	15/1	92	98
7	1	2	1	15/1	91	98
8	0.5	1	3	12/1	75	95
9	0.3	0.6	5	12/1	54	93
10	0.1	0.2	12	10/1	33	91

*Reaction conditions: ligand **7a**, $Cu(CH_3CN)_4PF_6$, iminoester **4a** (1.3 equivalents), Et_3N (20 mol%) and dipolarophile **3a** (1 equivalent) in THF (1 ml) at ambient temperature. †See Supplementary Information for details; further diastereomers were formed in only very minor amounts and not characterized. ‡Isolated yields of the major diastereomer **5a** after column chromatography. §Determined by chiral HPLC analysis.

ylide from the least hindered face, that is, from behind (as indicated by the arrow), thereby avoiding unfavourable steric interaction with the bulky PPh_2 group that points to the front. The carbonyl group of the oxindole **3a** may form a hydrogen bond with the amino group of the ligand **7a** and thereby stabilize the transition state. In ligands **7b** and **7c** the amino group is replaced by a dialkylamino group (Supplementary Fig. S1), and the hydrogen bond cannot be formed. Instead the alkyl groups will cause unfavourable steric interactions. Thereby the attack of the oxindole is directed to the front and the direction of the enantioselection is reversed (Supplementary Table S1). According to this model, protic additives should compete with hydrogen-bond formation in intermediate **8** and consequently lead to reduced enantioselectivity. Therefore, we performed cycloadditions of **4a** to **3a** in the presence of different alcohols and acetic acid (Supplementary Table S2). The cycloaddition products were indeed formed with reduced enantioselectivity and the degree of reduction correlated with the concentration and pK_a of the additives. In addition, replacement of the glycine ester imine **4a** with derivatives of β-alanine did not lead to the formation of the corresponding cycloadducts, presumably because complexes analogous to **8** cannot be formed and deprotonated to yield dipoles under these reaction conditions. This model is in agreement with previous studies on the use of ferrocenyl ligands in asymmetric 1,3-dipolar cycloadditions[20,21].

The regioselectivity observed in the enantioselective 1,3-dipolar cycloaddition reaction catalysed by the copper catalysts used here is opposite to the selectivity observed in analogous enantioselective proton-acid-catalysed 1,3-dipolar cycloadditions to nitrogen-protected methyleneindolinones[16]. This difference has been rationalized by the involvement of a zwitterionic azomethine ylide resonance form that is not usually present in 1,3-dipolar cycloadditions. Reversed regioselectivity is thought to result from the stabilization stemming from the favourable π–π stacking interaction between the oxo-indole ring and the conjugated esters, on the basis of theoretical calculations of the transition state[16].

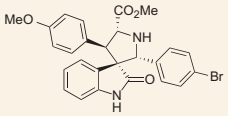
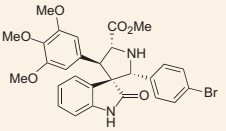
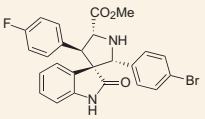
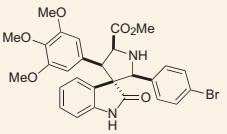
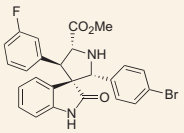
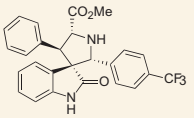
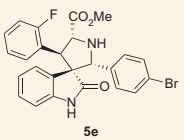
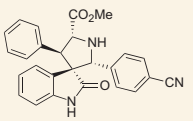
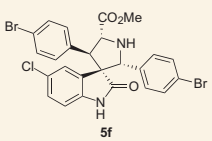
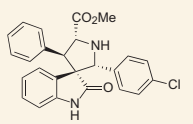
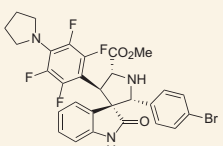
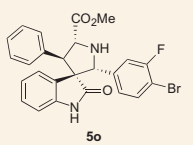
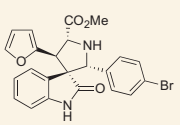
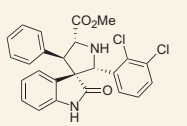
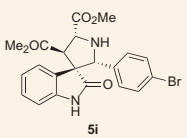
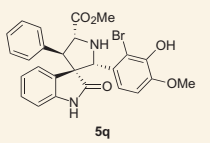
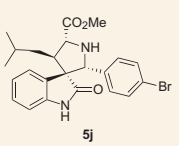
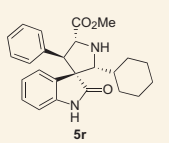
To further explore the scope of the reaction the structures of the dipolarophile and the 1,3-dipole were varied. Benzylidene indolinones with varying substituents and a heteroaromatic analogue yielded the desired cycloadducts in rapid reactions that proceeded in high yield and with excellent diastereoselectivity and enantioselectivity (Table 2, entries 1–10; diastereomeric ratio (d.r.) > 10–15:1). Replacement of the aromatic substituent on the double bond of the dipolarophile with an ester led to moderate decrease of enantioselectivity (Table 2, entry 8); however, after a single recrystallization the cycloadduct was obtained nearly enantiomerically pure. Alkylideneindolinone **5j** was formed under optimized conditions with 90% e.e. and in moderate yield (Table 2, entry 9). Spirooxindole **5g** (Table 2, entry 6) provides a particularly remarkable case as it contains a penta-substituted aromatic ring, and is therefore a significant challenge for an enantioselectively catalysed reaction. This example demonstrates that it is not necessary to use pure (*E*)-configured dipolarophiles in the synthesis sequence. The corresponding benzylidene-indolinone was used as a 2:1 mixture of the (*E*)- and (*Z*)-isomers, which equilibrates under the reaction conditions[24,25] and from which the (*E*)-isomer reacts more rapidly. The corresponding product **5g** was obtained with 84% yield and 97% e.e. However, if the benzylidene-indolinone is equipped with electron-donating substituents, as is the case for the 3,4,5-trimethoxyphenyl-substituted indolinone, the equilibration of the (*Z*)- and the (*E*)-isomer is slowed down[24,25] and both isomers can be separated and investigated independently. As expected, the (*E*)-isomer reacts rapidly with *p*-bromobenzylidene glycine ester under the optimized conditions to yield the cycloadduct **5k** with an e.e. of 97% (Table 2, entry 10). But, under the same conditions the corresponding (*Z*)-isomer gave only trace amounts of the expected cycloadducts. Instead, with silver acetate as the catalyst, the diastereomeric pyrrolidinyl-indolinone **6k** was obtained as a racemate (Table 2, entry 11). We assume that the complex formed between the imino ester, the Cu^+ and the chiral ligand cannot undergo a productive interaction with the (*Z*)-configured olefin because of unfavourable steric interactions between the ligand and the bulky trimethoxyphenyl group. In the presence of the silver catalyst, however, product formation most likely occurs by means of a stepwise sequence including formation of an intermediate Michael adduct **9** (Fig. 2c) followed by a fast Mannich reaction in analogy to the reaction of azomethine ylides with nitroalkenes[23,26].

In a second series of experiments the substitution pattern in the glycine ester imines was varied. In these cases the enantioselective catalysis results in high levels of enantioselectivity and fast reactions for monosubstituted benzylideneglycine esters bearing electron-withdrawing and -donating substituents and for multiply substituted benzylideneglycine esters (Table 2, entries 12–18). Notably the transformation proceeds smoothly in the presence of acidic groups; for example, the phenol embedded in **5q** (Table 2, entry 17). An imine derived from an aliphatic aldehyde was not reactive even at high catalyst loading (Table 2, entry 18).

In light of the finding that 3,3-pyrrolidino-spirooxindoles induce mitotic arrest by interfering with the p53-MDM2 interaction[13] we investigated whether the cycloadducts obtained as described above share this bioactivity. To this end, the compound collection was screened at a concentration of 30 μM for phenotypic changes associated with mitotic arrest in BSC-1 cells. From the 39 compounds tested only cycloadduct **6k** induced phenotypic changes, such as accumulation of round-shaped cells with condensed DNA, which are indicative of mitotic arrest (Supplementary Fig. S3). Compound **6k** differs from the other screened compounds by its relative configuration. In order to determine whether absolute configuration is also important for bioactivity, both enantiomers of **6k** were subjected to fluorescence activated cell sorting (FACS) analysis. This revealed that **(−)-6k** but not **(+)-6k** caused accumulation of cells in the G2/M phase in BSC-1, HCT116 p53 +/+ and p53−/−, and HeLa cells (Fig. 3a, Supplementary Table S5). At a 10 μM concentration virtually all cells were arrested in G2/M phase. In HeLa cells an increase of cells in the G2/M phase was observed at concentrations as low as 2 μM.

Unexpectedly, both enantiomers of **6k** were inactive in an enzyme-linked immunosorbent assay monitoring the formation of the p53-MDM2 complex, which indicates that the observed bioactivity does not correlate with inhibition of the p53-MDM2 interaction (Supplementary Fig. S4). This finding is supported by the fact that arrest in the G2/M phase by **(−)-6k** is not dependant on

Table 2 | Lewis acid catalysed synthesis of 3,3′-pyrrolidinyl-spirooxindoles.

Entry	Product	Time (h)	Yield (%)*	e.e. (%)†	Entry	Product	Time (h)	Yield (%)*	e.e. (%)†
1	 **5b**	4	80	**96**	10	 **5k**	2	85	**97**
2	 **5c**	3	82	**98**	11	 **6k**	4	41	–[¶]
3	 **5d**	2	89	**98**	12	 **5l**	1	95	**94**
4	 **5e**	2	81	**98**	13	 **5m**	2	94	**96**
5	 **5f**	1	65	**97**	14	 **5n**	0.5	84	**91**[‖]
6	 **5g**	3	84	**97**[‡]	15	 **5o**	4	89	**84**[‖]
7	 **5h**	3	82	**95**	16	 **5p**	0.5	93	**84**[‖]
8	 **5i**	1	97	**85 (99**[§]**)**	17	 **5q**	18	58	**96**[‖]
9	 **5j**	4	50	**90**[‖]	18	 **5r**	36	n.d.	–[#]

For reaction conditions see Supplementary Information. *Isolated yields of the major diastereomer after column chromatography. †Determined by chiral HPLC analysis. ‡After single crystallization. §A mixture of stereoisomeric oxindoles ($E/Z = 2/1$) was used. ‖3 mol% of catalyst and 6 mol% of ligand **7a** were used. ¶The reaction was performed with the (*Z*)-isomer of the arylidene-oxindole using AgOAc as catalyst. #5 mol% of catalyst and 10 mol% of ligand **7a** were used. n.d. – not detected.

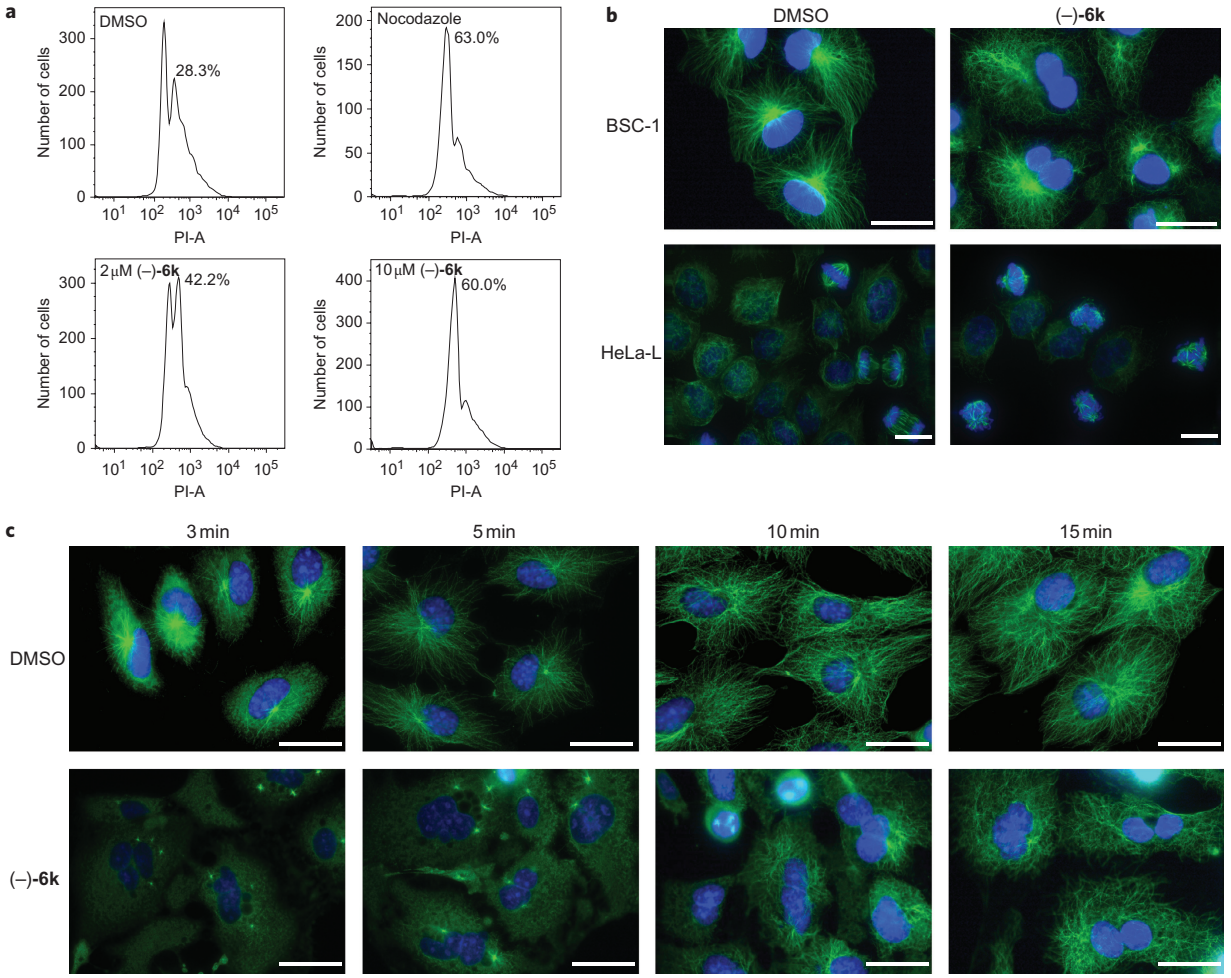


Figure 3 | Biological evaluation of cycloadduct (−)-6k. a, FACS analysis of HeLa cells treated with **(−)-6k**. Cells were incubated for 20 h with **2**, 10 μM **(−)-6k** or 1 μM nocodazole and DMSO as controls prior to staining with propidium iodide. Numbers show the percentage of cells in the G2/M phase. PI-A, propidium iodide fluorescence intensity. **b**, Influence of **(−)-6k** on BSC-1 and HeLa-L cells. Cells were treated for 20 h with 10 μM **(−)-6k** or DMSO as a control. Cells were then fixed and stained with an fluorescein isothiocyanate-coupled anti-α-tubulin antibody and DAPI/Hoechst for visualizing the DNA. Scale bar: 20 μm. **c**, Microtubule regrowth assay after cold treatment in BSC-1 cells. Cells were incubated for 20 h with 10 μM **(−)-6k** or DMSO as a control. Microtubule repolymerization was detected at the given time points after reheating, fixation and staining with an anti-α-tubulin antibody and Alexa Fluor 488-coupled secondary antibody, and 4′,6-diamidino-2-phenylindole (DAPI) for visualizing the DNA. Scale bar: 20 μm.

the presence of wild type p53 as shown for HCT116 p53 +/+ and p53−/− cell lines.

Further microscopic analysis in BSC-1 cells revealed that **(−)-6k** influences microtubules and results in a diffuse tubulin distribution (Fig. 3b). Interestingly, this phenotype might not be caused by a direct interference with the tubulin cytoskeleton because **(−)-6k** at concentrations as high as 50 μM did not significantly affect the *in vitro* polymerization of tubulin under the experimental conditions used. Furthermore, **(−)-6k** inhibited microtubule regrowth in BSC-1 cells after depolymerization by cold treatment at 10 μM (Fig. 3c and Supplementary Fig. S6), but **(+)-6k** did not. In cells treated with dimethylsulfoxide (DMSO), microtubule organizing centres were detected just 1 min after reheating and mictrotubule polymerization was completed after 15 min. **(−)-6k** caused a significant delay in the microtubule repolymerization and more than one microtubule organizing centre per cell appeared 2 min after reheating. Microtubules failed to repolymerize after 5 min, but after 10 min the formation of poorly organized microtubules was detected. Consistent with the presence of more than one microtubule organizing centre per cell, treatment of BSC-1 or HeLa-L cells with **(−)-6k** resulted in the formation of multipolar spindles and chromosome congression defects in mitotic cells (Fig. 3b; for higher magnification see Supplementary Fig. S7). Furthermore, a considerable number of BSC-1 cells were polynucleated, which is indicative of incomplete cell division.

Unlike the findings reported for related 3,3-spirooxindoles[13], our results demonstrate that **(−)-6k** does not act by inhibition of the p53-MDM2 interaction, but rather via an interference with microtubule polymerization. Ultimately this impairs the formation of the mitotic spindle leading to multipolar spindles, lagging chromosomes, mitotic arrest or incomplete mitosis. Notably, this difference in bioactivity may be linked to the very different spatial arrangement of functional groups for stereoisomer **6k** as compared with the diastereomeric spirooxindole-based p53-MDM2 inhibtors[11] (Supplementary Fig. S8).

The mode of action of compound **(−)-6k** also differs from the biological function of the related 3,3′-pyrrolidinyl-spirotryprostatin **2**. This spiro compound arrests the cell cycle at the G2/M phase but unlike cycloadduct **(−)-6k** it directly inhibits tubulin polymerization. In addition, formation of multipolar spindles and more than one microtubule organizing centre per cell has not been described for the spirotryprostatins[27,28].

In conclusion, we developed a highly enantioselective Lewis acid-catalysed 1,3-dipolar cycloaddition for the synthesis of biologically relevant natural-product-inspired 3,3′-pyrrolidinyl-spirooxindoles from simple and readily available starting materials. For the first

time we discovered a non-linear effect in the ligand/Cu^{+} ratio in the asymmetric 1,3-dipolar cycloaddition. This enantioselective synthesis efficiently gives access to spirocycles with an all-carbon quaternary spirocentre and three tertiary centres in one step using low catalyst loading. Our findings support the concept that compound collections based on natural-product-inspired scaffolds constructed with complex stereochemistry and decorated with assorted substituents will be a rich source of compounds with diverse bioactivity.

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Author contributions

A.P.A. designed and carried out chemical experiments. C.G-R. and M.C. performed biological and biochemical experiments. M.S. and H.P. carried out the X-ray crystallographic analysis. H.W., D.R. and S.Z. designed experiments and supervised the project. All authors discussed the results and commented on the manuscript. H.W., D.R., S.Z. and A.P.A. wrote the manuscript.

Additional information

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