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Original article

Cytotoxic ring A-modified steroid analogues derived from Grundmann's ketone

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Dedicated to Prof. Dr. Gunther Seitz, Marburg, at the occasion of his 75th birthday.

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ABSTRACT

A series of steroid and azasteroid analogues containing a six-membered ring A with various functionalities were synthesized. Furthermore, the syntheses of tetracyclic analogues bearing a five-membered A-ring and the syntheses of a number of bicyclic secosteroid analogues were carried out. All compounds were tested for their antibacterial, antifungal and cytotoxic activities. Among all tested compounds **7** and **9** showed outstanding cytotoxic activities but were devoid of antimicrobial activities. The cytotoxic activities of compounds **7**, **9** and **10** were initially verified by the National Cancer Institute (NCI) in a one-dose 60 cell assay. In accordance with our results **7** and **9** satisfied pre-determined threshold inhibition criteria for progression to the 5-dose NCI screening, which revealed a selective activity profile for both candidates.

1. Introduction

Steroids play pivotal roles in human metabolism by acting as hormones (androgens, estrogens, gestagens, glucocorticoids, mineralocorticoids) or essential parts of cell membranes (cholesterol). Typically, ring A of these physiological sterols consists of a 3-hydroxycyclohexane moiety (cholesterol), a cyclohexenone (androgens, gestagens, glucocorticoids, mineralocorticoids) or a phenol ring (estrogens). Introduction of other structural motifs into ring A of sterols frequently results in enhanced or completely different biological activities. In glucocorticoids an additional double bond (Δ1,2) results in enhanced potency and receptor selectivity [1], whereas the potent antiandrogen and gestagen cyproterone acetate contains a cyclopropane ring annulated at the 1,2-bond [1]. Additional amino groups at ring A are found in the plakinamines, a class of marine steroid alkaloids with cytotoxic and antifungal activities [2], the 2-hydroxy-3-aminosteroid amafolone shows antiarrhythmic activity [3], the muscle relaxant pancuronium bromide contains a quaternary ammonium group at C-2 [1]. Among the large group of biologically active heterosteroids the 5α-reductase inhibitors finasteride and dutasteride (4-azasteroids) gained particular importance in the treatment of benign prostatic hyperplasia and alopecia [4].

A large number of bioactive sterols oxidized in various rings have been isolated from marine organisms [5], and related semisynthetic epoxysterols show potent cytotoxicity [6]. Recently, the withanolides [7], a class of more than 300 oxidized sterols isolated from plants largely belonging to the *Solanaceae* family, have attracted considerable attention due to their cytotoxic [8,9], antifungal [10], leishmanicidal, trypanocidal [11], insecticidal, anti-inflammatory, anti-Alzheimer [12] and other activities. The vast majority of bioactive withanolides, e.g. withanolide D (Fig. 1) [10], contains an enone structure in ring A of the steroidal ring system, frequently accompanied by an epoxide group at ring B.

Among the metabolites of estradiol, both 2-hydroxy- and 4-hydroxyestradiol can be oxidized to the corresponding *ortho*-quinones (Fig. 1) in vivo and then undergo Michael-type additions with physiological nucleophiles like thiols and amino groups [13,14] and DNA bases like adenine [15].

The evident bioreactivity of Michael acceptor systems in the ring A of steroids prompted us to investigate novel steroid and azasteroid analogues with miscellaneous functionalities in ring A.

2. Results and discussion

2.1. Chemistry

Steroid analogues with a high structural diversity in ring A were prepared following a protocol worked out by De Riccardis et al. [16,17] based on fundamental investigations of Alberola et al. [18].

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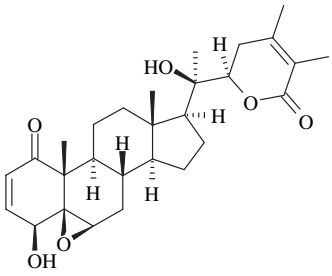
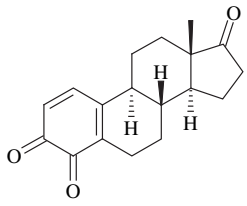


withanolide D

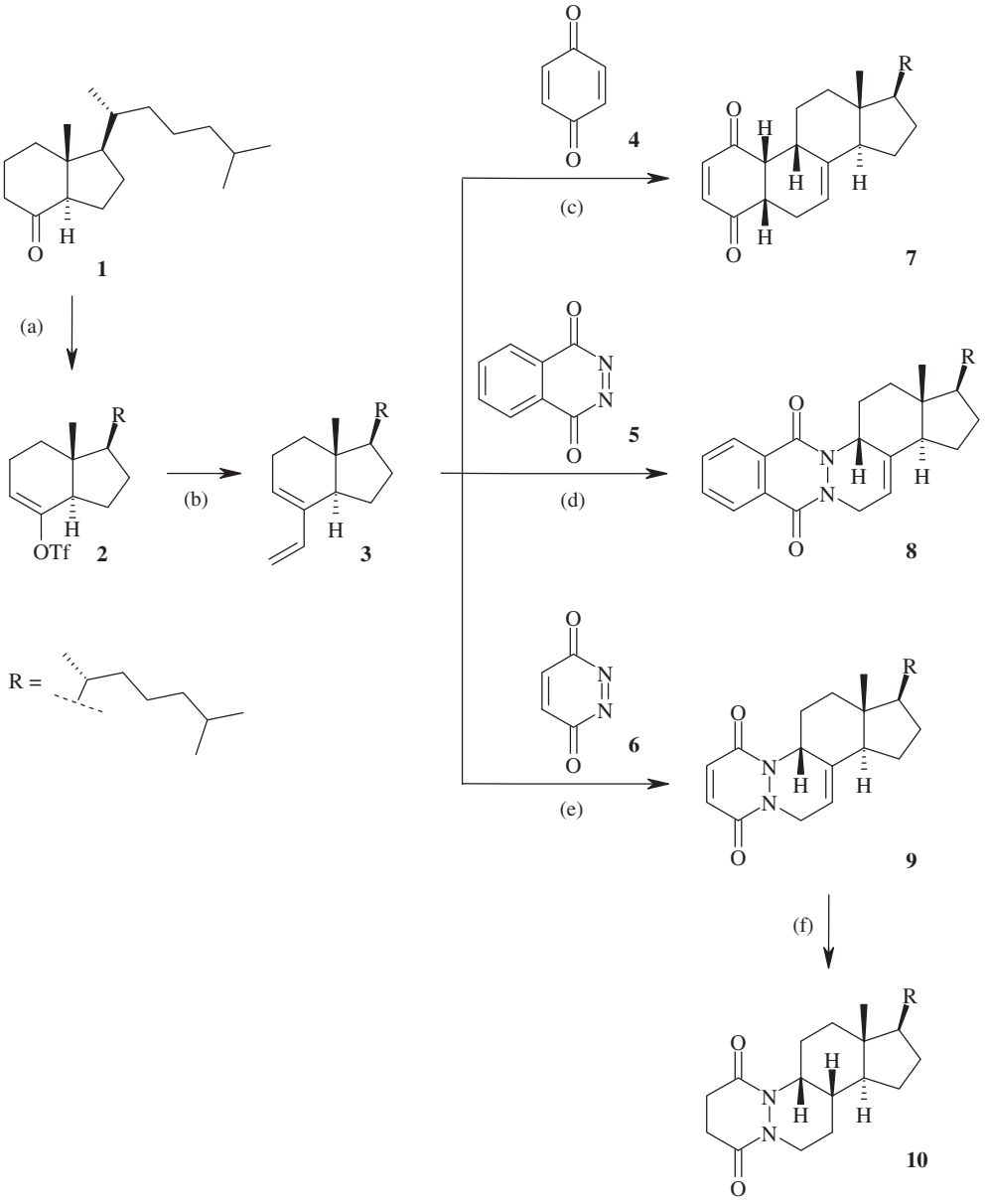


3,4-estrone-*o*-quinone

Fig. 1. Structures of withanolide D [10] and 3,4-estrone-*o*-quinone [15].

Thus, Grundmann's ketone (**1**), readily available by ozonolysis of vitamine D_3, was converted to the vinyl triflate **2** under kinetic control, the latter then converted to the 1,3-diene **3** by Stille coupling with vinyltributyltin, catalyzed by tetrakis(triphenylphosphine)-palladium(0). This diene had already been converted to the 1,4-dioxocholestane **7** by Diels-Alder cycloaddition with *p*-benzoquinone (**4**) [16] and to several moderately cytotoxic steroid-anthraquinone hybrids by cycloaddition/oxidation with naphthoquinones by De Riccardis (Scheme 1) [17].

In order to supply the compound for screenings, we prepared product **7** following the reported protocol [17]. The stereochemistry of the single isomer formed in this cycloaddition was confirmed by extensive NOE experiments (Supplementary data, Fig. 1). Further, we prepared a series of heterosteroidal congeners of **7** by cycloaddition of **3** with various cyclic dienophiles and diazadienophiles. Phthalazine-1,4-dione (**5**), prepared *in situ* by oxidation of phthalhydrazide with lead tetraacetate [19], gave the pentacyclic



Scheme 1. Reagents and conditions: (a) Sodium bis(trimethylsilyl)amide, THF, −78 °C, 1 h, then *N*-phenyl-bis(trifluoromethanesulfonimide), −78 °C to r.t., 2.2 h (67%); (b) $(PPh_3)_4Pd$, LiCl, vinyltributyltin, THF, 75 °C, 2 h (98%); (c) *p*-benzoquinone, toluene, 120 °C, 12 h (36%); (d) phthalhydrazide, $Pb(OAc)_4$, toluene, 120 °C, 12 h (76%); (e) 3,6-pyridazinedione (prepared *in situ* [20]), acetone, −78 °C to r.t., 1 h (20%); (f) H_2, PtO_2, MeOH, r.t., 20 bar, 48 h (76%).

Diels-Alder product **8**. For preparing the tetracyclic diazacholestane analogue **9**, the diazadienophile 3,6-pyridazinedione (**6**) had to be prepared freshly by *tert*-butylhypochlorite oxidation of the monopotassium salt of 3,6-dihydroxypyridazine [20]. Subsequent cycloaddition with diene **3** at low temperature gave product **9** in poor yield (20%). Comparably low yields in cycloadditions with 3,6-pyridazinedione had been reported by others [21]. Since **9** was identified as being highly cytotoxic in early investigations, and we supposed that the Michael acceptor system in ring A of this compound might be crucial for this activity, we converted **9** to its tetrahydro analogue **10** by catalytic hydrogenation of both olefinic moieties (Scheme 1). Once again the exact stereochemistry of the product was elucidated by extensive NMR experiments (Supplementary data, Figure 2).

A second series of compounds consisted in Diels-Alder products of diene **3** with various five-membered dienophiles (**11**, **12a**–**e**). Maleic anhydride [22], maleimide, as well as its *N*-methyl, *N*-ethyl and *N*-propyl derivatives underwent smooth cycloaddition with **3** to give the tetracycles **13** and **14a**–**d**. In an analogous manner 4-methyl-1,2,4-triazoline-3,5-dione [23] was converted to the triaza tetracycle **14e** (Scheme 2).

Since the cycloadducts **7** and **9**, standing out due to the Michael acceptor systems in ring A, showed by far the highest cytotoxic activities of all compounds prepared until then (Table 1), we prepared a third series of compounds. These products are characterized by having a maleimide group attached by a rigid or flexible C_3- or C_4-spacer to a bicyclic ring system derived from vinyl triflate **2**, the latter resembling sterol rings C and D and the lipophilic sterol side chain. The target compounds still are Michael acceptors, but do no longer contain the complete, rigid sterol backbone (Scheme 3).

Heck reaction [24,25] of **2** and allyl alcohol catalyzed by tri-*o*-tolylphosphine and palladium(II) acetate under microwave irradiation gave the hydroxymethyl-1,3-diene **15** in moderate yield. In contrast acetylenic alcohol **16** was obtained in excellent yield by Sonogashira coupling [26] of **2** and 3-butyn-1-ol catalyzed by $(PPh_3)_2PdCl_2$ under microwave conditions. Catalytic hydrogenation of **16** gave an α/β epimeric mixture of the fully saturated alcohol **17**, which was not separable by column chromatography. Thus, the crude mixture was used in the next step. The maleimides **18**, **19** and **20** were prepared from the corresponding alcohols **15**, **16** and **17** and maleimide in poor to moderate yields by Mitsunobu reaction under microwave conditions [27]. Under conventional Mitsunobu conditions (maleimide, DIAD, triphenylphosphine and the appropriate alcohol) no conversions were recorded at all.

Finally, we intended to elucidate whether or not the complete steroid-like tetracyclic ring system is required for the extraordinary biological activity of diazasteroid **9**. For this purpose the natural terpene *β*-myrcene (**21**) was selected as a suitable diene component, and reacted with 3,6-pyridazinedione (6) to give the bicyclic pyridazino[1,2-a]pyridazine-1,4-dione **22**. This product contains the ring A + B motif of **9**, but rings C and D of the steroid and the side chain are only adumbrated by a flexible side chain (Scheme 4).

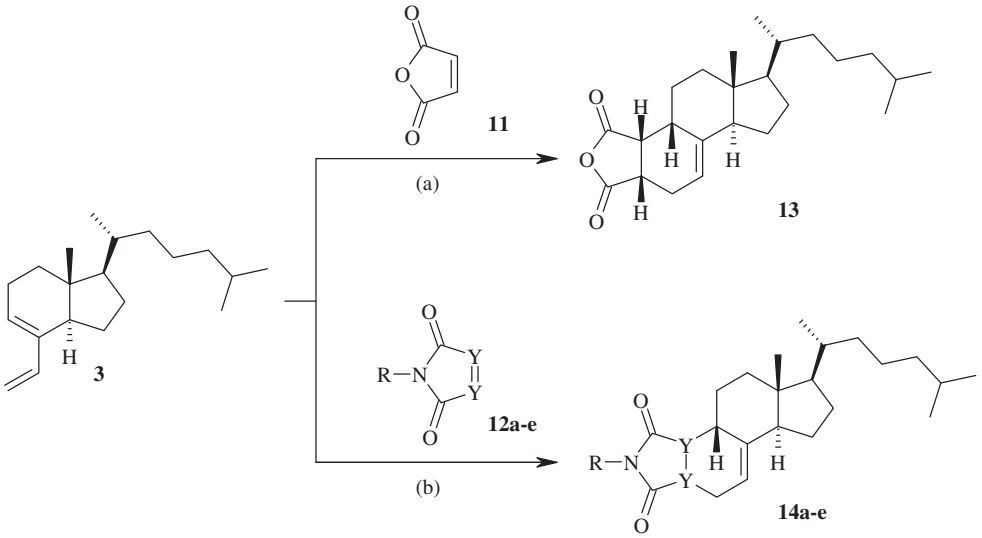
2.2. Pharmacology

2.2.1. Cytotoxic activity

A first screening for cytotoxic activities of the compounds was performed in a MTT assay [28] on human leukemia HL-60 cells. Cisplatin was used as reference. The results are shown in Table 1. Significant cytotoxicities ($IC_{50} < 10$ μM) were recorded only for three tetracyclic compounds: The *N*-propylmaleimide adduct **14d** showed cytotoxic potency comparable to cisplatin, whereas the diones **7** and **9** stand out with IC_{50}-values of 0.7 μM and 0.03 μM.

2.2.2. Antimicrobial activity

The compounds were subjected to a standard disc diffusion antimicrobial sensitivity test (Agar diffusion assay). Table 2 summarizes the results obtained for the zones of inhibition of all tested compounds against different strains of Gram-positive bacteria,



14 a: R = H; Y = CH
14 b: R = CH_3; Y = CH
14 c: R = C_2H_5; Y = CH
14 d: R = C_3H_7; Y = CH
14 e: R = CH_3; Y = N

Scheme 2. Reagents and conditions: (a) maleic anhydride, toluene, 120 °C, 12 h (77%); (b) dienophile (**a** = maleimide, **b** = *N*-methylmaleimide, **c** = *N*-ethylmaleimide, **d** = *N*-propylmaleimide, **e** = 4-methyl-1,2,4-triazoline-3,5-dione), toluene, 120 °C, 12 h (41–81%).

Table 1
Cytotoxic activities of investigated compounds against HL-60 cells determined by MTT test.

Compound	Molecular formula	IC_{50} [μM]
Cisplatin	$Cl_2H_6N_2Pt$	5
3	$C_{20}H_{34}$	>100
7	$C_{26}H_{38}O_2$	**0.7**
8	$C_{28}H_{38}N_2O_2$	17
9	$C_{24}H_{36}N_2O_2$	**0.03**
10	$C_{24}H_{40}N_2O_2$	30
13	$C_{24}H_{36}O_3$	30
14a	$C_{24}H_{37}NO_2$	16
14b	$C_{25}H_{39}NO_2$	15
14c	$C_{26}H_{41}NO_2$	13
14d	$C_{27}H_{43}NO_2$	7
14e	$C_{23}H_{37}N_3O_2$	13
15	$C_{21}H_{36}O$	32
16	$C_{22}H_{36}O$	38
17	$C_{22}H_{42}O$	13
18	$C_{25}H_{37}NO_2$	32
19	$C_{26}H_{37}NO_2$	46
20	$C_{26}H_{43}NO_2$	14
22	$C_{14}H_{18}N_2O_2$	41

different strains of Gram-negative bacteria, yeasts, a mould and a dermatophyte. The results are expressed as diameter of zone (mm). Tetracycline (antibiotic) and clotrimazole (antimycotic) were used as references.

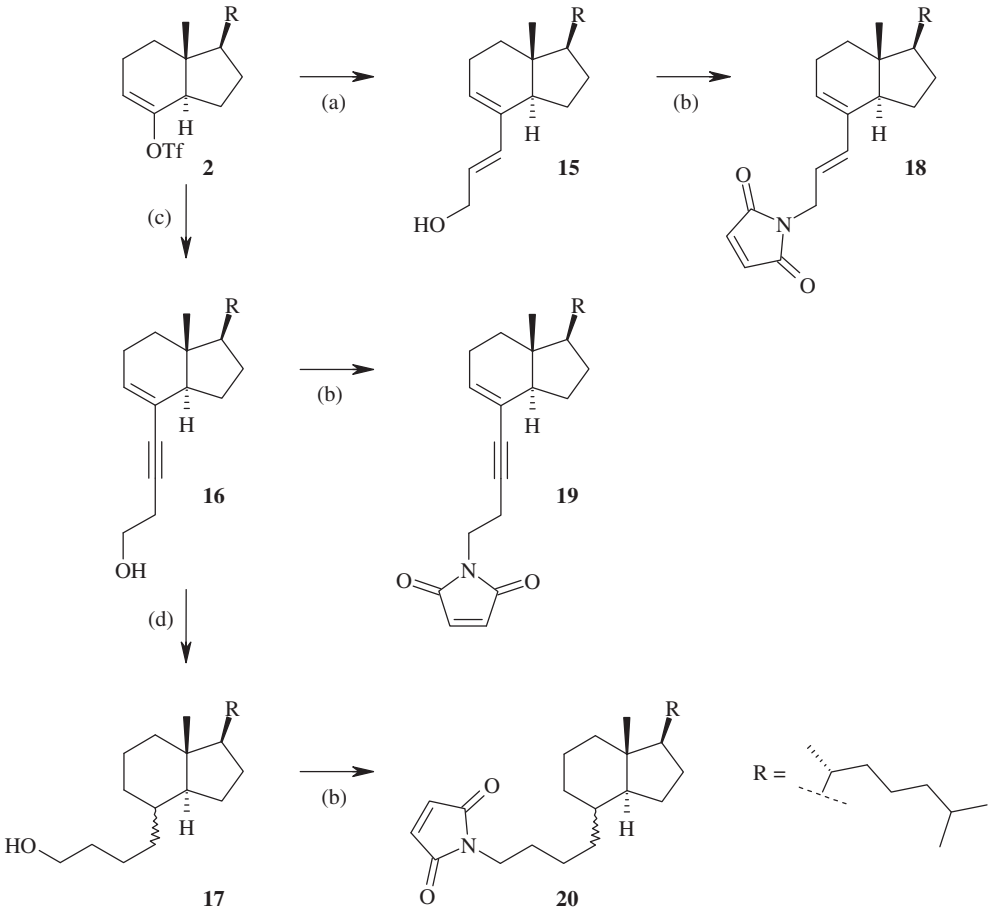
The Diels–Alder products **7**, **8** and **9** did not show noteworthy antimicrobial activity, whereas the saturated product **10** has broad activity. Among the maleimides only **14a** showed good activity. The open-chain compounds **15**–**20** showed moderate antimicrobial activities. Bicyclic compound **22** was completely inactive.

2.2.3. Primary single high dose (10^{-5} M) full NCI 60 cell panel in vitro assay

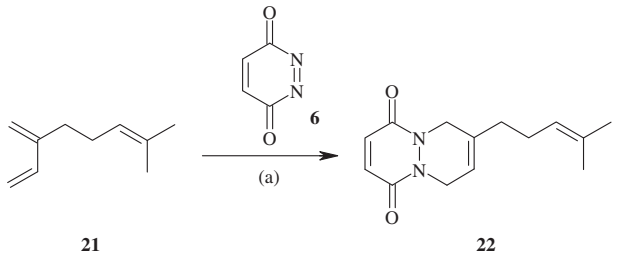
A selection of compounds described above (**7**, **9**, **10**) was accepted by the National Cancer Institute (NCI, USA) for testing initially at a single high dose cell assay. This assay was performed in a 60 cell panel representing leukemia, melanoma and cancers of lung, colon, brain, breast, ovary, kidney and prostate in accordance with the protocol of the NCI [29,30]. The compounds were incubated at a single concentration (10^{-5} M) for 48 h, end point determinations were made with a protein binding dye, sulforhodamine B (SRB). Results for each compound were reported as a Mean Graph of the growth percent of the treated cells when compared to the untreated control cells (Supplementary data, Table 1). After analysis of historical Developmental Therapeutics Program (DTP) compounds **7** and **9**, which satisfied pre-determined threshold inhibition criteria were selected for the NCI full panel 5-dose assay.

2.2.4. In vitro 5-dose full NCI 60 cell panel assay

The 60 cell lines, representing nine tumor subpanels, were incubated at five different concentrations (10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} and 10^{-4} M) with the compounds and log concentration versus % growth inhibition curves and three response parameters (GI_{50}, TGI and LC_{50}) were calculated from the outcomes for each cell line. The GI_{50} value (growth inhibitory activity) corresponds to the concentration of the



Scheme 3. Reagents and conditions: (a) allyl alcohol, tributylamine, tri-*o*-tolylphosphine, $Pd(OAc)_2$, MW: 80 W, 150 °C, 10 min (36%); (b) PPh_3, maleimide, THF, DIAD, MW: 50 W, 76 °C, 30 min (8–44%); (c) 3-butyn-1-ol, diethylamine, DMF, CuI, $(PPh_3)_2PdCl_2$, MW: 300 W, 120 °C, 5 min (98%); (d) H_2, PtO_2, ethyl acetate, r.t., 48 h (95%).



Scheme 4. Reagents and conditions: (a) 3,6-pyridazinedione (prepared *in situ*), acetone, −78 °C to r.t., 1 h (11%).

compound causing 50% decrease in net cell growth, the TGI value (cytostatic activity) is the concentration of the compound resulting in total growth inhibition and LC_{50} value (cytotoxic activity) is the concentration of the compound causing net 50% loss of initial cells at the end of the incubation period of 48 h.

The results of the assays are summarized as GI_{50}-, TGI- and LC_{50}-values of each cell line in Table 3. A cell line, which found to be insensitive at the highest tested concentration (100 μM), is signed with ">" as prefix to the concentration.

The observed GI_{50} values of compound **7** are, with only two exceptions, below 1 μM for all tested cell lines. Very high activities were recorded against the non-small cell lung cancer cells A549/ATCC and HOP-92, the colon cancers COLO 205 and HCT-116, the ovarian cancer OVCAR-8, the renal cancer UO-31 and the prostate cancer PC-3 with GI_{50} values in a low nM range (<100 nM). The obtained TGI-data for compound **7** represent a characteristic sensitivity profile that showed 13 cell lines with total growth inhibitory values less than 1 μM. Notable are the LC_{50}-values of the cell lines HCT-116, LOX IMVI, OVCAR-4, OVCAR-8 and UO-31 ranging from 8.10 μM to 1.75 μM. The highest cytotoxic activity of compound **7** was observed against the colon cancer cell line COLO 205 with a LC_{50} value of 0.54 μM.

The diaza analogue **9** showed even higher activity against all cell lines with GI_{50} values between 1.8 μM and 110 nM. The obtained TGI values of **9** are in the range of 4.79 μM to 0.29 μM. Neither the GI_{50}- nor the TGI Mean Graph shows any characteristic fingerprint of sensitivity. The cytotoxic activity of compound **9** is remarkable and exhibits a selective activity profile. Very low LC_{50}-values between 9.0 μM and 0.6 μM could be observed against all cell lines except the complete leukemia sub panel and some very few other cell lines (HCT-15, OVCAR-8 and HS 578T).

Table 2
Antimicrobial activities of investigated compounds determined by disk diffusion sensitivity test expressed as zone of inhibition (diameter of zone in mm).

	Ec	*Pa*	*Seq*	*Sen*	*Yl*	*Cg*	*An*	*Hb*
Tetracycline	30	25	30	22	n.i.	n.i.	n.i.	n.i.
Clotrimazole	n.i.	n.i.	n.i.	n.i.	18	18	13	20
3	–	–	10	9	–	–	–	–
7	–	–	–	–	–	–	–	–
8	–	7	–	–	–	10	–	7
9	–	8	–	7	7	8	–	10
10	11	12	9	10	15	–	–	15
13	7	10	–	12	–	8	–	10
14a	24	11	14	15	–	15	–	–
14b	10	7	–	10	–	10	–	14
14c	7	7	8	11	–	8	–	7
14d	7	7	–	7	10	6	–	14
14e	–	–	10	–	–	–	–	–
15	10	18	12	14	–	18	–	22
16	10	11	6	14	–	10	–	11
18	9	10	11	15	–	7	–	8
19	15	13	11	15	–	10	–	12
20	10	9	11	12	–	8	–	9
22	–	–	–	–	–	–	–	–

Ec, Escherichia coli; Pa, Pseudomonas antimicrobia; Seq, Staphylococcus equorum; Sen, Streptococcus entericus; Yl, Yarrowia lipolytica; Cg, Candida glabrata; An, Aspergillus niger; Hb, Hyphopichia burtonii; n.i., not investigated; –, inactive; concentration, 1% (w/v) DMSO solution of the testing compound.

3. Conclusion

In conclusion, we have synthesized a number of steroid and secosteroid analogues starting from Grundmann's ketone (**1**), which itself is readily available from commercial vitamin D_3. Diels–Alder cycloadditions of the 1,3-diene **3** with (aza)dienophiles gave steroid analogues **7**–**9**, **13** and **14a**–**e**, all products containing two carbonyl groups in the ring A equivalent. Within this subclass compounds **7** and **9** stand out due to their Michael acceptor system in ring A. Seco analogues containing *N*-alkylmaleimide moieties, which are Michel acceptors, too, were prepared from vinyl triflate **2** via Pd-catalyzed coupling reactions with C_3 and C_4-spacers, followed by attaching the maleimide moiety under Mitsunobu conditions. Finally, a bicyclic analogon **22** of compound **9** was obtained by Diels–Alder cycloaddition of β-myrcene (**21**) with 3,6-pyridazinedione.

The compounds were tested for their antibacterial, antifungal and cytotoxic activities. The tetra- and pentacyclic Diels–Alder products **7**, **8** and **9** were almost inactive in the screenings for antimicrobial activities, the tetrahydro analogue **10** showed moderate activity. Maleimides **14a**–**e** and the open-chain analogues containing terminal maleimido groups also showed moderate antimicrobial activities.

In a preliminary in-house screening for cytotoxic activity on HL-60 cells the tetracyclic compounds **7** and **9** showed extremely promising effects. These results were reinforced by screening results at the NCI. The single high dose 60 cell panel assays revealed that the (diaza)quinone derivatives **7** and **9** exhibit remarkable cytotoxic activities, whereas the hydrogenated analogue **10** showed only moderate activity. Compounds **7** and **9** were further investigated in a 5-dose 60 cell panel assay, and the diazaquinone **9** was identified as being highly active against a broad panel of cancer cell lines, except leukemia cells.

Obviously a Michael acceptor system in ring A, which is exclusively found in compounds **7** and **9**, is essential for high cytotoxic activity in the classes of compounds investigated here. Benzoanullation (compound **8**) as well as hydrogenation of the double bond (**10**) lead to a drastic decrease of activity, and also the maleic anhydride (**13**) and maleimido adducts (**14a**–**e**) are by far less active.

Furthermore, an intact tetracyclic steroid-like ring system seems to be indispensable for cytotoxic activity. The secosteroids **18**–**20,** only consisting of the rings C + D fragment of the steroid backbone, which is attached to a Michael acceptor system via a flexible alkyl side chain to mimic the rings A + B fragment, showed only very poor cytotoxic activities. The β-myrcene cycloaddition product **22**, which compared to the most active compound **9** lacks the rings C and D of the steroid backbone, is completely inactive.

Further it is worth mentioning that the compounds **7** and **9** showing outstanding activities against tumor cell lines are almost inactive against bacteria and fungi, thus suggesting that these compounds act by a specific mechanism of action, and not by unselective cytotoxicity. First investigations aimed at the identification of the target revealed that the steroid analogues **7** and **9** have no effect on cholesterol biosynthesis in the human cell line HL 60 [31]. Further investigations are under way in order to elucidate the molecular mode of action of these compounds.

Table 3
NCI DTP in vitro testing results of compounds **7** and **9** (values expressed in μM).

Compound panel	Cell line	**7** (NSC 750440/1)			**9** (NSC 750441/1)		
		GI_{50}	TGI	LC_{50}	GI_{50}	TGI	LC_{50}
Leukemia	CCRF-CEM	0.189	>100	>100	0.360	>100	>100
	HL-60 (TB)	0.153	n.d.	>100	0.113	0.490	>100
	K-562	0.249	>100	>100	0.309	2.02	>100
	MOLT-4	0.312	>100	>100	0.545	4.13	>100
	RPMI-8226	0.107	2.23	>100	0.181	0.601	>100
	SR	0.207	>100	>100	0.137	0.758	>100
Non-small cell lung cancer	A549/ATCC	0.058	0.780	>100	0.325	1.24	4.04
	EKVX	0.545	>100	>100	1.03	2.75	7.33
	HOP-62	0.630	>100	>100	1.33	2.67	5.33
	HOP-92	0.028	0.086	n.d.	0.175	1.03	3.76
	NCI-H226	0.480	n.d.	>100	1.27	3.26	8.33
	NCI-H23	0.147	n.d.	>100	1.42	3.58	9.00
	NCI-H322M	0.305	0.849	>100	1.45	2.91	5.84
	NCI-H460	0.333	5.59	>100	0.739	2.25	5.73
	NCI-H522	0.803	>100	>100	0.175	0.459	1.54
Colon cancer	COLO 205	0.080	0.220	0.536	0.255	0.623	2.36
	HCC-2998	0.274	0.855	>100	0.431	1.64	4.99
	HCT-116	0.055	1.03	3.91	0.142	0.291	0.594
	HCT-15	0.212	n.d.	>100	0.418	2.22	>100
	HT29	0.839	>100	>100	0.616	2.07	5.51
	KM12	0.338	>100	>100	0.443	1.51	4.44
	SW-620	0.141	n.d.	>100	0.375	1.36	5.33
CNS cancer	SF-268	0.566	>100	>100	1.46	2.88	5.70
	SF-295	0.141	0.539	>100	0.110	0.627	3.76
	SF-539	0.478	n.d.	>100	1.47	2.89	5.64
	SNB-19	0.654	>100	>100	1.52	3.02	6.01
	U251	0.094	1.23	n.d.	0.438	1.59	4.18
Melanoma	LOX IMVI	0.227	1.17	4.10	0.288	1.05	3.52
	MALME-3M	0.399	2.00	>100	1.44	3.03	6.38
	M14	0.299	n.d.	>100	0.285	1.01	3.32
	MDA-MB-435	0.229	0.997	>100	0.354	1.78	7.68
	SK-MEL-2	3.37	>100	>100	0.286	0.774	3.39
	SK-MEL-28	0.302	n.d.	>100	0.512	1.65	4.14
	SK-MEL-5	0.309	1.00	n.d.	0.563	1.83	4.52
	UACC-257	0.230	0.610	>100	0.433	1.55	4.32
	UACC-62	0.752	>100	>100	0.544	1.95	5.45
Ovarian cancer	OVCAR-3	0.502	>100	>100	0.861	2.11	4.78
	OVCAR-4	0.159	0.498	2.65	0.686	2.11	5.63
	OVCAR-5	0.212	>100	>100	1.05	2.87	7.84
	OVCAR-8	0.058	1.18	8.12	0.317	1.58	>100
	NCI/ADR-RES	0.275	n.d.	>100	0.417	1.90	2.28
	SK-OV-3	0.688	n.d.	>100	1.63	2.99	5.47
Renal cancer	786-0	0.311	1.50	n.d.	0.713	1.95	4.43
	A498	0.384	1.12	n.d.	1.06	2.27	4.87
	ACHN	0.162	1.35	n.d.	1.04	2.32	5.19
	CAKI-1	0.279	0.633	n.d.	0.179	0.577	3.47
	RXF 393	0.201	0.995	n.d.	0.952	2.30	5.43
	SN12C	0.269	>100	>100	0.388	1.69	5.28
	TK-10	2.65	>100	>100	0.356	1.26	3.60
	UO-31	0.072	0.359	1.75	0.237	1.15	4.19
Prostate cancer	PC-3	0.059	1.57	>100	0.243	1.11	4.83
	DU-145	0.632	>100	>100	1.04	2.26	4.89
Breast cancer	MCF7	0.112	0.757	n.d.	0.285	1.24	4.26
	MDA-MB231/ATCC	0.343	1.76	>100	1.04	2.54	6.25
	HS 578T	0.817	>100	>100	1.80	4.79	>100
	BT-549	0.427	2.55	>100	0.312	0.876	2.97
	T-47D	0.337	>100	>100	0.255	1.62	6.29
	MDA-MB-468	0.215	0.462	n.d.	0.628	2.56	9.42
MID		0.269	6.45	63.1	0.489	1.73	7.76

n.d., not determined; MID, average sensitivity of all cell lines in μM.

4. Experimental

4.1. Chemistry

Melting points were determined by open tube capillary method on a Büchi melting point B-450 apparatus and are uncorrected. IR spectra were obtained on a Perkin Elmer FT-IR: Paragon 1000 spectrometer. NMR spectra were recorded on Jeol JNMR-GX 400 (400 MHz) and Jeol JNMR-GX 500 (500 MHz) spectrometers with tetramethylsilane as an internal standard. All spectra were recorded in $CDCl_3$ as solvent and chemical shifts are reported in parts per million (ppm, δ). *J* values are given in Hz. Mass spectra (MS) were run by chemical impact (CI): Hewlett Packard 5989 A Mass Spectrometer with 59980 B Particle Beam LC/MS Inerterface and by electron impact (EI): at 70 eV on a Jeol JMS GCmate II. Anhydrous reactions were carried out in a nitrogen atmosphere unless otherwise described. Solvents were of HPLC grade or p.a. grade, if not they were distilled before use. All chemicals were purchased from

Sigma–Aldrich (Schnelldorf, Germany) and Acros Organics (Geel, Belgium). Microwave-promoted syntheses were performed on a single-mode microwave reactor (Discover) equipped with an IR temperature sensor from CEM (Kamp-Lintfort, Germany). Reactions were monitored by thin-layer chromatography (TLC) using pre-coated plastic sheets POLYGRAM® SIL G/UV254 from Macherey-Nagel (Düren, Germany). Compounds on TLC plates were detected under UV light at 254 nm and visualized by immersion in a solution of 5% $(NH_4)_6Mo_7O_{24}\cdot 4\ H_2O$, 0.2% $Ce(SO_4)_2\cdot 4\ H_2O$ and 5% conc. H_2SO_4. Merck silica gel 60 was used as stationary phase for flash column chromatography. Purity of the synthesized compounds was >95% (HPLC).

General procedure for synthesis of Diels-Alder adducts **7, 13, 14a-e.** Diene **3** [17] (0.20 g, 0.73 mmol) and dienophile (0.73 mmol) were dissolved in toluene (2.2 mL) and heated under reflux at 120 °C for 12 h. The reaction mixture was concentrated under reduced pressure. Purification was performed as described below for the single compounds.

(5*S*,9*S*,10*R*,13*R*,14*R*,17*R*)-17-((*R*)-1,5-Dimethylhexyl)-13-methyl-6,9,10,11,12,13,14,15,16,17-decahydro-5*H*-cyclopenta[a]phenanthrene-1,4-dione **(7)** [16]. Dienophile: *p*-benzoquinone **(4)**. The product was purified by silica column chromatography (hexane/ethyl acetate 4:1) to give 0.10 g (36%) **7** as pale yellow solid. m.p. 134–135 °C (for a complete analytical characterization see Supplementary data).

(1*R*,3a*R*,11b*R*,13a*R*)-1-((*R*)-1,5-Dimethylhexyl)-13a-methyl-2,3,3a,5,11b,12,13,13a-octahydro-1*H*-5a,11a-diazaindeno[5,4-a]anthracene-6,11-dione **(8).** A suspension of diene **3** (0.27 g, 1.00 mmol), phthalhydrazide (0.11 g, 1.00 mmol), lead(IV) acetate (0.44 g, 1.00 mmol) and toluene (3.0 mL) was heated at 120 °C for 12 h. The reaction mixture was concentrated under reduced pressure. The product was purified by silica column chromatography (hexane/ethyl acetate 4:1) to give 0.33 g (76%) **8** as pale yellow solid. m.p. 123–124 °C; $[\alpha]_D^{20} = -222.6$; IR (KBr): $\tilde{\nu} = 3422, 2953, 1632, 1467, 1357, 1241, 1141, 790\ cm^{-1}$; ^1H NMR ($CDCl_3$, 500 MHz): $\delta = 8.28$ (dd, $J = 9.4/6.7$ Hz, 2 H), 7.79 (dd, $J = 9.4/6.7$ Hz, 2 H), 5.78 (dd, $J = 7.3/2.7$ Hz, 1 H), 5.32 (dd, $J = 16.4/7.0$ Hz, 1 H), 5.26 (d, $J = 12.5$ Hz, 1 H), 3.86 (dt, $J = 16.3/5.2$ Hz, 1 H), 2.61–2.54 (m, 2 H), 2.05–1.84 (m, 4 H), 1.56–0.98 (m, 12 H), 0.90 (d, $J = 6.3$ Hz, 3 H), 0.89 (s, 3 H), 0.87 (d, $J = 6.6$ Hz, 3 H), 0.86 (d, $J = 6.6$ Hz, 3 H); ^{13}C NMR ($CDCl_3$, 100 MHz): $\delta = 158.9, 158.1, 139.1, 133.4, 133.1, 129.8, 128.9, 127.8, 127.2, 114.3, 57.5, 55.8, 49.9, 44.2, 40.9, 39.5, 36.0, 35.9, 35.7, 28.7, 28.0, 25.1, 23.9, 22.8, 22.6$ (2 C), 19.0, 18.3; MS (CI): m/z (%) = 435 $[M+H]^+$ (100); HRMS (EI): $m/z = 434.2936$ $[M]^+$, calcd for $C_{28}H_{38}N_2O_2$: 434.2933.

(5a*R*,7a*R*,8*R*,10a*R*)-8-((*R*)-1,5-Dimethylhexyl)-7a-methyl-6,7,7a,8,9,10,10a,12-octahydro-5a*H*-cyclopenta[f]-pyridazino[1,2-a]cinnoline-1,4-dione **(9)**. The dienophile 3,6-pyridazinedione (**6**) was prepared freshly by oxidization of 3,6-dihydroxypyridazine potassium salt with *tert*-butylhypochlorite.

Preparation of *tert*-butylhypochlorite solution. Protected from light a cooled (10 °C) aqueous solution of sodium hypochlorite (13% active chlorine, 250 mL, 46.0 mmol) was added to a vigorously stirred mixture of *tert*-butanol (4.1 mL, 43.0 mmol) and glacial acetic acid (2.6 mL, 46.0 mmol). The reaction mixture was stirred for further 3 min, then the organic layer was separated and washed with sodium carbonate solution (10 mL, 10%) and water (10 mL). The dried (calcium chloride) organic layer was used immediately for oxidization of 3,6-dihydropyridazine potassium salt.

Synthesis of 3,6-dihydroxypyridazine potassium salt. A suspension of 3,6-dihydroxypyridazine (1.00 g, 8.92 mmol) in water (4.0 mL) was treated with potassium hydroxide (0.50 g, 8.91 mmol). The reaction mixture was stirred until the solution became clear. The solvent was removed under reduced pressure and the achieved 3, 6-dihydroxypyridazine potassium salt was finely pestled and dried in an exsiccator (drying agent: phosphorus pentoxide).

Synthesis of **9**. To a stirred suspension of 3,6-dihydroxypyridazine potassium salt (0.31 g, 2.10 mmol) and acetone (15 mL) was added *tert*-butylhypochlorite solution (0.24 mL, 2.10 mmol) at −78 °C. After stirring for 3 h a solution of diene **3** (0.55 g, 2.00 mmol) and acetone (5 mL) was added. The reaction mixture was slowly warmed at r.t., stirred for further 1 h and then quenched with water (50 mL). The volatile organic components were evaporated and the resulting aqueous residue was extracted with diethyl ether (3 × 20 mL), the combined organic layers were dried over Na_2SO_4 and concentrated under reduced pressure. The product was purified by silica column chromatography (ethyl acetate) to yield 0.15 g (20%) of **9** as a pale yellow solid. m.p. 148–150 °C; $[\alpha]_D^{20} = +339.2$; IR (KBr): $\tilde{\nu} = 3423, 2954, 1627, 1467, 1416, 1342, 1129, 857\ cm^{-1}$; ^1H NMR ($CDCl_3$, 400 MHz): $\delta = 6.89$ (d, $J = 9.9$ Hz, 1 H), 6.85 (d, $J = 9.9$ Hz, 1 H), 5.70 (m, 1 H), 5.14 (dd, $J = 16.8/6.8$ Hz, 1 H), 5.03 (d, $J = 12.2$ Hz, 1 H), 3.78 (d, $J = 16.8$ Hz, 1 H), 2.64 (m, 1 H), 2.55 (m, 1 H), 2.04–1.91 (m, 2 H, 6-H), 1.89–185 (m, 2 H), 1.57–0.97 (m, 11 H), 0.90 (d, $J = 6.1$ Hz, 3 H), 0.87 (d, $J = 6.5$ Hz, 3 H), 0.86 (d, $J = 6.5$ Hz, 3 H), 0.84 (s, 3 H); ^{13}C NMR ($CDCl_3$, 100 MHz): $\delta = 157.9, 157.4, 138.6, 135.5, 133.8, 113.6, 57.5, 56.1, 49.7, 43.8, 40.7, 39.4, 35.9, 35.8, 35.7, 28.7, 28.0, 24.8, 23.9, 22.8, 22.5, 22.7, 18.9, 18.2$; MS (CI): m/z (%) = 385 $[M+H]^+$ (29), 282 (100); HRMS (EI): $m/z = 384.2784$ $[M]^+$, calcd for $C_{24}H_{36}N_2O_2$: 384.2776.

(5a*R*,7a*R*,8*R*,10a*S*,10b*S*)-8-((*R*)-1,5-Dimethylhexyl)-7a-methyl-dodecahydro-cyclopenta[f]pyridazino[1,2-a]cinnoline-1,4-dione **(10)**. Platinum(IV) oxide (30 mg, 0.13 mmol) was added to a solution of **9** (0.13 g, 0.34 mmol) and methanol (10 mL). The reaction mixture was stirred for 48 h under hydrogen atmosphere (20 bar) using a hydrogenation set-up. The mixture was filtered (celite) and concentrated under reduced pressure. The product was purified by silica column chromatography (ethyl acetate) to give 0.10 g (76%) **10** as colourless oil. $[\alpha]_D^{20} = -152.5$; IR (NaCl): $\tilde{\nu} = 3568, 2949, 1667, 1467, 1384, 1269, 1181, 717\ cm^{-1}$; ^1H NMR ($CDCl_3$, 400 MHz): $\delta = 4.57$ (dt, $J = 6.6/5.5$ Hz, 1 H), 3.97 (dt, $J = 12.5/8.8$ Hz, 1 H), 3.48 (dt, $J = 12.5/4.6$ Hz, 1 H), 2.62–2.51 (m, 4 H), 2.03–1.98 (m, 2 H), 1.93–1.75 (m, 4 H), 1.72–1.47 (m, 4 H), 1.41–0.98 (m, 11 H), 0.90 (d, $J = 6.0$ Hz, 3 H), 0.87 (d, $J = 6.6$ Hz, 3 H), 0.86 (d, $J = 6.6$ Hz, 3 H), 0.75 (s, 3 H); ^{13}C NMR ($CDCl_3$, 100 MHz): $\delta = 169.0, 168.6, 56.3, 52.8, 47.1, 41.5, 41.3, 39.4, 35.9, 35.8, 35.6, 31.4, 29.6, 29.1, 28.1, 28.0, 25.1, 25.0, 23.7, 23.0, 22.8, 22.6, 18.2, 12.9$; MS (CI): m/z (%) = 389 $[M+H]^+$ (100); HRMS (EI): $m/z = 388.3095$ $[M]^+$, calcd for $C_{24}H_{40}N_2O_2$: 388.3089.

(3a*R*,3b*S*,5a*R*,6*R*,8a*R*,10a*S*)-6-((*R*)-1,5-Dimethylhexyl)-5a-methyl-3b,4,5,5a,6,7,8,8a,10,10a-decahydro-3a*H*-indeno[5,4-e]isobenzofuran-1,3-dione **(13)**. Dienophile: maleic anhydride. The product was purified by silica column chromatography (hexane/ethyl acetate 4:1) to give 0.21 g (77%) **13** as a white solid. m.p. 139–141 °C; $[\alpha]_D^{20} = +53.6$; IR (KBr): $\tilde{\nu} = 2957, 1710, 1466, 1376, 1193, 926, 813\ cm^{-1}$; ^1H NMR ($CDCl_3$, 500 MHz): $\delta = 5.50$ (m, 1 H), 3.40 (dt, $J = 9.6/1.7$ Hz, 1 H), 3.31 (dd, $J = 9.6/6.5$ Hz, 1 H), 2.75 (dd, $J = 15.1/7.0$ Hz, 1 H), 2.42–2.31 (m, 2 H), 2.29–2.16 (m, 2 H), 2.03–1.92 (m, 2 H), 1.76 (m, 1 H), 1.62–0.98 (m, 13 H), 0.90 (d, $J = 6.5$ Hz, 3 H), 0.87 (d, $J = 6.6$ Hz, 3 H), 0.86 (d, $J = 6.6$ Hz, 3 H), 0.52 (s, 3 H); ^{13}C NMR ($CDCl_3$, 125 MHz): $\delta = 174.6, 171.8, 145.6, 116.8, 56.3, 48.9, 44.2, 42.6, 40.9, 39.5, 36.2, 36.2, 35.9, 34.2, 28.9, 28.0, 24.0, 23.9, 23.0, 22.8, 22.5, 21.6, 18.4, 18.3$; MS (CI): m/z (%) = 373 $[M+H]^+$ (100); HRMS (EI): $m/z = 372.2658$ $[M]^+$, calcd for $C_{24}H_{36}O_3$: 372.2665.

(3a*R*,3b*S*,5a*R*,6*R*,8a*R*,10a*S*)-6-((*R*)-1,5-Dimethylhexyl)-5a-methyl-3b,4,5,5a,6,7,8,8a,10,10a-decahydro-3a*H*-indeno[5,4-e]isoindol-1,3-dione **(14a)**. Dienophile: maleimide. The product was purified by silica column chromatography (hexane/ethyl acetate 4:1) to give 0.22 g (81%) **14a** as a white solid. m.p. 148–150 °C; $[\alpha]_D^{20} = -125.2$; IR (KBr): $\tilde{\nu} = 3190, 2957, 1711, 1466, 1354, 1196, 1003, 814\ cm^{-1}$; ^1H NMR ($CDCl_3$, 400 MHz): $\delta = 7.87$ (s, 1 H), 5.44 (m, 1 H), 3.16–3.07 (m, 2 H), 2.70 (dd, $J = 15.1/7.0$ Hz, 1 H), 2.40–2.25 (m, 3 H), 2.12 (m, 1 H),

2.00–1.90 (m, 2 H), 1.74 (m, 1 H), 1.58–0.99 (m, 13 H), 0.90 (d, J = 6.2 Hz, 3 H), 0.88 (d, J = 6.6 Hz, 3 H), 0.86 (d, J = 6.6 Hz, 3 H), 0.51 (s, 3 H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 180.1, 178.2, 144.9, 116.3, 56.2, 48.9, 44.6, 42.7, 41.6, 39.5, 36.4, 36.2, 35.9, 34.7, 28.9, 28.0, 23.9, 23.8, 23.1, 22.8, 22.5, 21.6, 18.4, 18.3; MS (CI): m/z (%) = 372 $[M+H]^+$ (100); HRMS (EI): m/z = 371.2864 $[M]^+$, calcd for $C_{24}H_{37}NO_3$: 371.2824.

(3a*R*,3b*S*,5a*R*,6*R*,8a*R*,10a*S*)-6-((*R*)-1,5-Dimethylhexyl)-2,5a-dimethyl-3b,4,5,5a,6,7,8,8a,10,10a-decahydro-3a*H*-indeno[5,4-e]isoindol-1,3-dione (**14b**). Dienophile: *N*-methylmaleimide. The product was purified by silica column chromatography (hexane/ethyl acetate 4:1) to give 0.22 g (78%) **14b** as colourless oil. $[\alpha]_D^{20}$ = –118.9; IR (KBr): $\tilde{\nu}$ = 3952, 1699, 1436, 1381, 1286, 1128, 1033, 990 cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz): δ = 5.37 (m, 1 H), 3.09–3.02 (m, 2 H), 2.90 (s, 3 H), 2.72 (dd, J = 15.0/7.2 Hz, 1 H), 2.40–2.32 (m, 3 H), 2.13 (m, 1 H), 1.99–1.89 (m, 2 H), 1.78 (m, 1 H), 1.56–0.97 (m, 13 H), 0.90 (d, J = 6.2 Hz, 3 H), 0.88 (d, J = 6.6 Hz, 3 H), 0.86 (d, J = 6.6 Hz, 3 H), 0.50 (s, 3 H); ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 180.1, 178.1, 144.6, 115.9, 56.0, 48.6, 43.2, 42.5, 39.9, 39.2, 36.2, 35.9, 35.6, 34.5, 28.7, 27.8, 24.5, 23.7, 23.6, 23.1, 22.5, 22.3, 21.4, 18.2, 18.0; MS (CI): m/z (%) = 386 $[M+H]^+$ (100); HRMS (EI): m/z = 385.2937 $[M]^+$, calcd for $C_{25}H_{39}NO_2$: 385.2981.

(3a*R*,3b*S*,5a*R*,6*R*,8a*R*,10a*S*)-6-((*R*)-1,5-Dimethylhexyl)-2-ethyl-5a-methyl-3b,4,5,5a,6,7,8,8a,10,10a-decahydro-3a*H*-indeno[5,4-e]isoindol-1,3-dione (**14c**). Dienophile: *N*-ethylmaleimide. The product was purified by silica column chromatography (hexane/ethyl acetate 4:1) to give 0.12 g (41%) **14c** as colourless oil. $[\alpha]_D^{20}$ = + 97.5; IR (KBr): $\tilde{\nu}$ = 3446, 2952, 1697, 1457, 1403, 1228, 1138 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 5.44 (m, 1 H), 3.46 (q, J = 7.3 Hz, 2 H), 3.07–2.94 (m, 2 H), 2.70 (dd, J = 15.1/7.0 Hz, 1 H), 2.41–2.27 (m, 3 H), 2.11 (m, 1 H), 2.00–1.88 (m, 2 H), 1.76 (m, 1 H), 1.60–0.96 (m, 16 H), 0.90 (d, J = 6.1 Hz, 3 H), 0.88 (d, J = 6.6 Hz, 3 H), 0.86 (d, J = 6.6 Hz, 3 H), 0.50 (s, 3 H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 180.0, 178.0, 144.7, 116.1, 56.2, 48.9, 43.3, 42.7, 40.2, 39.5, 36.5, 36.2, 35.9, 34.8, 33.5, 28.9, 28.0, 24.0, 23.9, 23.2, 22.8, 22.5, 21.6, 18.4, 18.3, 13.1; MS (CI): m/z (%) = 400 $[M+H]^+$ (100); HRMS (EI): m/z = 399.3138 $[M]^+$, calcd for $C_{26}H_{41}NO_2$: 399.3137.

(3a*R*,3b*S*,5a*R*,6*R*,8a*R*,10a*S*)-6-((*R*)-1,5-Dimethylhexyl)-5a-methyl-2-propyl-3b,4,5,5a,6,7,8,8a,10,10a-decahydro-3a*H*-indeno[5,4-e]isoindol-1,3-dione (**14d**). Dienophile: *N*-propylmaleimide. The product was purified by silica column chromatography (hexane/ethyl acetate 4:1) to give 0.16 g (53%) **14d** as colourless oil. $[\alpha]_D^{20}$ = +55.0; IR (KBr): $\tilde{\nu}$ = 3373, 2956, 1699, 1404, 1205, 1045, 830 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 5.40 (m, 1 H), 3.40 (t, J = 7.2 Hz, 2 H), 3.07–2.99 (m, 2 H), 2.73 (dd, J = 15.1/7.0 Hz, 1 H), 2.38–2.27 (m, 3 H), 2.12 (m, 1 H), 2.01–1.89 (m, 2 H), 1.77 (m, 1 H), 1.65–0.96 (m, 15 H), 0.93–0.85 (m, 12 H), 0.51 (s, 3 H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 180.3, 178.3, 144.8, 116.3, 56.2, 48.9, 43.3, 42.7, 40.2, 40.1, 39.5, 36.4, 36.2, 35.9, 34.8, 28.9, 28.0, 24.1, 23.9, 23.2, 22.8, 22.5, 21.9, 20.1, 18.4, 18.3, 11.2; MS (CI): m/z (%) = 386 $[M+H]^+$ (100); HRMS (EI): m/z = 413.3342 $[M]^+$, calcd for $C_{27}H_{43}NO_2$: 413.3293.

(3b*R*,5a*R*,6*R*,8a*R*)-6-((*R*)-1,5-Dimethylhexyl)-2,5a-dimethyl-4,5,5a,6,7,8,8a,10-octahydro-3b*H*-2,3a,10a-triazadicyclopenta[a,f]naphthalen-1,3-dione (**14e**). Dienophile: 4-methyl-1,2,4-triazoline-3,5-dione. The product was purified by silica column chromatography (hexane/ethyl acetate 4:1) to give 0.22 g (78%) **14e** as white solid. m.p. 150–151 °C; IR (KBr): $\tilde{\nu}$ = 3459, 2954, 1772, 1702, 1466, 1261, 1013, 759 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 5.43 (m, 1 H), 4.36–4.27 (m, 2 H), 3.91 (m, 1 H), 3.07 (s, 3 H), 3.01 (m, 1 H), 2.54 (m, 1 H), 2.00–1.70 (m, 5 H), 1.52 (sept, J = 6.6 Hz, 1 H), 1.40–0.97 (m, 10 H), 0.90 (d, J = 5.5 Hz, 3 H), 0.87 (d, J = 6.6 Hz, 3 H), 0.86 (d, J = 6.6 Hz, 3 H), 0.74 (s, 3 H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 155.3, 152.8, 138.6, 113.4, 57.5, 56.5, 48.4, 42.9, 40.5, 39.5, 35.9, 35.7, 35.6, 28.5, 28.0, 24.9, 24.2, 23.9, 23.4, 22.8, 22.5 (2 C), 18.7, 18.3; MS (CI): m/z (%) = 388 $[M+H]^+$ (100); HRMS (EI): m/z = 387.2918 $[M]^+$, calcd for $C_{23}H_{37}N_3O_2$: 387.2885.

(*E*)-3-[(1*R*,3a*R*,7a*R*)-1-((*R*)-1,5-Dimethylhexyl)-7a-methyl-2,3,3a,6,7,7a-hexahydro-1*H*-inden-4-yl]-prop-2-en-1-ol (**15**). To a solution of triflate **2** [17] (0.20 g, 0.50 mmol), acetonitrile (1.0 mL) and tri-*n*-butylamine (1.7 mL) was added tri-*o*-tolylphosphine (4.3 mg, 14 μmol), palladium(II) acetate (1.6 mg, 7.0 μmol) and allyl alcohol (34 μL, 0.50 mmol). The reaction was performed in a sealed vessel in a single-mode microwave reactor at a maximum output of 80 W, a maximum temperature of 150 °C and a reaction time of 10 min. The resulting mixture was diluted with diethyl ether (20 mL) and washed with water (2 × 20 mL) and brine (2 × 20 mL). The organic layer was dried with $MgSO_4$ and concentrated under reduced pressure. The product was purified by silica column chromatography (hexane/ethyl acetate 10:0 → 8:2) to give 54 mg (36%) **15** as yellow oil. $[\alpha]_D^{20}$ = +28.9; IR (NaCl): $\tilde{\nu}$ = 3328, 2956, 1467, 1377, 1181, 1082, 965, 803 cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz): δ = 6.01 (d, J = 15.7 Hz, 1 H), 5.85 (dt, J = 15.7/6.1 Hz, 1 H), 5.68 (dd, J = 6.6/3.3 Hz, 1 H), 4.14 (d, J = 6.2 Hz, 2 H), 2.41 (s_{br}, 1 H), 2.33 (m, 1 H), 2.20–2.16 (m, 2 H), 2.01–1.93 (m, 2 H), 1.74 (m, 1 H), 1.52 (m, 1 H), 1.45–1.08 (m, 10 H), 1.01 (m, 1 H), 0.94 (d, J = 6.6 Hz, 3 H), 0.87 (d, J = 6.6 Hz, 3 H), 0.86 (d, J = 6.6 Hz, 3 H), 0.70 (s, 3 H); ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 137.0, 133.8, 126.4, 125.9, 64.4, 54.0, 49.9, 42.7, 39.5, 36.2, 36.1, 35.9, 28.0, 27.9, 24.9, 24.1, 23.9, 22.8, 22.6, 18.8, 11.2; MS (CI): m/z (%) = 305 $[M+H]^+$ (34), 287 (100); HRMS (EI): m/z = 304.2791 $[M]^+$, calcd for $C_{21}H_{36}O$: 304.2766.

4-[(1*R*,3a*R*,7a*R*)-1-((*R*)-1,5-Dimethylhexyl)-7a-methyl-2,3,3a,6,7,7a-hexahydro-1*H*-inden-4-yl]-but-3-yn-1-ol (**16**). To a solution of triflate **2** [17] (0.35 g, 0.90 mmol) and diethylamine (1.5 mL) in DMF (0.5 mL) copper(I) iodide (6.9 mg, 0.04 mmol), bis-(triphenylphosphine)palladium(II) dichloride (12.8 mg, 18.2 μmol) and 3-butyn-1-ol (0.07 mL, 1.0 mmol) was added. The reaction mixture was irradiated in a single-mode microwave reactor in a sealed vessel at a maximum output of 300 W and a maximum temperature of 120 °C for 5 min. The resulting mixture was diluted with diethyl ether (20 mL) and washed with water (2 × 20 mL) and brine (2 × 20 mL). The organic layer was dried with $MgSO_4$ and concentrated under reduced pressure. The product was purified by silica column chromatography (hexane/ethyl acetate 10:0 → 9:1) to give 0.24 g (98%) **16** as yellow oil. $[\alpha]_D^{20}$ = + 29.7; IR (NaCl): $\tilde{\nu}$ = 3342, 2953, 1428, 1378, 1366 1044, 944 cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz): δ = 5.90 (dd, J = 6.8/3.3 Hz, 1 H), 3.71 (q, J = 5.7 Hz, 2 H), 2.57 (t, J = 6.1 Hz, 2 H), 2.20–2.12 (m, 3 H), 2.01–1.88 (m, 2 H), 1.80–1.73 (m, 2 H), 1.52 (m, 1 H), 1.44–1.08 (m, 10 H), 1.00 (m, 1 H), 0.93 (d, J = 6.6 Hz, 3 H), 0.87 (d, J = 6.6 Hz, 3 H), 0.86 (d, J = 6.6 Hz, 3 H), 0.68 (s, 3 H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 133.2, 122.1, 84.2, 82.8, 61.3, 54.8, 50.0, 41.8, 39.5, 36.2, 36.1, 35.9, 28.0, 27.9, 25.0, 24.1, 23.9, 23.8, 22.8, 22.5, 18.7, 11.0; MS (CI): m/z (%) = 317 $[M+H]^+$ (100); HRMS (EI): m/z = 316.2749 $[M]^+$, calcd for $C_{22}H_{36}O$: 316.2766.

4-[(1*R*,3a*S*,4*RS*,7a*R*)-1-((*R*)-1,5-Dimethylhexyl)-7a-methyl-octahydroinden-4-yl]-butan-1-ol (**17**). Platinum(IV) oxide (15 mg, 0.17 mmol) was added to a solution of alkynol **16** (1.00 g, 3.10 mmol) and ethyl acetate (30 mL). The reaction mixture was stirred at r.t. for 48 h under hydrogen atmosphere at ambient pressure. The reaction mixture was filtered (celite) and concentrated under reduced pressure. The raw product (0.95 g, 95%, colourless oil) was used directly for the next reaction step without further purification.

General procedure for synthesis of N-substituted maleimides **18**–**20**. Triphenylphosphine (0.83 g, 3.16 mmol) and maleimide (0.31 g, 3.16 mmol) were dissolved in THF (12.6 mL). The corresponding alcohol (**15/16/17**) (1.56 mmol) and diisopropyl azodicarboxylate (DIAD) (0.62 mL, 3.16 mmol) was added. The reaction was performed in open flask fitted with a reflux condenser in a single-mode microwave reactor. The reaction mixture was irradiated with a maximum output of 50 W and a maximum temperature of 76 °C for 30 min. After removal of the solvent, the product was purified by silica column chromatography (hexane/ethyl acetate 4:1) to yield the N-substituted maleimides **18**–**20**.

1-(*E*)-{3-[(1*R*,3a*R*,7a*R*)-1-((*R*)-1,5-Dimethylhexyl)-7a-methyl-2,3,3a,6,7,7a-hexahydro-1*H*-inden-4-yl]-prop-2-enyl}-pyrrole-2,5-dione (**18**). Yield: 20 mg (8%), pale yellow solid; m.p. 81–82 °C; $[\alpha]_D^{20} = +$ 22.7; IR (KBr): $\tilde{\nu} = 2936, 1708, 1462, 1385, 1375, 1244, 972\ cm^{-1}$; ^1H NMR ($CDCl_3$, 500 MHz): $\delta = 6.70$ (s, 2 H), 6.21 (d, $J = 6.3$ Hz, 1 H), 5.86–5.78 (m, 2 H), 4.16 (dd, $J = 5.5/1.4$ Hz, 2 H), 2.23–2.08 (m, 3 H), 2.01–1.92 (m, 2 H), 1.68 (m, 1 H), 1.55–0.98 (m, 12 H), 0.91 (d, $J = 6.4$ Hz, 3 H), 0.88 (d, $J = 6.5$ Hz, 3 H), 0.87 (d, $J = 6.6$ Hz, 3 H), 0.66 (s, 3 H); ^{13}C NMR ($CDCl_3$, 125 MHz): $\delta = 171.2$ (2 C), 136.0 (2 C), 132.9, 131.2, 131.1, 124.7, 55.1, 49.3, 40.9, 40.5, 39.4, 36.0, 35.9, 35.8, 28.0, 27.9, 25.2, 23.9, 23.8, 22.7, 22.6, 18.6, 11.4; MS (CI): m/z (%) = 384 $[M+H]^+$ (100); HRMS (EI): $m/z = 383.2836$ $[M]^+$, calcd for $C_{25}H_{37}NO_2$: 383.2824.

1-{4-[(1*R*,3a*R*,7a*R*)-1-((*R*)-1,5-Dimethylhexyl)-7a-methyl-2,3,3a,6,7,7a-hexahydro-1*H*-inden-4-yl]-but-3-ynyl}-pyrrole-2,5-dione (**19**). Yield: 0.27 g (44%), pale yellow solid; m.p. 81–83 °C; $[\alpha]_D^{20} = +18.3$; IR (KBr): $\tilde{\nu} = 3453, 2954, 1703, 1444, 1384, 1147, 842, 692\ cm^{-1}$; ^1H NMR ($CDCl_3$, 400 MHz): $\delta = 6.71$ (s, 2 H), 5.86 (dd, $J = 6.3/3.3$ Hz, 1 H), 3.70 (t, $J = 7.0$ Hz, 2 H), 2.57 (t, $J = 7.0$ Hz, 2 H), 2.17–2.05 (m, 3 H), 1.99–1.87 (m, 2 H), 1.69 (m, 1 H), 1.50 (m, 1 H), 1.44–1.08 (m, 10 H), 1.00 (m, 1 H), 0.92 (d, $J = 6.5$ Hz, 3 H), 0.87 (d, $J = 6.6$ Hz, 3 H), 0.86 (d, $J = 6.6$ Hz, 3 H), 0.66 (s, 3 H); ^{13}C NMR ($CDCl_3$, 100 MHz): $\delta = 170.5$ (2 C), 134.1 (2 C), 133.3, 122.1, 83.5, 82.4, 54.7, 49.9, 41.8, 39.5, 36.8, 36.2, 36.1, 35.9, 28.0, 27.9, 25.0, 24.0, 23.9, 22.8, 22.6, 19.2, 18.7, 11.0; MS (CI): m/z (%) = 396 $[M+H]^+$ (20), 163 (100); HRMS (EI): $m/z = 395.2820$ $[M]^+$, calcd for $C_{26}H_{37}NO_2$: 395.2824.

1-{4-[(1*R*,3a*S*,4*RS*,7a*R*)-1-((*R*)-1,5-Dimethylhexyl)-7a-methyl-octahydro-inden-4-yl]-butyl}-pyrrole-2,5-dione (**20**). Yield: 0.12 g (20%), pale yellow solid; m.p. 78–80 °C; $[\alpha]_D^{20} = +39.8$; IR (KBr): $\tilde{\nu} = 2936, 1708, 1467, 1408, 1375, 1244, 1098\ cm^{-1}$; ^1H NMR ($CDCl_3$, 500 MHz): $\delta = 6.68$ (s, 2 H), 3.51 (dt, $J = 7.3/3.0$ Hz, 2 H), 1.90 (m, 1 H), 1.80–0.96 (m, 26 H), 0.91 (d, $J = 6.7$ Hz, 3 H), 0.87 (d, $J = 6.6$ Hz, 3 H), 0.86 (d, $J = 6.6$ Hz, 3 H), 0.69 (s, 3 H); ^{13}C NMR ($CDCl_3$, 125 MHz): $\delta = 170.9$ (2 C), 134.1 (2 C), 57.1, 52.6, 42.2, 41.0, 39.5, 38.0, 37.0, 36.2, 36.0, 35.6, 29.9, 28.8, 28.0, 27.6, 27.4, 23.8, 23.7, 22.8, 22.5, 20.7, 18.7, 14.0; MS (CI): m/z (%) = 402 $[M+H]^+$ (100); HRMS (EI): $m/z = 401.3250$ $[M]^+$, calcd for $C_{26}H_{43}NO_2$: 401.3294.

7-(4-Methylpent-3-enyl)pyridazino[1,2a]pyridazin-1,4(6*H*,9*H*)-dione (**22**). The dienophile 3,6-pyridazinedione was prepared freshly as follows: To a stirred suspension of 3,6-dihydroxypyridazine potassium salt (1.00 g, 6.66 mmol) and acetone (25 mL) at −78 °C was added *tert*-butylhypochlorite solution (0.75 mL, 6.66 mmol). After stirring for 3 h a solution of **21** (β-myrcene) (1.00 mL, 6.00 mmol) was added. The reaction mixture was slowly warmed at r.t., stirred for further 1 h and then quenched with water (30 mL). The mixture was extracted with diethyl ether (3 × 20 mL). The combined organic layers were washed with saturated sodium chloride solution, dried over Na_2SO_4 and concentrated under reduced pressure. The product was purified by silica column chromatography (silica gel 60, ethyl acetae) to give 0.17 g (11%) **22** as yellow solid. IR (KBr): $\tilde{\nu} = 3335, 2966, 2924, 2856, 1637, 1436, 1334, 1136\ cm^{-1}$; ^1H NMR ($CDCl_3$): $\delta = 6.94$ (s, 2 H), 5.74 (s, 1 H), 5.08 (s, 1 H), 4.51 (s, 2 H), 4.43 (s, 2 H), 2.19 (s, 4 H), 1.70 (s, 3 H), 1.61 (s, 3 H); ^{13}C NMR ($CDCl_3$): $\delta = 156.4, 156.3, 134.3$ (2 C), 133.4, 133.0, 122.6, 114.5, 46.3, 43.9, 34.2, 25.7, 25.6, 17.8; MS (CI): m/z (%) = 247 $[M+H]^+$ (100); HRMS (EI): $m/z = 246.1370$ $[M]^+$, calcd for $C_{14}H_{18}N_2O_2$: 246.1368.

4.2. Biological assays

HL 60 cells (DSMZ, German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) were maintained in RPMI 1640 medium (PAA Laboratories, Cölbe, Germany) containing 10% fetal bovine serum (FBS, PAA Laboratories, Cölbe, Germany) without antibiotics at 37 °C in a humidified atmosphere containing 5% CO_2.

4.2.1. MTT assay

Cytotoxicity of synthesized compounds was determined by MTT test according to the method of Mosmann [28]. Solutions of the compounds in DMSO (1 μL, concentrations ranging from 10^{-9} to 10^{-4} mol/L) were incubated with 99 μL of a suspension of HL 60 cells (9×10^5 cells/mL) in RPMI 1640 medium with 10% FBS in 96 well plates for 24 h at 37 °C. Then, 10 μL of MTT solution in PBS (5 mg/mL) were added and the plate was incubated for another 2 h. The cells were quenched with 190 μL DMSO and after 1 h of continuously shaking of the plates a photometric evaluation on an ELISA plate reader MRX II (Dynex Technologies, Denkendorf, Germany; Software: Revelation 4.06) using the wavelength of 550 nm followed. The IC_{50}-values were calculated by using Prism 4 (GraphPad Software, La Jolla, USA).

4.2.2. Assay for antibacterial and antifungal activities

All the work was performed under sterile conditions in a laminar flow cabinet (HeraSafe 12, Heraeus Instruments, Hanau, Germany). The bacteria and fungi (see Table 2) were cultivated on an AC agar (Sigma–Aldrich) except *Aspergillus niger*, which was cultivated on a potato dextrose broth agar (Sigma–Aldrich). Each strain was spread evenly on the agar medium. To prepare the drug formulations 1% (w/v) DMSO solutions of the testing compound were made. Three paper disks were prepared with 5 μL of each test solution. As a control a further paper disk was prepared with 5 μL of DMSO. All paper discs were placed evenly separated on a culture plate. The plates were covered and incubated for 36 h at 32 °C (bacteria) and 28 °C (fungi), respectively. After incubation the diameters of the zones of inhibition (defined as diameter of inhibited bacterial growth) were measured in millimeters.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejmech.2011.04.036.

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