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Synthesis and antiproliferative activity of new tonantzitlolone-derived diterpene derivatives†

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The synthesis of the diterpene (+)-tonantzitlolone A and a series of derivatives is reported. The study includes the determination of their antiproliferative activities against selected cancer cell lines.

1. Introduction

The endemic Mexican medical plant *Stillingia sanguinolenta* has been found to be a rich source for a wide range of terpenes.[1] Structurally, the two diterpenes tonantzitlolone A (**1**) and B (**2**; OAc at C-4′ of side chain) are the most remarkable secondary metabolites from *Stillingia sanguinolenta*. Noteworthy, diterpene cyclases rarely generate 15-membered cyclization products – the backbone is commonly named the flexibilan skeleton (**3**) – as found in tonantzitlolones A (**1**) and B (**2**) (Fig. 1).

The roots of *S. sanguinolenta* are used in poultices after childbirth, and northern Mexican natives recommend infusions of leaves for the treatment of pulmonary ailments. Native American tribes of Navajos and Creek used the closely related *S. sylvatica* in a similar fashion.[2,3] Indeed, tonantzitlolone also occurs in the roots of *S. sylvatica* and in *Sebastiana macrocarpa.* Importantly, the isolation of six additional tonantzitlolone derivatives from the endemic Mascarene species *Stillingia lineata* was reported recently by M. Litaudon and coworkers.[4]

We showed that tonantzitlolone A and its enantiomer exert cytostatic activity on tumor cells.[5] They induce monoastral half-spindle formation suggesting inhibition of the mitotic motor protein kinesin-5. Interestingly, they are no general kinesin inhibitors as they do not affect kinesin-1 function. This was an important finding, also because motor proteins are involved in many fundamental eukaryotic cell processes such as the transport of vesicles and cell organelles, as well as

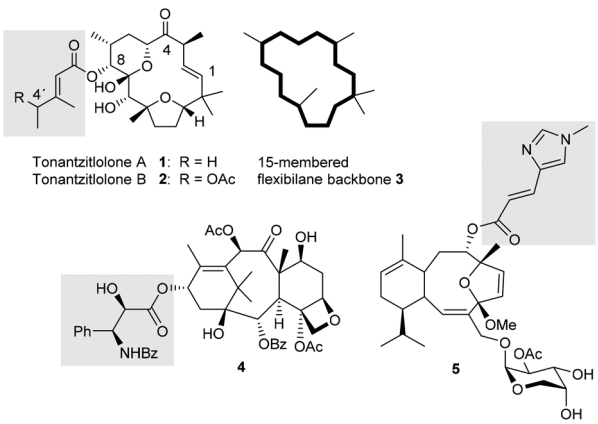

Fig. 1 Structures of tonantzitlolone A (**1**) and B (**2**), paclitaxel (**4**) and eleutherobin (**5**) as well as schematic representation of the flexibilane backbone (**3**) (numbering of the tonantzitlolones as defined in the original paper on the isolation and structure elucidation).

the organisation and structure of the spindle apparatus during cell devision.[6] Consequently, targeting of kinesins with small molecules is regarded to be an important strategy in antitumor research and monastrol as well as HR22C16 are classical examples of such inhibitors.[7]

Tonantzitlolone is one diterpene example[8] that exerts cytostatic activity, the most famous being paclitaxel (**4**), first isolated from the bark of the yew tree *Taxus brevifolia* found on the Northpacific coast. It is used as a clinical drug (Taxol®) against several solid tumors (*e.g.* mamma, prostate, ovarian cancer). Secondly, eleutherobin (**5**) is a marine diterpene glycoside collected from the soft coral *Eleutherobia cf. albiflora* which exerts strong antiproliferative activity against cancer cell lines (IC_{50} = 10–15 nM) by stabilizing microtubules like paclitaxel (**4**).

Although the diterpenoid core structures of these three diterpenes differ, they all contain a small ester side chain (marked in grey) that is essential for the biological activity.[7] Based on our important findings that tonantzitlolone A (**1**) can

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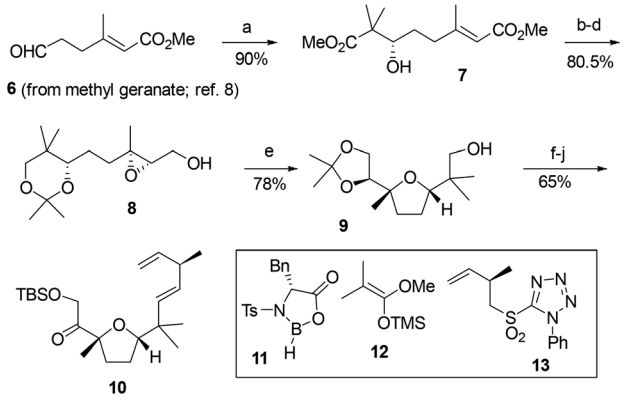
act on mitotic motor protein kinesin-5 an important target in cancer therapy we extended our investigations on the preparation of tonantzitlolone A (**1**) and new derivatives that differ in the side chain including the incorporation of the side chains found in paclitaxel (**4**) and eleutherobine (**5**) thereby creating derivatives that are occasionally called hybrids composed of structural elements found in two different natural products or drugs.[9] Synthetically, we made use of the principal approach reported in our first successful total synthesis of the enantiomer of tonantzitlolone A (*ent*-**1**).[10] It was supposed to provide sufficient amounts of the diterpene.

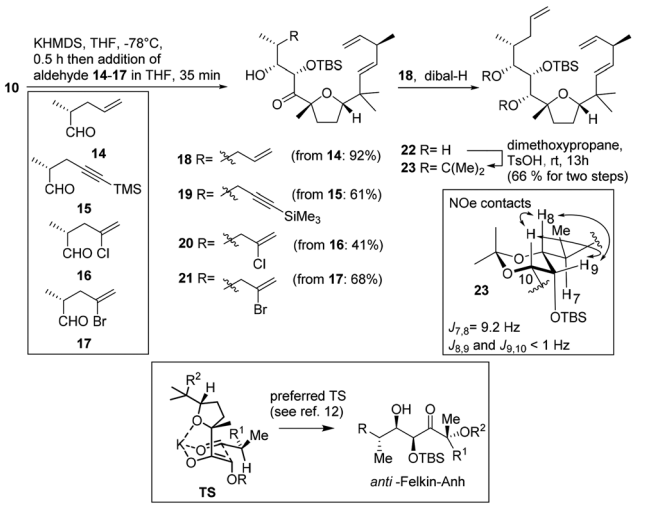
2. Results and discussion

Synthesis of tonantzitlolone

The synthesis of tonantzitlolone started from methyl geranate which was transformed into diester **7** *via* aldehyde **6** using the *O*,*O*-ketene acetal **12** in the Kiyooka aldol reaction (Scheme 1).[11] Yields and enantiocontrol were excellent. From here epoxy alcohol **8** was prepared utilizing the Sharpless-epoxidation as the key step. Next, acid catalyzed acetonide cleavage initiated tetrahydrofuran formation which was followed by reacetalization of the 1,2-diol moiety, overall with a high degree of stereo-, regio and chemoselectivity. The next key step of the synthesis was the aldol reaction that proceeded with high *anti* Felkin control when the potassium enolate was employed. Thus, ketone **10** was treated with KHMDS and in the reaction with aldehyde **14** aldol product **18** was formed (dr = 18 : 1) *via* a transition state (TS) that supposedly relies on chelation of the furan oxygen atom (Scheme 2).[12]



Scheme 1 Synthesis of ketone **10**. Reagents and conditions: (a) **11** and **12**, CH_2Cl_2, −85 °C to −40 °C, 0.5 h (90%, 99% ee); (b) $LiAlH_4$, Et_2O, 0 °C to rt; (c) 2,2-dimethoxy propane, *p*-TsOH, 2.5 h, rt, H_2O, 35 min (83% for two steps); (d) L-(+)-diethyl tartrate, $Ti(OiPr)_4$, *t*-BuOOH, molecular sieves 4 Å, CH_2Cl_2, −15 °C, 0.5 h, then cooling to −25 °C and addition of alkene, CH_2Cl_2, 20 h, 97%; (e) CH_2Cl_2, THF, $(HOCH_2)_2$, *p*-TsOH, 55 min, then 2,2-dimethoxy propane, rt, 16 h, then addition of H_2O, 15 min (78%); (f) TPAP, NMO, molecular sieves 4 Å, CH_2Cl_2, rt, 2.5 h (91%); (g) **13**, LDA, THF, −78 °C, then addition of aldehyde, −78 °C to rt, 39 h, Δ, 4.5 h (79%); (h) *p*-TsOH, MeOH/H_2O (1 : 1), 6.5 h, 50 °C (>99%); (i) TBSCl, imidazole, 4-DMAP, CH_2Cl_2, rt, 3 h (97%); (j) Dess–Martin-periodinane, CH_2Cl_2, 0 °C to rt, 6.5 h (94%).



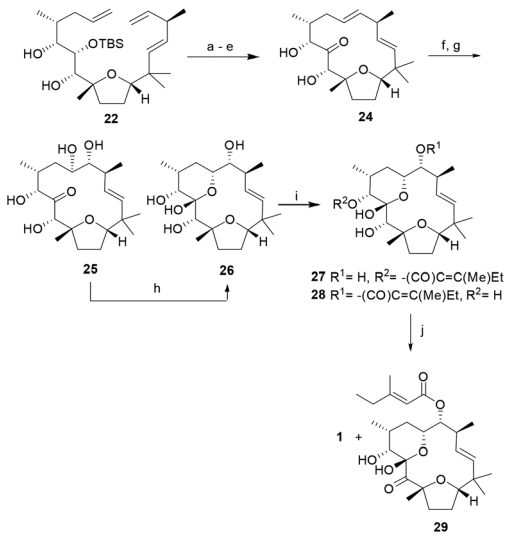
Scheme 2 Aldol reaction with aldehydes **14–17** yield potential RCM precursors **18–21**. Reagents and conditions: (a) KHMDS, THF, −78 °C, 0.5 h then addition of aldehyde **14–17** in THF, 35 min; (b) **18**, dibal-H; (c) dimethoxypropane, TsOH, rt, 13 h (66% for two steps).

The relative stereochemistry was determined after dibal-H reduction to yield the 1,3-*syn* diol **22**. Protection provided acetonide **23** that allowed us to prove the stereochemistry of the stereotriade by determining key coupling constants (*J*) and NOe-contacts (Scheme 2).

The unexpected stereochemical outcome of this aldol reaction is general. Indeed, we also utilized aldehydes **15–17**,[13] that contain a second alkene moiety or an alkyne group, respectively, to furnish aldol products **19–21** again with excellent diastereoselectivity (Scheme 2).

We included these aldol products into our studies because we planned to utilize them in ring closing metatheses to follow. These would result in macrocycles with a functionalized olefinic double bond at C-4 and C-5 that could directly be transformed into the targeted hydroxy ketone upon dihydroxylation without tedious differentiation of the diol moiety. However, at this point it needs to be noted that all efforts to enforce ring closing metathesis using different Grubbs catalysts failed.[14] Problems of achieving olefin metathesis with halogenated substrates have been discussed before.[15]

Aldol product **22** was converted into the corresponding hydroxy ketone by a series of functional group manipulations (desilylation, doubly silylation with TES-Cl, oxidation and desilylation) that smoothly underwent ring closing metathesis to yield macrocycle **24** (Scheme 3). Importantly, the RCM provided the (*E*)-alkene as major diastereoisomer. It allowed the regioselective dihydroxylation of this less hindered olefin at C4/C5 after TMS-protection of the 1,3-diol unit. This silylation turned out to be beneficial for the step to follow as the yield and the selectivity increased. Best facial selectivity in favour of the desired (4*R*,5*R*)-diol **25** was achieved under Sharpless dihydroxylation conditions using the simpler α-DHQ-CLB ligand. It has to be noted that the 4 : 1 ratio represents the mismatched case. The *Z*-diastereoisomer remained untouched under the



Scheme 3 Total synthesis of tonantzitlolone (**1**). Reagents and conditions: (a) TBAF 3 H_2O, THF, rt, 45 min (>99%); (b) TES-Cl (excess), imidazole, DMF, rt, 21 h then 50 °C, 3 h (89%); (c) Dess–Martin periodinane, CH_2Cl_2, 0 °C to rt, 3 h (93%), then TBAF 3 H_2O, THF, 0 °C, 20 min (92%); (e) 10 mol% Grubbs-II, CH_2Cl_2, Δ, 1.5 h, (93%; *E*/*Z* ~ 4 : 1); (f) TMS-Cl (excess), Et_3N, DMF, 0 °C, (93%); (g) 10 mol% OsO_4, 36 mol% α-DHQ-CLB, K_2CO_3, $MeSO_2NH_2$, $K_3Fe(CN)_6$, *t*BuOH, H_2O, 0 °C, 3 h, then TBAF, THF, 0 °C, 15 min (97%, dr ~ 4 : 1 for favored (4*R*,5*R*)-diastereoisomer); (h) PPTS, CH_2Cl_2, rt, 20 min (51% and 36% of a mixture with starting material 4*R*,5*R* and 4*S*,5*S*); (i) $HO_2CC{=}C(Me)Et$, DIC, DMAP, CH_2Cl_2, rt, 17 h (45%); (j) TPAP (cat.), NMO, 4 Å MS, CH_2Cl_2, rt, 40 min (**1**: 21%, **29**: 21%).

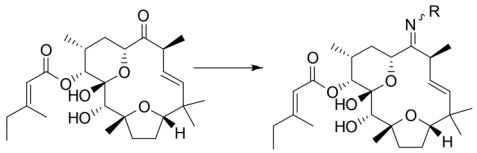
dihydroxylation conditions. The equilibrium between ketone **25** obtained after removal of the TMS-protection and semiacetal **26** could only be shifted towards the desired pyranol under acidic conditions. Next, the introduction of (*E*)-3-methylpent-2-enoic acid became possible. This step was not regioselective and provided ester **27** and **28** with preference for the C-4 position. The hydroxy group at C10 remained untouched. The two regioisomers could not be separated at this point so that this step was coupled with the Ley–Griffith oxidation. This two step protocol provided two final products: (a) tonantzitlolone **1** and its regioisomer **29** in which the ester side chain is located at C4 and C8, respectively while C10 remained untouched. Depending on the location of the side chain, either C4 or C10 are oxidized in the final step. The isomers could be separated by column chromatography. The synthetic sample of tonantzitlolone (**1**) was identical with an authentic sample including optical rotations: $[\alpha]_{20}^{D}$ = +116° (*c* 0.5 in $CHCl_3$) (authentic material: $[\alpha]_{20}^{D}$ = +134° (*c* 0.25 in $CHCl_3$, ref. 1 and 10)).

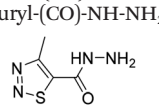
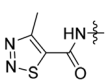
Synthesis of tonantzitlolone derivatives, modified at C4

Tonantzitlolone A (**1**) served as a starting point for the preparation of a series of derivatives. With these we could carry out preliminary structure–activity studies for this diterpene, especially in view of it potency to inhibit the mitotic motor protein kinesin-5.[4]

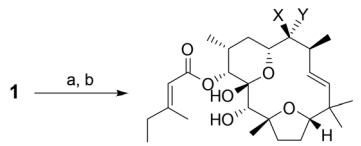
The keto group at C-4 can be condensed with several amino containing agents to yield C=N derivatives thereby preserving the sp^2-character at C-4. However, simple amines like methyl amine cannot be used as amino donors, because they yield complex product mixtures. From these we could identify products that had undergone double bond migration to the corresponding enone (C2–C4) as well as lactol opening with transacylation and migration of the ester side chain most likely to C-5.[16,17] In contrast, two diastereomeric oximes **30a,b** become available after treatment of **1** with hydroxyl amine (Table 1, entry 1). The *E*/*Z*-isomers are configurationally stable and can easily be separated chromatographically. Their acyla-

Table 1 Synthesis of tonantzitlolone derivatives **30–36**, structure of derivative **37** and stereochemical assignments by NOe measurements



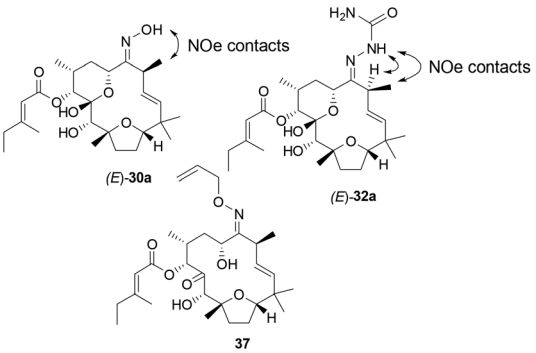
Entry	Conditions	Product, R	*E*/*Z*-ratio	Yield [%]
1	H_2NOH, pr, rt, 12h	**30a,b**-OH	2 : 1	80
2	H_2NO-allyl, pr, rt, 12h	**31a,b**-*O*-Allyl	6 : 1	35[a]
3	$H_2N(CO)NH\text{-}NH_3Cl$ (10 eq.), py, rt, 12h	**32a,b**-$NH(CO)NH_2$	10 : 1	91
4	*p*H_2NPh-$(CO)NHNH_2$, py, 1N HCl, rt, 36 h	**33a,b**-$NH(CO)$*p*$PhNH_2$	1 : 5	76
5	*p*HOPh-$(CO)NHNH_2$, py, 1N HCl, rt, 4d	**34a,b**-$NH(CO)$*p*PhOH	1 : 9	79
6	3-Furyl-(CO)-NH-NH_2, CH_2Cl_2, PTSA, MS 4A, 24 h, 40 °C	**35a,b**, –NH(CO)–3-furyl	1 : 4.	67[b]
7	 py, 1N HCl, rt,10d,	 **36a,b**	1 : 7	12

[a] Hydroxyketone **37** formed which could in part be ring closed to the lactol [PPTS, CH_2Cl_2, rt, 6 h (35% **31a,b**, 55% **37**)]. [b] 19% starting material reisolated.



Scheme 4 Reduction of the keto group at C-4. Reagents and conditions: (a) dibal-H, CH_2Cl_2, −78 °C, (82%), or (b) polystyrene-$CH_2NMe_3BH_4$, MeOH, rt (90%).

tion turned out to be difficult and no defined products were isolated.

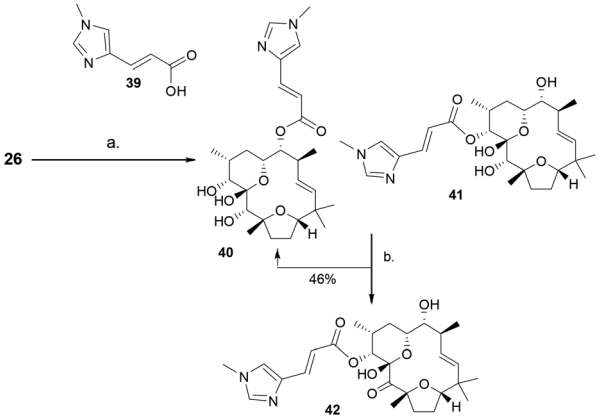


Also allyl oximes **31a,b** were prepared from tonantzitlolone **1** but here the condensation reaction yielded the ring-opened acetal **37**. Ringclosure to the hemiacetals **31a,b** could only be enforced under acidic conditions (see also **25** → **26**). Additionally, also semicarbazones **32a,b** and acyl hydrazides **33a,b–36a,b** were prepared under standard condensation conditions. The ratio of *E*/*Z*-diastereomers differed. Oximes and semicarbazones **32a,b** preferentially yielded *E*-configured isomers, while the reaction with acyl hydrazines strongly favoured the formation of *Z*-acyl hydrazides **33–36** as judged from NOe-measurements.

The epimer **38** of dihydro tonantzitlolone **27** becomes available by reducing the keto group at C-4 in **1**. Both diastereomeric hydro-tonantzitlolone A derivatives **27** and **38** are generated with moderate substrate control, depending on the reducing agent employed (Scheme 4).

Synthesis of tonantzitlolone derivatives with different ester side chains

Tonantzitlolone precursor **26**, in which the side chain is missing, can be used to introduce other ester side chains (Scheme 5). *E.g.*, coupling with carboxylic acid **39**,[18] the side chain in eleutherobin (**5**), under standard condensation conditions yielded two unseparable regioisomeric esters **40** and **41**[19] similar to the transformation **26** → **27**/**28** (Scheme 3). Carefully conducted Ley–Griffith oxidation of this mixture only exerted the transformation of regioisomer **41**, while ester **40** was reisolated. Unexpectedly, the oxidation took place at C10 and not at C4 thereby yielding ketone **42**. This observation is

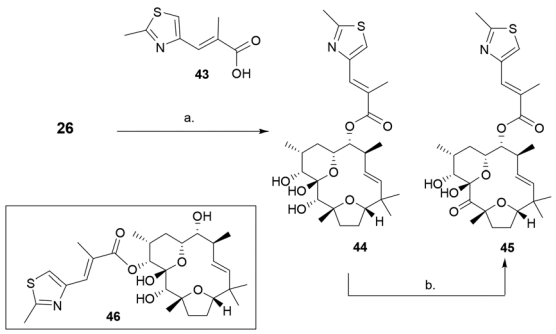


Scheme 5 Preparation of tonantzitlolone derivatives **40–42**. Reagents and conditions: (a) CH_2Cl_2, DIC, rt, 1 h, then DMAP, 19 h (72%; **40** : **41** = 2 : 1); (b) TPAP, NMO, MS 4 Å, CH_2Cl_2, rt, 1.5 h (36%).

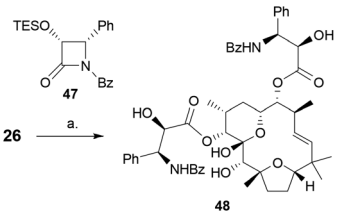
difficult to rationalize. One may discuss two effects: (a) shielding of position 4 by the side chain or (b) precoordination of the oxidant to the imidazol moiety thereby directing it close to the C10 position.

Next, we probed the introduction of the terminal polyketide element of the antitumor agent epothilone (Scheme 6).[20] (*E*)-2-Methyl-3-(2-methylthiazol-4-yl)acrylic acid **43**[21] was coupled with tonantzitlolone derivative **26** to exclusively yield the regioisomer **44** even when a 9-fold excess (with respect to **26**) of the carboxylic acid **43** was employed. The formation of the regioisomeric ester **46** could not be detected. Ley–Griffith oxidation again selectively occurred at C10 to furnish ketone **45**.

Finally, we also introduced the α-hydroxy-β-amino acid present in the diterpene paclitaxel **4** utilizing the activated β-lactam **47**[22] (Scheme 7). In this case only the diester **48** could be isolated in small amounts after extensive HPLC purification. The reaction proceeded rapidly under basic conditions but the product mixture was complex and NMR analysis was hampered by line broadening. The small isolated amounts of **48** stopped us to conduct the Ley–Griffith oxidation.



Scheme 6 Preparation of tonantzitlolone derivatives **44–46**. Reagents and conditions: (a) **43**, CH_2Cl_2, DIC, rt, 16 h, then DMAP, 29 h (53%); (b) TPAP, NMO, MS 4 Å, CH_2Cl_2, rt, 3.5 h (78%).



Scheme 7 Preparation of tonantzitlolone derivative **48**. Reagents and conditions: (a) NaHMDS, THF, 0 °C, 0.5 h, then THF, TBAF* 3 H_2O, 0 °C, 20 min (7%).

The latter series of experiments reveal the difficulties in predicting and rationalizing the regio- and chemoselectivity of even simple transformations on the periphery of multifunctionalized natural product scaffolds or backbones.[2,3]

Biological evaluation

For a first biological evaluation tonantzitlolone A (**1**) and its derivatives were subjected to *in vitro* biological testing of their anti-proliferative activity on the mouse fibroblasts cell line L-929, the *Potorous tridactylus* cell line PtK2, and the human breast cancer cell line MCF-7. This biological evaluation indicated moderate low-μM activity against cultured mammalian cells of different origin. The results from these tests are given as values for the half-maximal cell growth inhibitory concentration (IC_{50}, Table 2).

Our finding that tonantzitlolone and its enantiomer are able to block kinesin-5 was based on an earlier observation. When PtK_2 potoroo kidney cells were incubated for 18 h with 21.5 μM of **1** dissolved in methanol, ~20% of mitotic cells were arrested in mitosis and showed an unphysiological monoastral half spindle instead of a normal bipolar spindle apparatus. Such a monoastral phenotype is associated with the inhibition of the molecular motor protein kinesin-5 (also known as Kif11, Eg5 or kinesin spindle protein KSP).[6]

Table 2 Half-maximal anti-proliferative activity IC_{50} [μM] of **1**, **25**, **26**, **27**, **29–38**, **40**, **42**, **44**, **45** and **48** with mammalian cell lines. Values shown are means of two determinations in parallel; L-292 (mouse fibroblasts), PtK2 (kidney cell line of a potoroo), MCF-7 (human breast cancer cell line)

	1	25	26[a]	27	29[b]	30	31 (37[c])	32	33
L-929	>86	54	94	43	11	31	13 (58)	10	32
PtK2	43	>108	22	—	11	—	6 (6)	4	37
MCF-7	—	>108	>108	—	—	>83	9 (—)	15	14

	34	35	36	38	40	42	44	45	48
L-929	15	31	46	>86	69	33	7	7	9
PtK2	13	17	10	—	30	13	24	37	8
MCF-7	8	11	12	—	>79	>66	17	—	12

[a] *ent*-**26** (IC_{50} with L-929 > 108 μM). [b] *ent*-**29** (IC_{50} with L-929 > 86 μM). [c] Ring opened lactol.

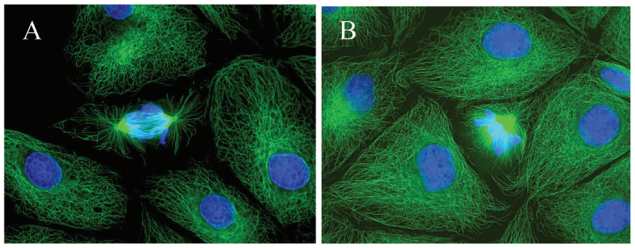


Fig. 2 Derivatives of tonantzitlolone induce half-spindle formation as was observed with the parent compound. (A) PtK_2 potoroo kidney cells with a mitotic cell in the centre showing a bipolar spindle. (B) A monopolar half-spindle was induced after incubation with **25**. Since we did not find a significant correlation between growth inhibitory activity and influence on spindle formation there may be additional biological targets involved. Microtubules are shown in green, DAPI labeled DNA is shown in blue.

In order to link the anti-proliferative activity of the new derivatives with the established biological target found for tonantzitlolone (**1**), we also studied the effect of tonantzitlolone derivatives **25**, **29**, **32**, **33**, **34**, **37**, **42**, and **48** on mitotic spindle formation of PtK_2 potoroo kidney cells. While normal spindles show a symmetrical bipolar structure, tonantzitlolone and also derivatives of it induced the formation of monopolar half-spindles. This effect was most pronounced with derivative **32**, which was also the most active anti-proliferative one. One third of the mitotic spindles counted had a half-spindle shape at a given concentration of 19 μM. But our preliminary investigation on the relation between IC_{50} and the degree of half-spindle formation could not show a significant correlation of these two parameters. Thus, we cannot exclude additional effects (Fig. 2).[5]

Conclusions

In summary, the present work discloses the total synthesis of tonantzitlolone A and the preparation of a small library of several tonantzitlolone derivatives modified at C4, C8 and C10, respectively. Compared to tonantzitlolone A, many of these new derivatives show similar or improved antiproliferative activity towards mammalian cell lines in the lower micromolecular range. Particularly, the allyl oxime **31** and the semicarbazone **32** showed clearly higher activities than the parent natural product. **32** was found to be 10-fold more potent. In this case we linked the activity to a high degree of monoastral half-spindle formation which suggests inhibition of the mitotic motor protein kinesin-5 as reported before.[5] A more reliable statement about the link between IC_{50} and kinesin-5 inhibition can only be made after measuring the inhibitory activity on the target itself. Although the antiproliferative activities are not in the range of what one expects from chemotherapeutics the study reveals that tonantzitlolone can be modified in various positions with improved activity which paves the way for finding more potent derivatives in the future.

Experimental

The description of the synthetic protocols, the analytic data and the procedure for the biological evaluations are found in the ESI.†

Acknowledgements

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