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Natural product–inspired cascade synthesis yields modulators of centrosome integrity

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In biology-oriented synthesis, the scaffolds of biologically relevant compound classes inspire the synthesis of focused compound collections enriched in bioactivity. This criterion is, in particular, met by the scaffolds of natural products selected in evolution. The synthesis of natural product–inspired compound collections calls for efficient reaction sequences that preferably combine multiple individual transformations in one operation. Here we report the development of a one-pot, twelve-step cascade reaction sequence that includes nine different reactions and two opposing kinds of organocatalysis. The cascade sequence proceeds within 10–30 min and transforms readily available substrates into complex indoloquinolizines that resemble the core tetracyclic scaffold of numerous polycyclic indole alkaloids. Biological investigation of a corresponding focused compound collection revealed modulators of centrosome integrity, termed centrocountins, which caused fragmented and supernumerary centrosomes, chromosome congression defects, multipolar mitotic spindles, acentrosomal spindle poles and multipolar cell division by targeting the centrosome-associated proteins nucleophosmin and Crm1.

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Biology-oriented synthesis uses biological relevance as the key criterion for the synthesis of focused compound collections for chemical biology and medicinal chemistry research[1–4]. The underlying scaffolds of natural product classes define the areas of chemical space explored by nature in evolution, and natural product–inspired compound collections[1] are expected to be biologically relevant and therefore enriched in biological activity[5]. Besides their role in drug discovery[6], natural products have been instrumental[7] in elucidating various biological processes related to cancer, particularly mitosis. The proteins involved in this highly regulated process seem to be particularly responsive to modulation by small molecules[8–10]; for instance, indole alkaloids have delivered many interesting candidates targeting mitosis (**Fig. 1**)[11,12]. The structural complexity and diversity of natural products, and consequently also that of natural product–inspired compound collections, calls for the development of efficient synthesis methods that enable the flexible assembly of structurally diverse compound collections based on natural product scaffolds[1]. Cascade reaction sequences in which several chemical reactions proceed consecutively in one pot can rapidly generate molecular complexity[13–15]. They require starting materials with multiple functional groups to facilitate different chemical transformations[16]. Here we report on the development of a highly efficient cascade reaction sequence of unprecedented length that rapidly proceeds via 12 consecutive chemical transformations in one pot, includes nine different chemical reactions (conjugate additions of P-, O-, N- and C-nucleophiles; acyclic and cyclic aminal formation; enamine condensation to form dihydropyridines; the aza-Claisen reaction; Pictet-Spengler cyclization; and chromone ring opening) and two opposing types of organocatalysis (Brønsted acid and phosphine catalysis), and terminates in the formation of tetracyclic tetrahydroindolo[2,3-a]quinolizines, which resemble the core scaffold of numerous polycyclic indole alkaloids (**Fig. 1**)[17]. A corresponding focused compound collection yielded modulators of centrosome integrity that induce fragmented and supernumerary centrosomes, formation of multipolar mitotic spindles and acentrosomal spindle poles, defects in metaphase plate formation, multipolar cell division, and mitotic arrest and apoptosis, and that exert their biological activity by binding to the centrosome-associated proteins nucleophosmin (NPM1, which is also known as B23 or numatrin)[18] and Crm1 (ref. 19).

RESULTS

Synthesis of a natural product–inspired compound collection

Upon treatment with an organocatalyst-like triphenylphosphine, electron-poor 3-formyl chromones **1** and alkynes **2** undergo a [4 + 2] annulation reaction to yield natural product–inspired tricyclic benzopyrones **5**. The three-step, one-pot reaction proceeds via conjugate addition of the phosphine to the triple bond, conjugate addition of the resulting zwitterion enolate **3** to the chromone and subsequent annulation by conjugate addition of the oxygen anion to an α,β-unsaturated ester (**Scheme 1**)[20]. The resulting tricyclic benzopyrones **5** incorporate two electrophilic α,β-unsaturated carbonyl groups and a pronucleophilic benzopyrone ring, which may open up to generate a nucleophilic phenol. Thus, if combined with reaction partners that also contain multiple functional groups, and if in the presence of suitable catalysts, they might serve as versatile starting points for new multistep reaction cascades. On the basis of this reasoning and with polycyclic indole alkaloid scaffolds in mind,

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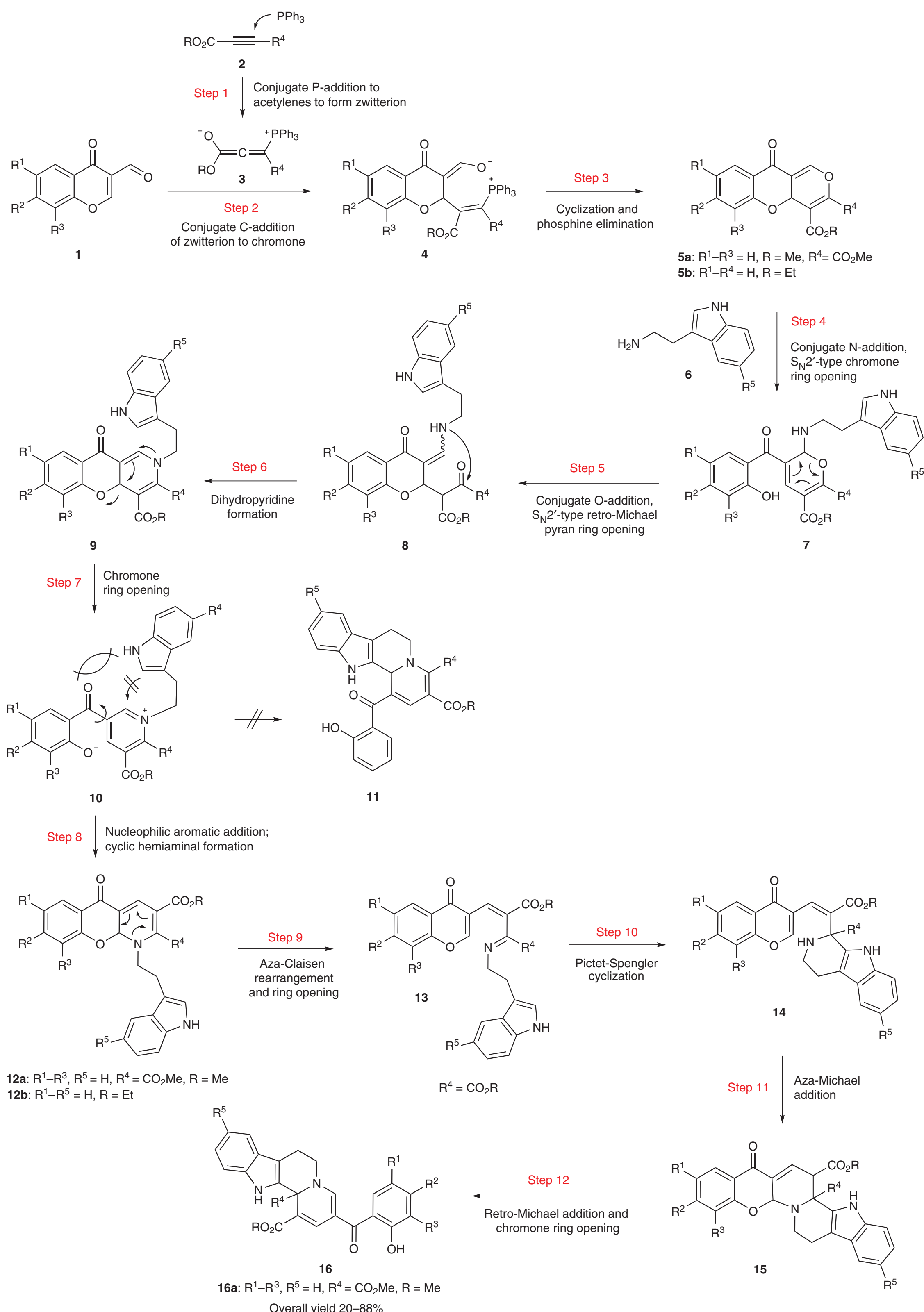
we chose to combine the triphenylphosphine-catalyzed formation of tricyclic benzopyrones with tryptamine derivatives in the presence of an acid.

We envisioned that under suitable reaction conditions both natural product–based fragments equipped with more than one site of complementary reactivity could establish a cascade reaction that might yield unique natural product–inspired compound classes in a concise and efficient manner (**Fig. 1b**). In particular, it was considered possible that under suitable conditions the cascade sequence might lead to the formation of polycyclic indole derivatives such as indoloquinolizines and related heterocycles. The indoloquinolizine scaffold is characteristic of numerous polycyclic monoterpene



Scheme 1 | Cascade synthesis of indoloquinolizines 16a–16z using easily accessible substrates. Further details are in the text.

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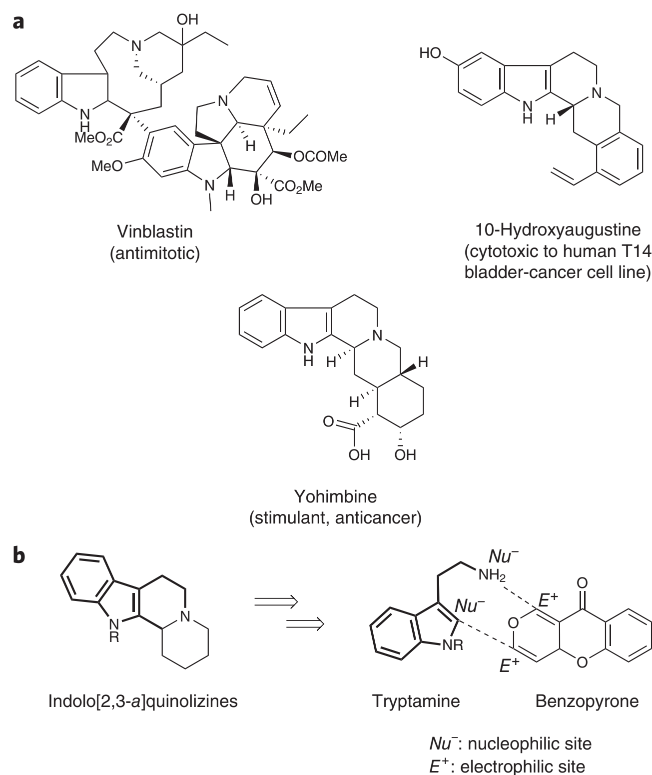

Figure 1 | Inspiration and synthesis design for indoloquinolizine compound collections. (**a**) Indole alkaloid natural products that have diverse biological activities. (**b**) A cascade reaction–based retrosynthetic analysis suggests using tryptamine and benzopyrones as cascade substrates to yield indoloquinolizines.

indole alkaloids with diverse biological activities (for example, yohimbine, hirsutine or augustin; **Fig. 1a**).

For reaction development, benzopyrone **5a** was treated with tryptamine **6** for a few minutes at 80 °C followed by addition of camphorsulfonic acid, and the reaction mixture was stirred for an additional 30 min. Regioselective S_N2'-type addition to the less substituted α,β-unsaturated vinylogous ester would result in chromone ring opening to yield aminal **7**. Conjugate addition of the intermediary phenol with concomitant retro-Michael pyran ring opening would lead to enamine **8**. The subsequent nucleophilic attack of the enamine on the neighboring α-ketoester was expected to result in formation of dihydropyridine **9**. Ring opening of chromones[21] was expected to lead to the formation of a pyridinium intermediate **10**, which then might have been trapped by the electron-rich indole to terminate the cascade, yielding indoloquinolizine **11**. However, compound **16a** was isolated, and its structure was unambiguously proven by crystal structure analysis (**Supplementary Methods**). Insight into the cascade reaction sequence emerged from the successful isolation of intermediates **12a** and **12b** (an X-ray crystal structure of **12b** and spectroscopic characteristics of **12a** are shown in **Supplementary Methods**).

We presume that the intermediates **12** are formed from **10** by addition of the phenol to the pyridinium ion and undergo subsequent [3,3]-sigmatropic aza-Claisen ring opening to yield α-imino esters **13**, which are subject to acid-catalyzed Pictet-Spengler cyclization. The resulting secondary amines **14** then may form hexacyclic compounds **15** by another conjugate addition. Chromone ring opening, with the phenol serving as a good leaving group, finally results in the formation of the tetrahydroindolo[2,3-a]quinolizines **16**. Under acidic conditions, indole rings do not add to the pyridinium cations[22,23], and therefore formation of **16** or **11** directly from **10** is very unlikely. We assume that the ester group ($R^4 = CO_2R$) attached to the enamine moiety in **12a** lowers its lowest unoccupied molecular orbital and therefore facilitates the aza-Claisen rearrangement to yield imino-esters **13**, which then enter the further cascade reaction sequence (steps 10 to 12) that terminates in indoloquinolizines **16**. Further support for the proposed cascade mechanism was obtained from a separate experiment in which **12a** was converted almost quantitatively to **16a** upon exposure to acid, whereas **12b** remained unchanged under these conditions (**Scheme 1** and **Supplementary Methods**). Although these results lend very strong support to our mechanistic proposal, we note that an alternative plausible formation of **16a** via a different, more direct mechanism cannot be completely ruled out.

This acid-catalyzed conversion of **5** into **16** in one pot could successfully be combined with the phosphine-catalyzed formation of **5** from **1**. The desired indoloquinolizines **16a–16z** are formed within 10–30 min (**Table 1**) in an efficient one-pot procedure by treating a mixture of **1**, **2** and triphenylphosphine at 80 °C in toluene with tryptamine (slow addition, 2–5 min) followed by addition of camphorsulfonic acid. The results given in **Table 1** show that the reaction is widely applicable and is notably high yielding. Using exclusively commercially available starting materials, the entire one-pot cascade reaction sequence proceeds via twelve different reaction steps, including nine different chemical transformations and two opposing types of catalysis. To the best of our knowledge, this is the longest cascade reaction sequence known[13–15]. The resulting heterocycles resemble the core scaffold structure of numerous alkaloids such as the yohimbines[12].

Investigation of mitosis modulation

Given the responsiveness of mitosis to small molecules, in particular indole alkaloids[8,24], we then tested our focused collection of indoloquinolizines for possible modulation of cell division using a phenotypic screen in BSC-1 cells[3]. Out of all of the tested compounds, **16a** led to the accumulation of round-shaped cells with condensed DNA, characteristics that are indicative of mitotic cells (**Supplementary Results** and **Supplementary Fig. 1**). Moreover, the mitotic cells showed impaired mitotic spindles.

Treatment of HeLa cells overexpressing GFP-tubulin and U2OS cells overexpressing mCherry–α-tubulin with **16a** and analogs thereof induced multipolar cell division and abundant formation of three daughter cells during mitosis (**Supplementary Movies 1** and **2**). Impairment of mitosis in HeLa cells (**Fig. 2** and **Supplementary Fig. 2**) and other cell lines (**Supplementary Fig. 3**) is dependent on both the concentration and structure of the compound, with **16a** and **16h** being the most potent compounds (mitotic defects can already be detected at a concentration of 1.5 μM; detailed in **Supplementary Fig. 4** and **Supplementary Results**) and (*R*)-**16a** and (*R*)-**16h** being more potent than (*S*)-**16a** and (*S*)-**16h** (**Supplementary Results** and **Supplementary Fig. 4**; determination of the absolute configuration of **16a** is detailed in **Supplementary Methods**). The substitution pattern in the aromatic ketone substructure and in the quinolizine cannot be changed substantially without loss of activity (**Table 1**). However, variation of R^5 in the phenyl ring of the indole was tolerated (**Table 1**, entries 8, 22, 23 and 25).

(*R*)-**16a** and (*R*)-**16h** induced chromosome congression defects and tri- or even multipolarization of the mitotic spindle (**Supplementary Fig. 4** and **Supplementary Movies 3** and **4**). In the parental HeLa cell line, centrosomes were visualized using γ-tubulin–specific antibody, and centrioles were stained with cep135–specific antibody. Inspection of the images shown in **Figure 2a** and **Supplementary Figure 2** revealed the formation of different spindle poles that were either stained for both γ-tubulin and cep135, contained only one of both markers or represented acentrosomal poles. In addition, the number of stained centrosomes did not always match the expected number of centrioles.

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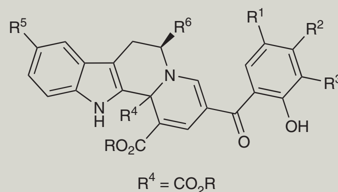
Table 1 | Synthesis and biological evaluation of indoloquinolizines.



Entry	Product	R	R^1	R^2	R^3	R^5	R^6	Percentage yield	Activity[a]
1	**16a**	Me	H	H	H	H	H	58	++
2	**16b**	Et	H	H	H	H	H	88	–
3	**16c**	Et	Me	H	H	H	H	65	–
4	**16d**	Me	Me	H	H	H	H	56	–
5	**16e**	Me	Br	H	H	OMe	H	62	–
6	**16f**	Me	Cl	H	H	H	H	39	–
7	**16g**	Me	Br	H	H	H	H	39	–
8	**16h**	Me	H	H	H	OMe	H	76	++
9	**16i**	Me	^{i}Pr	H	H	H	H	66	–
10	**16j**	Me	Cl	H	Cl	H	H	20	–
11	**16k**	Me	Br	H	Br	H	H	20	–
12	**16l**	Me	Cl	Me	H	H	H	60	–
13	**16m**	Et	H	H	H	OMe	H	76	–
14	**16n**	Et	Br	H	H	H	H	52	–
15	**16o**	Et	Me	H	H	OMe	H	45	–
16	**16p**	Et	^{i}Pr	H	H	OMe	H	65	–
17	**16q**	Et	Cl	Me	H	H	H	40	–
18	**16r**	Et	Cl	H	H	H	H	65	–
19	**16s**	Et	Cl	H	H	OMe	H	63	–
20	**16t**	Et	Br	H	H	OMe	H	54	–
21	**16u**	Me	Phenyl	Phenyl	H	H	H	52	–
22	**16v**	Me	H	H	H	Br	H	73[b]	+
23	**16w**	Me	H	H	H	Me	H	74[b]	+
24	**16x**	Me	H	OBn	Me	H	H	59	–
25	**16y**	Me	H	H	H	OH	H	67[c]	+
26	**16z**	Me	H	H	H	H	CO_2Me	91[b,c]	–

[a]A qualitative phenotypic analysis using HeLa cells treated with 25 μM of 16a–z was done, and molecules causing chromosomal misalignments were considered active ones (++, >90 % of all mitotic cells; +, > 30% of all mitotic cells; –, <10% of all mitotic cells). [b]The reaction was carried out from the tricyclic benzopyrone **5** (**Supplementary Methods**, general procedure 2). [c]The product was obtained as mixture of isomers. ^{i}Pr, isopropyl; OBn, O-benzyl.

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These findings indicate that centrosomes may have been fragmented or that supernumerary centrosomes were formed.

Indoloquinolizines (*R*)-**16a** and (*R*)-**16h** inhibit cell proliferation, induce apoptosis as monitored by caspase 3 and caspase 7 activation (**Supplementary Figs. 3b** and **5** and **Supplementary Table 1**) and induce a pronounced mitotic delay in HeLa GFP-H2B cells (**Supplementary Fig. 6**) with cell cycle arrest at the M stage (**Fig. 2b**,**c** and **Supplementary Fig. 7**).

Identification of cellular targets

On the basis of this structure-activity relationship, we synthesized indoloquinolizine probes **17** and **18** (negative control) (**Fig. 3a**) for target identification by means of a chemical proteomics approach. Compound **17** was immobilized on Sepharose beads and exposed to HeLa cell lysates, and bound proteins were released by elution with a ten-fold excess of **16a** followed by MS determination of the bound proteins, revealing that probe **17**, but not control probe **18**, binds the centrosomal protein NPM and U2 small ribonuclear protein (**Supplementary Fig. 8**).

Knockdown of NPM by small interfering RNA induces fragmentation of centrosomes and impairs chromosome congression and mitotic spindle formation in HeLa cells[25]. NPM is involved in the regulation of centrosome duplication during mitosis and also promotes ribosome biogenesis, such that U2 small ribonuclear protein could be pulled down in complex with NPM. On the basis of these data, NPM was validated as a target protein. Reversible binding of NPM to **17** was confirmed by western blotting with an NPM-specific antibody and by concentration-dependent competition between immobilized and nonimmobilized ligand (**Fig. 3b**). Regulation of centrosome duplication by NPM includes complex formation with the nuclear export receptor Crm1 (ref. 26). Crm1 knockdown as well as Crm1 inhibition by leptomycin B leads to defects in chromosome alignment and spindle assembly[26,27]. Reinvestigation of the affinity pull-down experiment by immunoblotting with a Crm1-specific antibody indeed revealed reversible, competitive binding of Crm1 to immobilized ligand **17** (**Fig. 3b**). Binding of both NPM and Crm1 was confirmed by means of fluorescence polarization measurements using fluorescent probe **19** (**Fig. 3a**), which revealed an apparent K_d of 25.4 ± 1.9 μM for histidine-tagged NPM (His-NPM) and 8.8 ± 1.6 μM for Crm1 (**Fig. 3c** and **Supplementary Fig. 10**). Direct interaction between indoloquinolizine probe **19** and NPM as well as Crm1 in HeLa cells

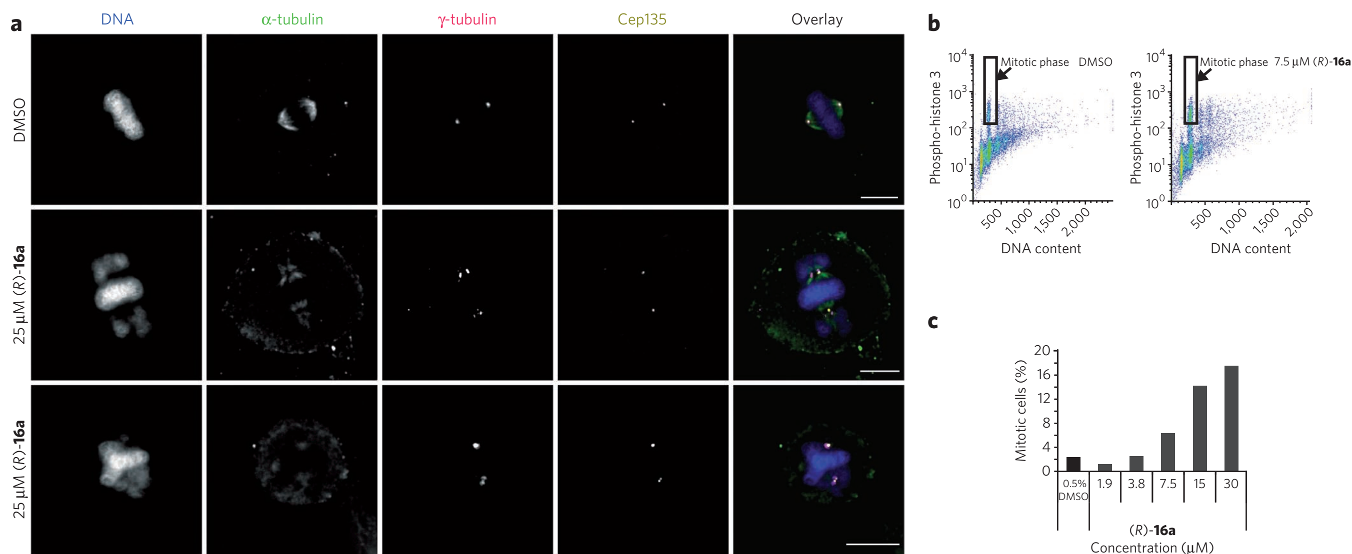

Figure 2 | Influence of (*R*)- 16a on chromosome congression, spindle pole formation and progression of mitosis in HeLa cells. (**a**) Multiple defects including chromosome congression defects (middle row) and aberrant spindle structures (bottom row) induced by (*R*)- **16a**. HeLa cells were treated with 25 µM (*R*)- **16a** for 18 h before staining with antibodies specific for α-tubulin, γ-tubulin, cep135 and DNA. Images are *Z* projections of image stacks done with ImageJ software. Representative images of mitotic cells are shown for cells treated with DMSO (top) and cells treated with 25 µM (*R*)- **16a** (middle and bottom). γ-Tubulin staining is indicative of centrosomes, and cep135-staining is indicative of centrioles. Scale bar, 10 µm. (**b**,**c**) FACS analysis of HeLa cells treated with (*R*)- **16a**. Cells were incubated for 18 h with different concentrations of (*R*)- **16a** and DMSO (as a control) before staining with phospho-histone 3–specific antibody, Alexa Fluor 488–labeled secondary antibody and propidium iodide. Representative histograms for DMSO and 7.5 µM (*R*)- **16a** (**b**) and dose-dependent accumulation of mitotic cells are shown (**c**).

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was proven by means of a significant ($P < 0.05$) decrease in the fluorescence lifetime of the donors NPM-citrine and enhanced yellow fluorescent protein (EYFP)-Crm1 after addition of the Cy3-labeled acceptor indoloquinolizine **19** (**Fig. 3d**,**e**). These findings reveal that both NPM and Crm1 independently bind the derivative **19**. Nuclear export mediated by Crm1 can be monitored by following the cellular localization of RanBP1, a cargo molecule for Crm1 (ref. 28). Whereas HeLa cells treated with the known nuclear export inhibitor leptomycin B showed accumulation of RanBP1 in the nucleus, (*R*)-**16a** did not influence the cellular distribution of RanBP1 up to a concentration of 25 µM (**Supplementary Fig. 11**). At 50 µM (*R*)-**16a**, the observed partial inhibition of nuclear export demonstrated that the indoloquinolizine targets Crm1. Notably, the indoloquinolizines are much less cytotoxic than leptomycin B (in cell viability assays, half-maximal inhibitory concentration (IC_{50}) values for centrocountins are in the micromolar range, whereas leptomycin B has IC_{50} values in the low nanomolar range[29]; **Supplementary Fig. 12**). These results indicate that the indoloquinolizines have a different mode of action than leptomycin B.

Our findings demonstrate that compound (*R*)-**16a** independently targets the centrosome-associated proteins NPM and Crm1 in cells. It thereby leads to impairment of centrosome and spindle integrity, chromosome congression defects, cell cycle arrest at the M stage and apoptosis.

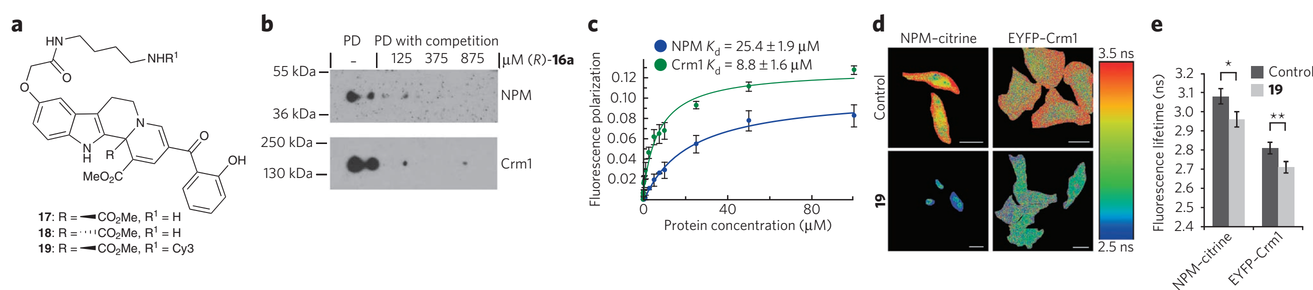

Figure 3 | Identification and validation of NPM and Crm1 as target proteins of 17. (**a**) Structure of probes used in affinity pull-down (PD) experiments, fluorescence lifetime experiments and determination of fluorescence polarization. (**b**) Immunodetection of NPM and Crm1 binding to immobilized **17**. After pull-down using immobilized **17**, proteins released by heating were resolved by SDS-PAGE, transferred to a polyvinylidene fluoride membrane and subsequently detected with NPM- and Crm1-specific antibodies. For competition experiments, HeLa cell lysates were preincubated with different concentrations of (*R*)- **16a**. Full-length blots are presented in **Supplementary Figure 9.** (**c**) Binding of **19** to NPM and Crm1 as determined by means of fluorescence polarization. The Cy3-labeled probe **19** was titrated with increasing concentrations of His-tagged NPM or Crm1 until saturation was reached. K_d values were determined from the fit of fluorescence polarization versus His-NPM or Crm1 concentration. (**d**) Fluorescence lifetime imaging microscopy showing specific binding of NPM-citrine and EYFP-Crm1 (donor) to Cy3-labeled **19** (acceptor). HeLa cells were transfected with NPM-citrine or EYFP-Crm1. Twenty-four hours later, 2.5 µM **19** was added. The decrease in the lifetime of the donor after addition of **19** is shown in the lifetime maps of cells. Scale bars, 30 µm. (**e**) Graph showing the decrease in the donor lifetime as compared to the control. Data are expressed as mean ($n > 15$) ± s.d. *, ***P* < 0.05. *P* values were determined following two-tailed Student's *t*-test. A *P* value of < 0.05 was considered significant.

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DISCUSSION

We demonstrate the development of an exceptionally versatile reaction cascade of unprecedented length and efficiency, giving access to a structurally complex natural product–inspired compound collection that yields new modulators of centrosome integrity and mitotic spindle pole formation by simultaneously targeting NPM and Crm1. On the basis of the induced phenotype, we term these molecules centrocountins, with compound **16a** being centrocountin 1. Although for chemical-biological analysis of Crm1 function leptomycin B is a widely used molecular probe[30], small molecules influencing NPM function have rarely been described, with the electrophilic and thereby nonselective natural product avrainvillamide being the most prominent case[31]. A dual NPM–Crm1 ligand is unique, and a molecular probe for the elucidation of NPM or NPM–Crm1 function is not available yet. NPM is involved in the establishment of many cancers, although its role is controversial[32]. In light of the efficient accessibility of the centrocountins and the fact that treatment of cancer cell lines with these compounds is antiproliferative and leads to mitotic arrest and apoptosis, the centrocountins and modulation of the activity of NPM or the NPM–Crm1 complex might inspire new drug discovery programs.

METHODS

General procedure for the cascade synthesis of indoloquinolizines 16. The 3-formylchromone (1 eq.) was dissolved in toluene (10 ml mmol^{-1}) by heating to 80 °C, and the acetylenedicarboxylate (1.3 eq.) and triphenylphosphine (0.6 eq.) were added. After 2–5 min, the tryptamine (1.1 eq.) was added, and, after the tryptamine dissolved, camphorsulfonic acid (1.5 eq.) was added. After 5–30 min, the solvent was evaporated, and the remaining residue was subjected to column chromatography on silica gel to yield the indoloquinolizine **16** as a yellow solid, which can be further purified by precipitation from methanol. Further details are available in **Supplementary Methods**.

Other methods. Cell lines and plasmids, imaging protocols, isolation of binding proteins and other cell-based procedures are detailed in **Supplementary Methods**.

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Author contributions

H.D., V.K. and H.B. designed and performed the synthesis experiments. V.P., S.M., A.W.B., Z.M. and S.Z. carried out the biological studies. P.J. and A.B. performed MS analysis. H.W., K.K., K.H., S.Z. and A.H. designed experiments. M.S. and H.P. carried out the X-ray crystallographic analysis. S.G. determined the absolute configuration of **16a**. H.W., K.K. and S.Z. supervised the project and wrote the manuscript. All authors discussed the results and commented on the manuscript.

Competing financial interests

The authors declare no competing financial interests.

Additional information

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