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Medicinal Chemistry

Preparation of Thermocleavable Conjugates Based on Ansamitocin and Superparamagnetic Nanostructured Particles by a Chemobiosynthetic Approach

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Abstract: A combination of mutasynthesis, precursor-directed biosynthesis and semisynthesis provides access to new ansamitocin derivatives including new nanostructured particle–drug conjugates. These conjugates are based on the toxin ansamitocin and superparamagnetic iron oxide–silica core shell particles. New ansamitocin derivatives that are functionalized either with alkynyl- or azido groups in the ester side chain at C-3 are attached to nanostructured iron oxide core–silica shell particles. Upon exposure to an oscillating electromagnetic field these conjugates heat up and the ansamitocin derivatives are released by a retro-Diels–Alder reaction. For example, one ansamitocin derivative exerts strong antiproliferative activity against various cancer cell lines in the lower nanomolar range while the corresponding nanostructured particle-drug conjugate is not toxic. Therefore, these new conjugates can serve as dormant toxins that can be employed simultaneously in hyperthermia and chemotherapy when external inductive heating is applied.

Introduction

Besides total and semisynthesis the combination of chemical synthesis with methods from the field of modern biotechnology and metabolic engineering have emerged as new strategies to carry out natural product synthesis with the aim to generate compound libraries of complex secondary metabolites.[1] In this context mutasynthesis,[2] a technique originating from precursor-directed biosynthesis,[3] and related concepts based on interference with biosynthesis are highly useful to create structural diversity, otherwise difficult to achieve purely by total or semisyntheses. Mutasynthesis is based on the generation of mutants of a producer organism blocked in the formation of a biosynthetic pathway intermediate. Mutasynthons, which are, chemically prepared, modified biosynthetic intermediates are administered to the blocked mutant thereby potentially restoring the biosynthetic flux to finally provide new metabolites. Our group and others have shown that mutasynthesis can ideally be combined with chemical synthesis to generate even greater structural diversity.[4,5] Precursor-directed biosynthesis (PDB) is related to mutasynthesis in that biosynthetic precursor-analogues are supplemented to the fermentation cultures, but here producer strains are used that do not depend on external supplementation of advanced key precursors. In contrast to mutasynthesis, yields of the desired analogues are often low due to the internal competition between natural and unnatural precursors and separation from the concomitantly formed natural derivatives poses extra difficulties.[2]

Among other natural products,[6] the ansamitocins (**3**–**5**),[8] being representatives of the group maytansinoids[7] that belong to the class of ansamycin antibiotics, have become a showcase for combining chemical synthesis with mutasynthesis. In this context, particularly mutants of the ansamitocin producer *Actinosynnema pretiosum*, blocked in the biosynthesis of the starting building block 3-amino-5-hydroxybenzoic acid (AHBA, **1**)[9,10] or late stage-blocked mutants have been employed.[11]

The ansamitocins are medically highly important natural products that inhibit growth of cancer cell lines as well as human solid tumors at subnanomolar concentrations.[12] The mode of action for this activity is the prevention of tubulin polymerization that subsequently leads to apoptosis.[13] The structural features essential for bioactivity have been identified as the presence of an ester side-chain at C-3, the α-orientation of the hydroxyl group at C-9 and the presence of the cyclic structure across C-7 to C-9.[11] Currently, the maytansinoids are one of the most intensely studied group of natural products. In early 2013 the antibody–maytansine conjugate Trastuzumab emtansine (T-DM1; tradename: Kadcyla) was approved by the

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FDA as chemotherapeutic agent against Her2 positive breast cancer.[14]

The maytansinoids are biosynthesized by a type I polyketide synthase (PKS) into which a nonribosomal peptide synthetase-like loading module introduces AHBA as starter building block.[16] Chain extension by seven consecutive PKS modules, followed by macrolactamization catalyzed by the amide synthase Asm9 provides proansamitocin (**2**; Scheme 1).[17,18] Six post-PKS tailoring enzymes furnish the ansamitocins **3**–**5**.[15] In vivo, the acyltransferase Asm19 transfers carboxylic acids such as isobutyrate **8** in form of its activated CoA-thioester onto the hydroxyl group at C-3. Ansamitocin P3 (**4**) is the main metabolite formed by *A. pretiosum* under most fermentation conditions. Isobutyrate (**8**) can be derived from L-valine (**7**) by oxidative deamination (by the branched-chain amino acid transaminase) and oxidative decarboxylation (by the branched-chain α-keto acid dehydrogenase enzyme complex).[19] Whether the apparent preference for the isobutyrate side chain can be attributed to the substrate preference of the enzyme or simply results from the abundant internal supply of this particular activated carboxylic acid, is unclear. Nevertheless, utilization of 3 g L^{-1} L-valine in the fermentation medium is known to stimulate ansamitocin production and to lead to almost exclusive formation of the P-3 type (**4**), greatly improving yields and simplifying chromatographic clean-up.[12f]

Modification of ansamitocins at C-3 has proven attractive, especially for conjugating tumor-specific ligands to an ansamitocin warhead.[20] This can be achieved by a semisynthetic approach starting from a mixture of ansamitocins produced by fermentation. After $LiAlH(OMe)_3$-promoted removal of the different ester side chains, the reagent system $DCC/ZnCl_2$ mediates re-esterification with the desired acyl side chain in about 30% yield over two steps.[21]

Here, we report, for the first time, on the introduction of ester side chains functionalized for further semisynthetic modifications during fermentation by precursor-directed biosynthesis (PDB) and show that this approach can be combined with mutasynthesis to achieve two structural changes in the ansamitocins during one bioprocess. Besides standard chemical manipulations and oligomerizations of ansamitocins, we also conjugate ansamitocin via the ester side chain to superparamagnetic nanostructured particles (size: 10–40 nm) called MAGSILICA employing a thermolabile linker (Figure 1).

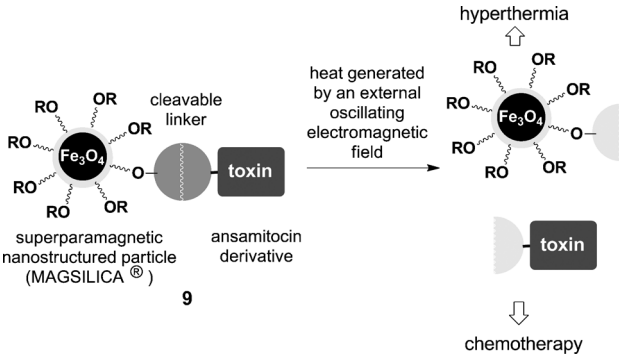
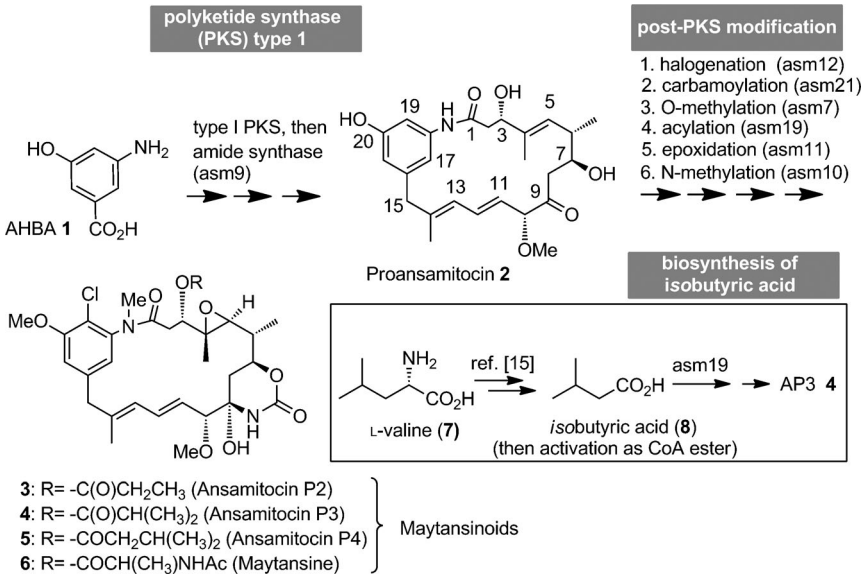


Figure 1. Concept for the combination of hyperthermia with chemotherapy using drug–nanostructured particle conjugates **9**.

The nanostructured MAGSILICA particles are composed of a ferritic core (maghemite γ-Fe_2O_3 and magnetite Fe_3O_4) encased in a silica shell and fulfil the requirements for being applied in the design of nanostructured–drug particle conjugates. The size is suitable for passing through blood vessels while the silica shell prevents agglomeration, ensures chemical resistance and provides options for chemical modification of its surface. A distinguishing feature of the particles is their superparamagnetism. When brought into close proximity of solid tumors, these new conjugates could induce apoptosis in two ways: 1) through hyperthermia[22] mediated by superparamagnetic nanostructured particles when an external oscillating electromagnetic field is applied, and 2) by the chemotoxic effect ensuing from thermal cleavage of the linker and liberation of the highly toxic ansamitocin derivative. While we prepared this report, Cheon et al. reported on the development of drug–nanostructured particle conjugate based on the toxin geldanamycin and a thermolabile linker that relies on a radical cleavage protocol.[23]



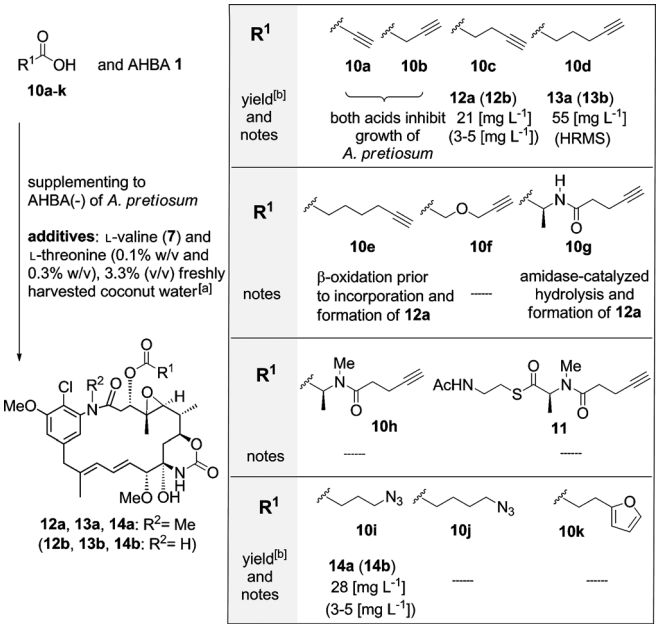
Scheme 1. Biosyntheses of ansamitocins P-2 (**3**), P-3 (**4**) and P-4 (**5**) via proansamitocin (**2**) (postketide transformations from **2** to **3**–**5** are listed along with gene annotations according to the predominant route of metabolic flux) and biosynthesis of isobutyrate (**8**) from L-valine (**7**).[15]

The present report covers two main topics: 1) the side chain modification and functionalization of ansamitocin by advanced PDB and mutasynthesis, and 2) the fusion with superparamagnetic nanostructured particles that can release the attached ansamitocin derivatives through inductive heating.

Results and Discussions

Side-chain modifications at C3 by PDB and mutasynthesis

In view of the fact that AP3 (**4**) is the major metabolite formed by *A. pretiosum* under standard fermentation conditions with 3 g L^{-1} L-valine (**7**) added as production stimulating and side chain-directing additive (for mechanism see Scheme 1), with yields usually ranging from 80–110 mg L^{-1}, we investigated the introduction of functionalized ester side chains at C-3 under altered fermentation conditions by supplementing the functionalized carboxylic acids **10** or, in case of the more complex amides, the corresponding SNAC ester **11**, a SCoA mimetic (Scheme 2).



Scheme 2. Side chain modification of ansamitocins by PDB. [a] Conditions: K-medium, AHBA **1** (1.25 mmol L^{-1}), **10** (12.5 mmol L^{-1}) both in H_2O/DMSO (1:1), 7–10 days of fermentation (see also the Supporting Information); [b] yields in mg L^{-1} refer to yields achieved under optimum conditions for isolated products.

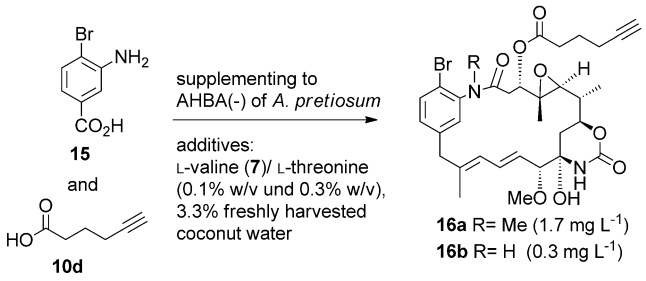
In addition to the toxic effects of alkynoic and azido carboxylic acids, neutralized prior to addition, the lack of the stimulating additive L-valine resulted in very low productivity in initial experiments. With a view on the studies carried out by Hatano et al.,[12e] different amino acid additives were investigated. A combination of L-valine and L-threonine added to a final concentration of 1 and 3 g L^{-1}, respectively, resulted in excellent stimulation of productivity, but did not favor the isobutyrate side chain unduly. However, addition of the toxic carboxylic acids still affected production strongly, resulting in yields estimated by analytical reversed phase HPLC to drop to about 10%. With a view on the potential symbiosis of maytansinoid-producing strains with certain plants,[7] fresh coconut water was investigated as an additive. Fresh coconut water is known as a stimulating additive in plant cell culture.[24] In our experiments it proved to be the additional key additive to apparently counter the toxic effects of the unnatural carboxylic acids when added to the fermentation broth in 3.3% (v/v). Furthermore, our usual mutasynthetic approach of using a drop-wise addition of precursors via syringe pump-driven feeding capillaries over 3 days proved essential for these experiments (see the Supporting Information).

Under the optimized fermentation conditions three new ester side chains could be introduced at C-3 and provided ansamitocin derivatives **12a**, **13a** and **14a** in excellent, synthetically useful amounts. To a small degree also derivatives not *N*-methylated by Asm10 (**12b**, **13b** and **14b**) were formed. The two shortest alkyne-functionalized carboxylic acids **10a** and **10b** proved pronouncedly toxic and inhibited cell growth, likely by disruption of fatty acid β-oxidation (vide infra). On the other hand, 6-heptynoic acid **10e**, which was not incorporated as such, first underwent β-oxidation to the SCoA-activated derivative of **10c**, so that ansamitocin **12a** was formed instead. Likewise, efforts to incorporate the maytansine side chain derivatives **10g**/**h** and **11** were not successful.[8a] In case of the N-demethylated precursor **10g**, first amide hydrolysis must have occurred because only formation of metabolite **12a** was detected. Neither acids **10f**, **10h**, **10j**, **10k**, nor SNAC ester **11** resulted in corresponding ansamitocin derivatives.

These results are remarkable since the xenobiotic carboxylic acids can exert inhibitory effects on fatty acid β-oxidation once being activated as CoA-thioesters, disrupting bacterial metabolism and growth. Particularly ω-alkynyl-functionalized carboxylates such as 4-pentynylate show broad toxicity.[25] To the best of our knowledge, the literature only provides one example for the successful incorporation of an alkynylate, but analysis was merely based on mass spectrometry.[26] In contrast to alkyne moieties, the azido group is not found in nature. Some reports note that organic azides may have mutagenic properties.[27]

We also achieved the first example of a combined mutasynthetic and precursor-directed biosynthetic (PDB) approach employing the AHBA(−) mutant strain of *A. pretiosum* supplemented with 5-hexynoic acid (**10c**) and aminobenzoate **15** (Scheme 3). Expectedly, isolated yields of new secondary metabolites **16a** and **16b** were lower than for fermentations with mutasynthon **15** under standard fermentation conditions (~17%).[5]

Under the optimized fermentation conditions three new ester side chains could be introduced at C-3 and provided ansamitocin derivatives **12a**, **13a** and **14a** in useful amounts. To a lesser extent also the N-demethylated metabolites (**12b**, **13b** and **14b**) were formed. The two shortest alkyne-functionalized carboxylic acids **10a** and **10b** inhibited cell growth that we ascribe to inhibition of fatty acid β-oxidation (vide supra). On the other hand, 6-heptynoic acid (**10e**) first underwent β-oxidation before being incorporated as activated SCoA-ester **10c**, so that



Scheme 3. Access to ansamitocin derivatives **16a**,**b** by combined mutasynthesis and PDB (see also Scheme 2 and the Supporting Information).

metabolite **12a** was formed instead. Likewise, efforts to incorporate the maytansine side chain were not successful.[8a] In case of the N-demethylated precursor **10g** first amide hydrolysis must have occurred because only formation of metabolite **12a** was detected. Neither acids **10f**, **10h**, **10j**, **10k** nor SNAC ester **11** were incorporated during fermentation.

On each stage of our studies towards conjugates composed of ansamitocin and nanostructured particles, we evaluated the antiproliferative profiles using cultured mouse fibroblast cells and different human tumor cell lines (Table 1). All new derivatives **12a**/**b**, **13a**, **14a** and **16a**/**b** with modified ester side chains show strong antiproliferative activity similar to AP-3 (**4**) towards mouse and human cell lines, the latter being a prerequisite for the development of tumor-targeting conjugates.

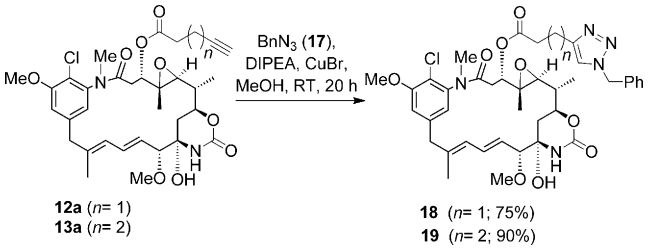
Table 1. Antiproliferative activities IC_{50} [nmol L^{-1}] of **12a**,**b**, **13a**, **14a** and **16a**,**b** and comparison with AP-3 (**4**) towards different mammalian cell lines.[a]

Cell line[b]	12a	12b	13a	14a	16a	16b	4
L-929	1.3	5.7	0.42	1.1	1.0	1.1	0.2
KB-3-1	–	0.11	–	–	0.04	0.05	0.17
U-937	0.03	0.11	0.02	0.08	0.04	0.05	0.01
A-431	0.16	0.2	0.08	0.12	0.1	0.1	0.08
SK-OV-3	0.1	0.35	0.05	0.07	0.09	0.17	0.05
PC-3	0.19	0.25	0.35	0.19	0.07	0.13	0.06
MCF-7	0.13	0.35	0.06	0.16	0.08	0.15	–
HUVEC	0.1	0.25	0.05	0.1	0.1	0.2	0.02

[a] Values shown are means of two determinations in parallel. [b] L-929 (mouse fibroblasts), KB-3-1 (cervix carcinoma), U-937 (histiocytic lymphoma), PC-3 (prostate adenocarcinoma), SK-OV-3 (ovary adenocarcinoma), A-431 (epidermoid carcinoma), MCF-7 (breast adenocarcinoma), HUVEC (umbilical vein endothelial cells).[28]

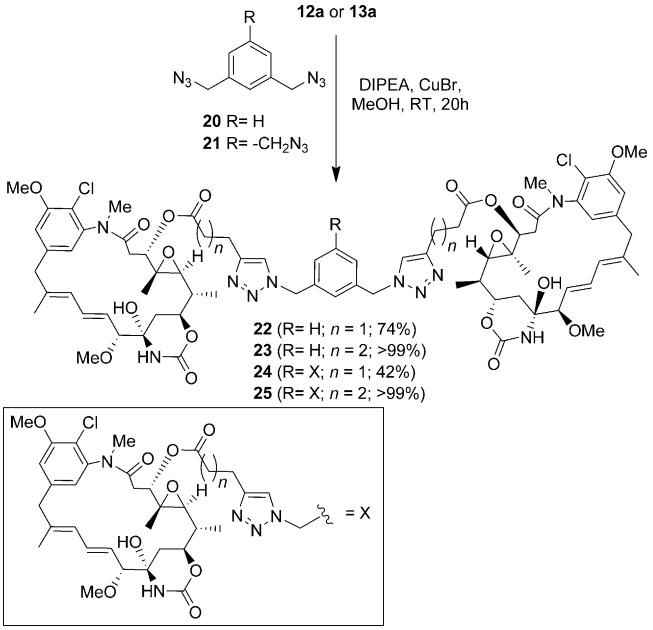
1,3-Dipolar cycloadditions with modified side chains

En route to new ansamitocin conjugates composed of side chain modified ansamitocins and superparamagnetic, nanostructured particles we first optimized the linker design which included investigations on 1,3-dipolar cycloadditions by making use of the additional chemical functionality introduced by PDB. Both ansamitocin derivatives **12a** and **13a** reacted smoothly with benzyl azide **17** in the presence of a stoichiometric amount of the copper(I) source and Hünig's base to yield triazoles **18** and **19**, respectively (Scheme 4).[29,30]



Scheme 4. 1,3-Dipolar cycloadditions of alkynes **12a** and **13a**, respectively, with benzyl azide (**17**).

We extended this semisynthetic concept to the preparation of dimeric ansamitocins **22** and **23** as well as trimers **24** and **25**, which were formed by cycloaddition of alkynes **12a** and **13a**, respectively, and the bis- as well as trisazide **20** and **21** (Scheme 5). The concept of creating multivalency by dimerizing complex natural products has been pursued for several drugs[31] including neomycin.[32,33]



Scheme 5. 1,3-Dipolar cycloadditions with bis- and trisazides **20** and **21**, respectively.

Antiproliferative properties of triazoles **18**, **19** and **22**–**25** were also determined and except for the cell line U-937 (histiocytic lymphoma, see Table 2), which still showed overall high sensitivity, biological activities were lower by a factor of about 10 (for the dimeric ansamitocins **22** and **23**), 100 (for benzyltriazoles **18** and **19**) and 1000 (for trimers **24** and **25**) compared to AP-3 (**4**). Still, all values are in the lower nanomolar range, a sufficiently low value for anticancer drugs. But it needs to be noted, that in the present case the concept of oligomerizing drugs and creating multivalency did not lead to improved biological activities.

Table 2. Antiproliferative activities IC_{50} [nmol L^{-1}] of **18**, **19** and **22**–**25**.[a]

Cell line	18	19	22	23	24	25
L-929	33	10.2	1.6	0.55	6.4	9.5
U-937	0.55	0.19	0.64	0.58	8.7	9.9
A-431	2.7	1.0	1.2	1.8	12.4	21
SK-OV-3	3.1	1.1	0.23	0.4	4.1	4.1
PC-3	5.3	1.6	0.66	6.6	50	72
MCF-7	0.81	1.5	0.95	1.8	15.1	17.1
HUVEC	2.3	1.1	0.74	1.1	3.8	2.8

[a] For details on cell lines see Table 1.

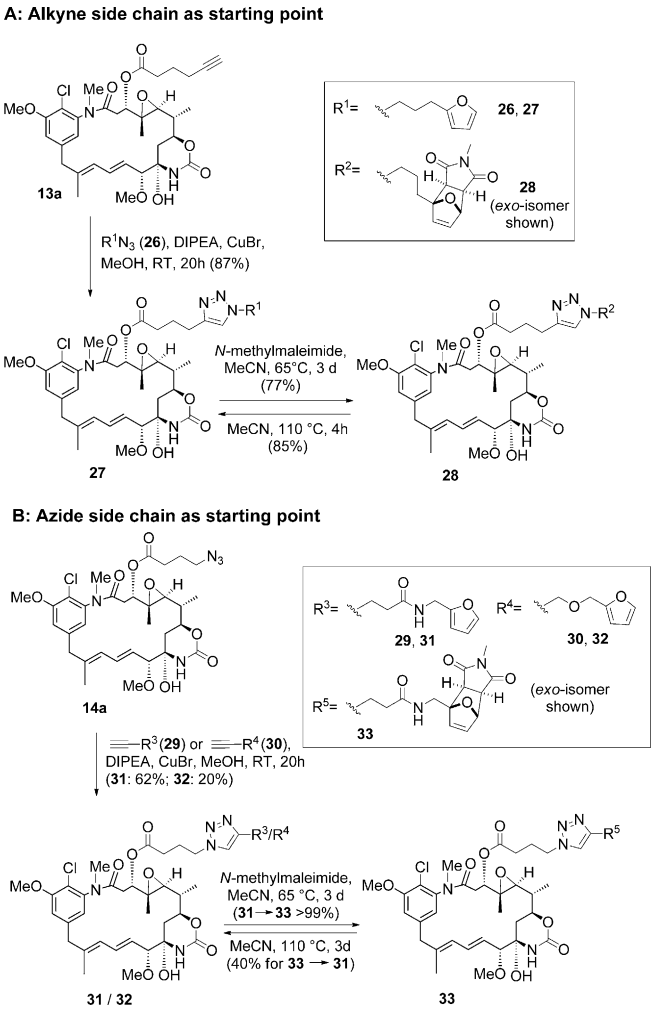
Development of nanostructured particle–ansamitocin conjugates

Having established a new biotechnological route for introducing functionalized ester side chains into ansamitocins and having demonstrated that these side chains can further be modified by 1,3-dipolar cycloadditions, we consequently pursued the conjugation of these ansamitocin derivatives with superparamagnetic nanostructured particles[34] while implementing a thermolabile linker at the same time. Approval of the antibody–drug conjugate Kadcyla by the FDA made it a frontrunner example for a general and strong trend in current anticancer drug development, the conjugation of extremely toxic molecules with tumor-specific ligands such as monoclonal antibodies,[35] vitamins[5,36] and epidermal growth factors.[37] These conjugates can guide the toxin to solid tumors with high selectivity. Commonly, they are internalized by endocytosis followed by liberation of the toxin inside the tumor cell.

Alternatively, the linker system can be designed in a way that allows specific cleavage of the drug outside the cancer cell by an external trigger. This can be the lower pH value in close vicinity of solid tumors,[38] enzyme-mediated hydrolysis[38–40] or heat.[23] This field of research is governed by: 1) the choice of the ideal toxin, 2) the development of a smart linker system that is cleavable under bioorthogonal conditions, and 3) the quest for a target specific delivery systems.[41]

In order to combine hyperthermia with chemotherapy based on toxins such as ansamitocin derivatives, a thermocleavable linker is required. The modified drug should be attached to the nanoparticle surface in a defined way and it must be efficiently released during exposure to an external oscillating electromagnetic field. Loading and unloading should proceed in bioorthogonal manner. Additionally, the drug must be thermally stable, a prerequisite fulfilled by the ansamitocins.[42]

A simple and reliable thermocleavable linker can be adapted from by a concept known from the field of self-healing polymers.[43] The concept relies on a Diels–Alder system composed of a maleimide and furan. The coupling occurs slowly at temperatures between 50–70 °C, while cleavage rapidly takes place between 110–120 °C. During the preparation of this paper, the combination of superparamagnetic nanostructured particles with this linker system able to undergo retro-Diels–Alder reaction when inductively heated was reported.[44] We chose to locate the furan ring in the toxin while the superparamagnetic



Scheme 6. Side-chain functionalization of alkyne **13a** and azide **14a**, respectively, by Huisgen-type 1,3-dipolar cycloadditions.

nanostructured particle was equipped with maleimide. For that purpose we coupled modified azide **26** with alkyne **13a** by a copper-mediated cycloaddition to furnish ansamitocin derivative **27** (Scheme 6).

Likewise, azido-functionalized ansamitocin derivative **14a** was modified with alkynes **29** and **30** to provide new ansamitocins **31** and **32**, respectively. As the reaction between azide **14a** and alkyne **30** proceeded in low yield, we restricted the Diels–Alder cycloaddition as well as retro Diels–Alder reaction studies to furan derivatives **27** and **31**. *N*-Methylmaleimide served as a model dienophile and yielded adducts **28** (from **27**) and **33** (from **31**) in good isolated yield under low temperature conditions (acetonitrile, 65 °C, 3 days). The retro-Diels–Alder reaction proceeded at elevated temperature (110 °C) in the same polar solvent within a few hours and furnished ansamitocin derivatives **27** and **31** in moderate to very good isolated yields (Scheme 6).

Next, the medical relevance of new ansamitocin derivatives **27**, **28** and **31**–**33** was investigated by determining their antiproliferative properties. In addition, the cytotoxic properties of functionalized nanostructured particles **38**, **41** and **42** were

tested (Table 3). Although ansamitocin derivatives **27, 31** and **32** substantially differ from AP3 (**4**) in the ester side chain at C-3, all derivatives that result from 1,3-dipolar cycloadditions showed strong or very strong antiproliferative activity against various cancer cell lines. Another important outcome of these antiproliferation assays is that the Diels–Alder cycloaddition test products **28** and **33** with extended side chain at C-3 as well as the modified nanostructured particles **38**, **41** and **42** are not cytotoxic. These latter results are another important prerequisite when utilizing these conjugates in a combined

Table 3. Antiproliferative activity IC_{50} [nmol L^{-1}] of **27**, **28** and **31**–**33** and comparison to AP3 (**4**).[a]

Cell line	27	28	31	32	33
L-929	4.9	>40000	250	7.4	2700
KB-3-1	0.41	–	4.9	3.2	190
A-431	0.77	–	14	6.9	330
PC-3	2.2	–	35	10.5	310
SK-OV-3	1.6	–	12	6.2	290

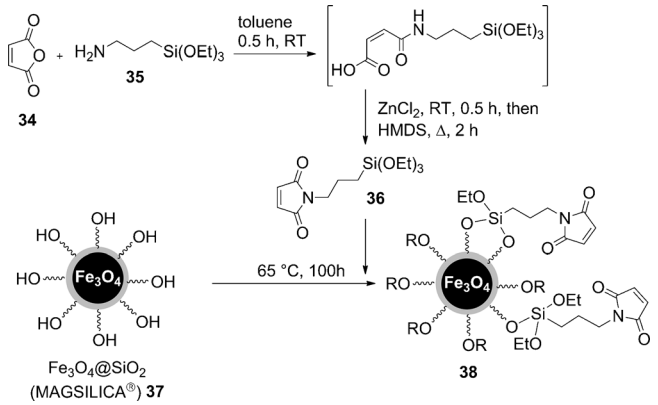
[a] Values shown are means of two determinations in parallel. Compounds **38**, **41** and **42** were tested with L-929 but no inhibitory effect was measured up to 10 μg mL^{-1}.

therapy based on hyperthermia and chemotherapy.

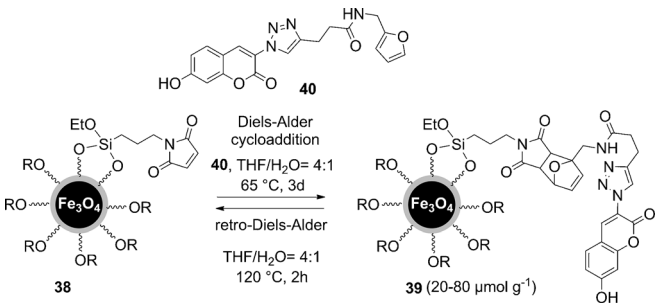
Having established the principal chemical and biological parameters, we turned our attention to the surface modification of MAGSILICA **37**. The preparation of the thermolabile linker to be attached to the nanostructured particle commenced with the coupling of maleic anhydride (**34**) and 3-aminopropyltriethoxysilane (APTES, **35**) to yield maleimide (**36**; Scheme 7). In order to prevent autopolymerization through the ethoxysilane moieties, the preparation of maleimide and functionalization of MAGSILICA **37** was carried out in a one-pot protocol. Formation of the open-chain form of maleimide was monitored by the disappearance of maleic anhydride. Subsequent ring closure to **36** proceeded in the presence of the Lewis acid zinc chloride and HMDS at 120 °C within 2 h.[45] Attachment to MAGSILICA **37** was completed after stirring at 65 °C for about four days. Noteworthy, the preparation of the Diels–Alder system in its detached form paves the way to rapidly create various surface-modified nanostructured particles and testing them independently with any other ansamitocin derivative.

XPS (X-ray photoelectron spectroscopy) measurements provided information on the amount of nitrogen present either as amine or as maleimide on the surface of modified MAGSILICA. We found that 88% of nitrogen on the silica surface of **38** was part of maleimide moieties.

These qualitative results were complemented with quantification of loading using fluorescent coumarin derivative **40** that was attached onto functionalized nanostructured particles **38** by Diels–Alder cycloaddition. Likewise the fluorescence label can be released by retro Diels–Alder reaction (Scheme 8). Thus, functionalized particles **38** were treated with furan **40** at 65 °C for three days and the solution was analyzed for remaining coumarin **40** by LC-MS (using AHBA hydrochloride (**1**) as an internal standard; see the Supporting Information). Then, surface-modified particles **39** (20–80 μmol g^{-1}) were heated at 120 °C for 2 h and the amount of coumarin derivative **40** released into solution was measured again. From these studies, the loading was determined to be in the range of 2 to 7 μmol g^{-1} for different samples. When these samples were heated up a second time at 120 °C for 2 h no detectable



Scheme 7. Functionalization of core shell nanostructured particles (MAGSILICA **37**); two modes of attachment (via one or two siloxy-linkages) are depicted.

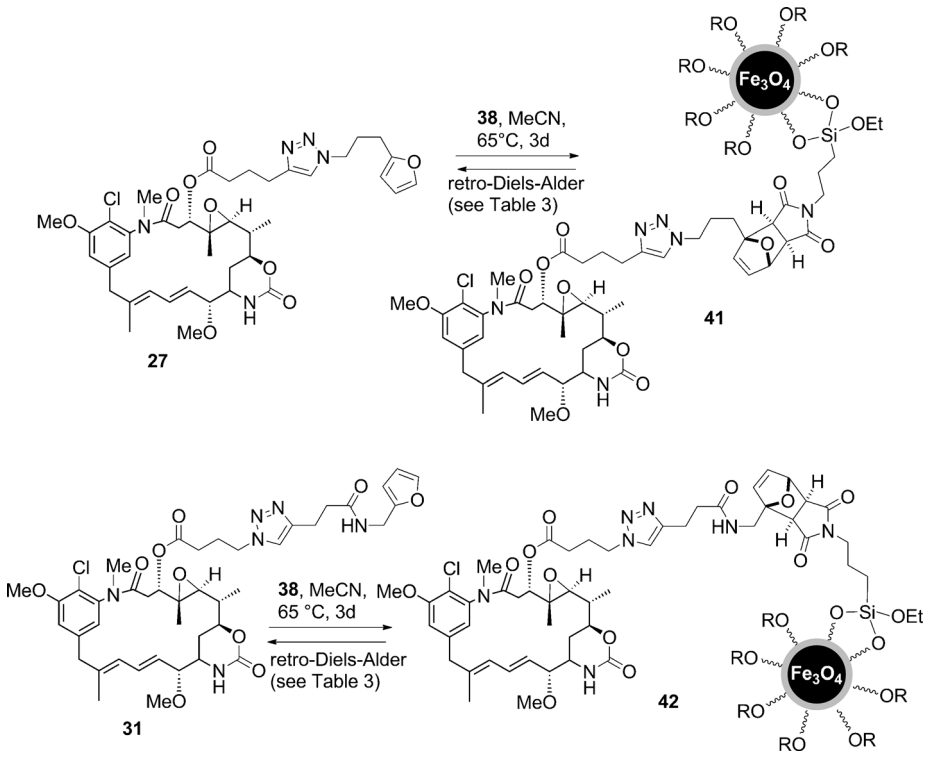


Scheme 8. Functionalization of core shell nanostructured particles **38** with coumarin derivative **40**.

amount of coumarin derivative **40** was found in the solution.

Next, the surface-modified nanostructured particles **38** underwent Diels–Alder cycloadditions in the presence of the furan-modified ansamitocin derivatives **27** and **31**, respectively, under conditions established for *N*-methylmaleimide (Scheme 6). The cycloadditions were monitored (by TLC and by HPLC) as the soluble ansamitocin derivatives slowly disappeared and functionalized nanostructured particles **41** and **42** formed (Scheme 9).

Finally, we studied the release of ansamitocin derivatives **27** and **31** from the modified nanostructured particles by: 1) heating with a conventional oil bath, or 2) in the presence of an external oscillating electromagnetic field (Table 4). In an oil bath at 110 °C complete release of ansamitocin derivatives **27** and **31**, respectively, was achieved within 4 h as judged by HPLC-MS analysis.[28] The reaction mixture was decanted from the magnetic nanostructured particles under the influence of



Scheme 9. Functionalization of nanostructured particles **38** with ansamitocin derivatives **27** and **31** and thermal cleavage by retro-Diels–Alder reaction (see also Table 3).

a magnet, the particles were washed with acetonitrile, taken up again in the same solvent and heated in the oil bath. Surprisingly, no traces of ansamitocin derivatives were detected in the supernatant. Conventional heating proved inefficient for releasing the toxins **27** or **31** under more physiological conditions such as in water or in the Ringer solution.[46]

Alternatively, superparamagnetic nanostructured particles can directly be heated under medium (mf = 15–25 kHz) or high frequency (hf = up to 780–850 kHz) conditions. The choice of frequency can have a dramatic effect on the efficiency of heating nanoparticles. A stronger magnetic field (*H*) and a higher frequency (*f*) induce more heat inside most conductive materials as well as superparamagnetic nanoparticles. Medium frequencies are preferentially employed when larger objects need to be heated, whereas high frequencies are ideally suited for smaller objects including superparamagnetic nanomaterials.[34,47]

The first experiments were carried out in acetonitrile at 15 kHz, which did not lead to cleavage of the toxin from modified MAGSILICA **41**. When increasing the oscillating field to 25 kHz a small amount of ansamitocin derivative **27** (2%) was released as judged by HPLC-MS. In contrast, 25% of ansamitocin derivative **31** was liberated from the nanostructured particle under the same conditions.

It needs to be stressed, that unlike conventional oil-bath heating, determination of the surface temperature of individual nanoparticles is a principal problem. Commonly, the temperature of the suspension is determined, which is much lower than the surface temperature of a suspended and inductively heated superparamagnetic nanoparticle. This fact explains why release of toxins from nanostructured particles like **41** proceeds more efficiently with inductive heating than in the oil bath and why high frequency conditions are preferred to medium frequencies (Table 3). About 11–14% of the theoretical amount of ansamitocin derivative **27** was released from conjugate **41** by inductive heating at 850 kHz. This corresponds to about 25–30 nmol per mg nanoparticle. If one takes the measured cytotoxicity of ansamitocin derivative **27** into account (Table 3) only 0.16–0.19 mg mL^{-1} of functionalized nanoparticle **41** should be sufficient to exert complete cell destruction under the conditions of assaying.

Table 4. Release of ansamitocin derivatives **27** and **31**, respectively, from functionalized nanostructured particles **41** and **42** using conventional and inductive heating.[a]

NP	retro-Diels–Alder reaction conditions (oil bath; solvent, temperature, reaction time)	*T* [°C][b]	Yield [%] **27**/**31**[c]
41	MeCN, 110 °C, 4 h	110	>99
42	MeCN, 110 °C, 4 h	110	>99
NP	retro-Diels–Alder reaction conditions (mf; solvent, power in %, sample, reaction time)	*T* [°C]	Yield [%] **27**/**31**[c]
41	MeCN, 100% mf, 50 mg mL^{-1}, 4 h	40	2
42	MeCN, 100% mf, 60 mg mL^{-1}, 4 h	40	25
NP	retro Diels–Alder reaction conditions (hf; solvent, power in %, sample, reaction time)[b]	*T* [°C]	Yield [%] **27**[c]
41	MeCN/*R*, 40% hf, 10 mg mL^{-1}, 1 h	49 (IR)	n.d.[d]
41	H_2O, 40% hf, 10 mg mL^{-1}, 1 h	50 (IR)	traces
41	MeCN/H_2O, 70% hf, 2.3 mg mL^{-1}, 1 h	31 (IR)	10
41	H_2O, 70% hf, 2.3 mg mL^{-1}, 1 h	32 (IR)	traces
41	H_2O, 90% hf, 2.4 mg mL^{-1}, 1 h	35 (IR)	traces
41	H_2O, 100% hf, 2.2 mg mL^{-1}, 1 h	36–39 (IR) 57[e]	11–14

[a] NP = functionalized nanostructured particle; mf = medium frequency (15–25 kHz); hf = high frequency (750–850 kHz); R = Ringer solution.[46] [b] Temperature of the solution was measured conventionally using a thermometer; [c] determined with a calibration curve (LC-MS-UV);[28] [d] quantification was not possible because of overlapping signals in the UV but release of toxin was detected; [e] temperature was measured in test tube.

Conclusions

In summary, we have reported on the development of new ansamitocin derivatives that are conjugated via a thermolabile linker to superparamagentic core-shell nanostructured particles based on a ferritic core (maghemite γ-Fe_2O_3 and magnetite Fe_3O_4) and a silica shell. Synthetically, a combination of mutasynthesis, precursor-directed biosynthesis and semisynthesis was pursued to access new ansamitocin derivatives that are either functionalized with an alkyno- or azido-function in the ester side chain at C-3. To the best of our knowledge, this is the first description of successful supplementation experiments with such toxic carboxylic acids, resulting in isolated and synthetically useful amounts of new ansamitocin derivatives. The essential synthetic feature of the linkage between the toxin and the nanostructured particle is a Diels–Alder cycloaddition between a furan and a maleimide group which undergoes a retro-Diels–Alder reaction upon heating. As the conjugates of ansamitocin and the nanostructured particle have superparamagnetic properties these can be heated in an oscillating electromagnetic field and the cytotoxic ansamitocin derivatives are released into solution. Most importantly, the ansamitocin derivatives liberated after heating show strong antiproliferative activity against several mammalian cancer cell lines, while the same cell lines were found to be insensitive towards nanostructured particle drug conjugates. As cleavage can also be induced in aqueous solutions, these conjugates can serve as dormant toxins that can be triggered through external inductive heating to act simultaneously in hyperthermia and in chemotherapy. Further studies in animal models are currently being pursued in our laboratories.

Experimental Section

The details provided below cover synthetic key protocols for preparing conjugate **27** composed of an ansamitocin derivative and a nanostructured particle. Additional synthetic procedures towards all other derivatives, relevant background information on fermentation experiments, testing of antiproliferative activities and copies of NMR spectra are found in the Supporting Information.

Preparation of ansamitocin derivatives 13 a/b using an AHBA(−) mutant strain of *A. pretiosum*

Actinosynnema pretiosum HGF073[17a] was stored as spore suspension in 40% (v/v) glycerol/water at −80 °C, and used for the inoculation of YMG agar plates. Following incubation of the plates for 4 days at 30 °C, 5–8 well-sporulated colonies were transferred to a 1.5 mL tube charged with 1 mL of sterile distilled water and filled to approximately 50% height with sterile glass beads (Ø = 2 mm, washed with dilute hydrochloric acid). After vortex-mixing, the resulting suspension was used for the inoculation of precultures in bottom-baffled 250 mL Erlenmeyer flasks charged with YMG medium (50 mL per flask, with additional steel spring). Precultures were shaken for 2 days at 29 °C before inoculation of main production cultures (1:15 dilution). Cultivations were performed in K medium with additives (37.5 mL K medium, basal composition + 3 mL amino acid solution [L-valine 1.5% (w/v), L-threonine 4.5% (w/v)] + 1.5 mL young coconut water [sterile filtered] + 3 mL preculture + 1 drop of SAG 471 anti-foam {GE Bayer Silicones}[48]) using non-baffled 250 mL Erlenmeyer flasks (final volume: 45 mL per flask, with additional steel spring). AHBA (**1**) and carboxylic acid derivative **10 d** were dissolved in DMSO/water and neutralized with $NaHCO_3$-(aq.) [1 M] [DMSO/water 1:1; volume of feeding solution should not exceed 10% (v/v) with respect to the recipient culture] and sterilized by filtration. Cultures were shaken for two days at 29 °C before continuous (drop-wise) addition of **1** and carboxylic acid derivative **10 d** over the time-course of 3 days was started using autoclavable, syringe pump-driven feeding capillaries (Braintree Scientific BS-9000-8 syringe pump with Upchurch Scientific high-purity Teflon PFA tubing {1/16" OD, 0.1" ID} and Tefzel connectors). Shaking was continued to a total cultivation time of 7–10 days. For detection of novel products from test cultures, samples of the culture broth (200 μL) were mixed with ethanol (200 μL), centrifuged (20 800 *g*, 3 min, 4 °C) and the clear supernatant was subjected to UPLC-ESI-MS analysis.

For isolation of product **13 a** from large-scale fermentation, the combined fermentation broth was extracted with ethyl acetate, and the crude extract was subjected to a sequence of chromatographic purifications as described in Table 5. Derivative **13 b** was detected by HRMS and MS-MS from fermentation supernatant.

Table 5. Chromatographic purification of ansamitocin derivative **13 a**.

Sample	Phase	Conditions of elution	Fractions
crude extract	SiO_2	ethyl acetate	F-1 (R_f (ethyl acetate) 0.5–0.2)
F-1	C18-$P_{[B]}$	H_2O [+0.1% formic acid]/MeOH [+0.1% formic acid] [A/B], flow rate = 15 mL min^{-1} gradient (t [min]/B [%]): (0/20) (5/20) (55/70) (99/85)	F-2 (t_R = 70.0–72.0 min)
F-2 (five portions)	CN-SP	H_2O/MeCN [A/B], flow rate = 2.5 mL min^{-1} gradient (t [min]/B [%]): (0/5) (5/5) (105/45)	**13 a** (t_R = 85.0 min)

13 a: ^{1}H NMR (400 MHz, [D_4]MeOH, CHD_2OD = 3.31 ppm): δ = 7.15 (d, J = 2.0 Hz, 1H, 21-H), 6.96 (d, J = 2.0 Hz, 1H, 17-H), 6.63 (dd, J = 15.4, 11.0 Hz, 1H, 12-H), 6.32 (d, J = 11.0 Hz, 1H, 13-H), 5.62 (dd, J = 15.4, 9.0 Hz, 1H, 11-H), 4.8 (dd, J = 12.0, 2.7 Hz, 1H, 3-H), 4.19 (ddd, J = 11.0, 10.9, 3.0 Hz, 1H, 7-H), 3.98 (s, 3H, 20-OMe), 3.58 (d, J = 9.0 Hz, 1H, 10-H), 3.56 (d, J = 13.0 Hz, 1H, 15-H_a), 3.37 (s, 3H, 10-OMe), 3.32 (d, J = 13.0 Hz, 1H, 15-H_b), 3.16 (s, 3H, *N*-Me), 2.76 (d, J = 9.6 Hz, 1H, 5-H), 2.69 (t, J = 7.3 Hz, 2H, 2′-H), 2.59 (dd, J = 13.9, 12.0 Hz, 1H, 2-H_a), 2.39 (t, J = 2.7 Hz, 1H, 6′-H), 2.37–2.21 (m, 2H, 4′-H), 2.14 (dd, J = 13.9, 2.7 Hz, 1H, 2-H_b), 1.91–1.83 (m, 2H, 3′-H), 1.73 (s, 3H, 14-Me), 1.61–1.48 (m, 1H, 6-H), 1.58 (dd, J = 13.8, 3.0 Hz, 1H, 8-H_a), 1.52 (dd, J = 13.8, 11.0 Hz, 1H, 8-H_b), 1.23 (d, J = 6.5 Hz, 3H, 6-Me), 0.88 ppm (s, 3H, 4-Me); ^{13}C NMR (100 MHz, [D_4]MeOH, [D_4]MeOH = 49.0 ppm): δ = 173.4 (s, C-1′), 171.4 (s, C-1), 157.5 (s, C-20), 155.3 (s, 7-OCONH), 143.1 (s, C-18), 142.7 (s, C-16), 141.1 (s, C-14), 133.8 (d, C-12), 130.0 (d, C-11), 126.0 (d, C-13), 123.3 (d, C-17), 119.8 (s, C-19), 114.9 (d, C-21), 89.7 (d, C-10), 84.7 (s, C-5′), 81.9 (s, C-9), 78.1 (d, C-3), 75.9 (d, C-7), 70.9 (d, C-6′), 67.9 (d, C-5), 61.8 (s, C-4), 57.2 (q, 20-OMe), 57.0 (q, 10-OMe), 47.5 (t, C-15), 39.2 (d, C-6), 37.5 (t, C-8), 36.2 (q, *N*-Me), 33.8 (t, C-2), 33.8 (t,C-2′), 24.9 (t, C-3′), 18.4 (t, C-4′), 15.8 (q, 14-Me), 14.7 (q, 6-Me), 12.4 ppm (q, 4-Me); UPLC-MS [MeOH] t_R = 2.42 min, [MeCN] t_R = 2.10 min; HRMS [ESI]

m/z for $C_{34}H_{43}ClN_2O_9Na$ $[M+Na]^+$: calcd 681.2555, found 681.2563; MS-MS {659.28 $[M+H]^+$}: 547.22 $[M-HOC(O)(CH_2)_3C{\equiv}CH+H]^+$.

13b: UPLC-MS [MeCN] $t_R = 1.92$ min; HRMS [ESI] *m/z* for $C_{33}H_{42}ClN_2O_9$ $[M+H]^+$: calcd 645.2579, found 645.2580; MS-MS {645.25 $[M+H]^+$}: 533.20 $[M-HOC(O)(CH_2)_3C{\equiv}CH+H]^+$.

Ansamitocin derivative 27

$Cu^{I}Br$ (8.5 mg, 59 μmol, 26.0 equiv) was dissolved in degassed methanol (3 mL) under argon atmosphere and DIPEA (30 μL, 0.16 mmol, 76.0 equiv) was added. The resulting yellowish suspension was degassed for 30 min under a stream of argon and with exclusion of light. Ansamitocin derivative **13a** (1.5 mg, 2.3 μmol, 1.0 equiv) was dissolved in degassed methanol (0.5 mL) under argon atmosphere and treated with 240 μL of the freshly prepared suspension of $Cu^{I}Br$-DIPEA and 2-(3-azidopropyl)furan **26** (0.8 mg, 5.3 μmol, 2.3 equiv). The reaction mixture was stirred at room temperature under argon atmosphere and with exclusion of light for 20 h. EDTA [pH 8.0, (0.5 M), 0.5 mL] was added and after being stirred for 5 min the reaction mixture was directly purified by HPLC (Trentec Reprosil 100 C18-ISIS 5 μm, 250 mm × 8 mm, with guard column, 40 mm; gradient H_2O/MeOH 95:5→90:10 over 10 min, then 90:10→40:60 over 50 min, 40:60→20:80 over 20 min and 20:80→100% MeOH over 10 min; flow 2.25 mL min^{-1}, t_R = 76.5 min). Furfurylansamitocin derivative **27** (1.6 mg, 1.97 μmol, 87%) was isolated as a colorless foam.

^{1}H NMR (500 MHz, [D_4]MeOH CD_2HCOD = 3.31 ppm): δ = 7.83 (s, 1H, 6′-H), 7.34 (d, J = 1.6 Hz, 1H, 13′-H), 7.12 (d, J = 1.6 Hz, 1H, 21-H), 6.80 (d, J = 1.6 Hz, 1H, 17-H), 6.57 (dd, J = 11.1, 15.4 Hz, 1H, 12-H), 6.32 (dd, J = 1.6, 3.1 Hz, 1H, 12′-H), 6.08 (d, J = 11.1 Hz, 1H, 13-H), 6.04 (d, J = 3.1 Hz, 1H, 11′-H), 5.37 (dd, J = 15.4, 8.9 Hz, 1H, 11-H), 4.76 (dd, J = 11.9, 2.8 Hz, 1H, 3-H), 4.39 (t, J = 7.2 Hz, 2H, 7′-H), 4.18 (td, J = 2.6, 10.9 Hz, 1H, 7-H), 3.97 (s, 3H, 20-OCH_3), 3.56 (d, J = 8.9 Hz, 1H, 10-H), 3.54 (m, 1H, 15-H_a), 3.35 (d, J = 2.2 Hz, 3H, 10-OCH_3), 3.26 (m, 1H, 15-H_b), 3.11 (s, 3H, N-CH_3), 2.82 (t, J = 7.3 Hz, 2H, 4′-H), 2.76 (d, J = 9.8 Hz, 1H, 5-H), 2.63 (t, J = 7.4 Hz, 2H, 9′-H), 2.57 (m, 3H, 3′-H, 2-H_a), 2.20 (q, J = 7.2 Hz, 2H, 8′-H), 2.12 (dd, J = 12.9 Hz, 1H, 2-H_b), 2.03 (m, 2H, 2′-H), 1.69 (s, 3H, 14-CH_3), 1.56–1.47 (m, 3H, 8-H_a, 8-H_b, 6-H), 1.22 (d, J = 6.4 Hz, 3H, 6-CH_3), 0.85 ppm (s, 3H, 4-CH_3); ^{13}C NMR (125 MHz, [D_4]MeOH, [D_4]MeOH = 49.0 ppm): δ = 173.5 (p, C-1), 171.4 (p, C-1′), 157.4 (p, C-20), 155.5 (p, OCONH), 155.3 (p, C-10′), 148.4 (p, C-5′), 142.9 (p, C-18), 142.7 (p, C-16), 142.5 (p, C-10′), 141.1 (p, C-14), 133.8 (s, C-12), 129.7 (s, C-11), 125.9 (s, C-13), 123.6 (s, C-6′), 123.2 (s, C-17), 119.7 (p, C-19), 114.9 (s, C-21), 111.2 (s, C-12′), 106.6 (s, C-11′), 89.7 (s, C-10), 81.8 (p, C-9), 78.1 (s, C-3), 75.9 (s, C-7), 67.9 (s, C-5), 61.8 (p, C-4), 57.2 (q, 20-OCH_3), 57.1 (q, 10-OCH_3), 50.6 (t, C-7′), 47.4 (t, C-15), 39.2 (t, C-8), 37.5 (d, C-6), 36.2 (q, N-CH_3), 34.3 (t, C-3′), 33.8 (t, C-2), 29.9 (t, C-8′), 25.8 (t, C-2′), 25.7 (t, C-4′), 25.5 (t, C-9′), 15.8 (q, 14-CH_3), 14.7 (q, 6-CH_3), 12.4 ppm (q, 4-CH_3); UPLC-MS t_R = 2.03 min; HRMS [ESI] *m/z* for $C_{41}H_{53}N_5O_{10}Cl$ $[M+H]^+$: calcd 810.3481, found 810.3450.

Preparation of surface-modified MAGSILICA 38

MAGSILICA 300 (**37**; 6.0 g) was dried at 120 °C for 6 h under high vacuum. Maleic anhydride (**34**; 1.72 g, 18 mmol, 3.0 mmol g^{-1}), 120 mL dry toluene and 3-aminopropyltriethoxy silane (**35**; 2.8 mL, 12 mmol, 2.0 mmol g^{-1}) were added consecutively and the suspension was gently stirred for 30 min at room temperature under an argon atmosphere. To this suspension $ZnCl_2$ (2.46 g, 18 mmol, 3.0 mmol g^{-1}) was added and gentle stirring was continued for 30 min at room temperature. Then, hexamethyldisilazane (5.0 mL, 24 mmol, 4.0 mmol g^{-1}) was added and the reaction mixture was heated under refluxing conditions for 2 h. The reaction vessel was sealed and stirred at 65 °C for 100 h. After completion the reaction mixture was decanted by fixing the nanostructured particles with an external magnet and the solid was washed 12 times with 50 mL methanol. The functionalized MAGSILICA **38** was dried under high vacuum.

Diels–Alder cycloaddition of modified MAGSILICA 38 with ansamitocin derivative 27 and formation of conjugate 41

Ansamitocin derivative **27** (1 mg, 1.24 μmol, 1.0 equiv) was dissolved in acetonitrile (1 mL) and modified MAGSILICA **38** (100 mg) was added. The mixture was heated at 65 °C and was stirred at this temperature for 4 days. After cooling to room temperature the supernatant was decanted using a pipette by fixation of modified MAGSILICA **41** with an external magnet. The particles were suspended in methanol several times until no ansamitocin derivative **27** could be detected as judged by LC/MS. The starting material was recovered and purified by HPLC (C-18-ISIS phase; gradient H_2O/MeOH 95:5→90:10 over 10 min, then 90:10→40:60 over 50 min, 40:60→20:80 over 20 min and 20:80→100% MeOH over 10 min; flow 2.25 mL min^{-1}; t_R = 68.5 min). Ansamitocin derivative **27** (0.4 mg, 0.49 μmol, 40%) was isolated as a colorless foam. Functionalized MAGSILICA **41** was dried in vacuo.

Retro Diels–Alder reaction of functionalized MAGSILICA 41

Functionalized MAGSILICA **41** was suspended in acetonitrile (5 mL) and heated at 110 °C for 4 h. The suspension was cooled to room temperature in an ice bath and after fixation of nanostructured particles with an external magnet the supernatant was decanted using a pipette. The particles were suspended with methanol several times. The particles were suspended in methanol several times until no ansamitocin derivative **27** could be detected as judged by LC/MS. The combined organic extracts were removed under reduced pressure and the residue was purified by HPLC (C-18-ISIS phase; gradient H_2O/MeOH 95:5→90:10 over 10 min, then 90:10→40:60 over 50 min, 40:60→20:80 over 20 min and 20:80→100% MeOH over 10 min; flow 2.25 mL min^{-1}; t_R = 68.5 min). Ansamitocin derivative **27** (0.6 mg, 0.74 μmol) was isolated as a colorless foam.

The analytical and spectroscopic data are listed above.

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FULL PAPER

Medicinal Chemistry

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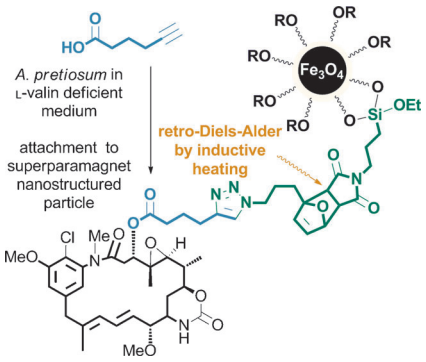
Preparation of Thermocleavable Conjugates Based on Ansamitocin and Superparamagnetic Nanostructured Particles by a Chemobiosynthetic Approach



A dual strategy to fight cancer: New ansamitocin derivatives were prepared by a combination of precursor-directed biosynthesis and semisynthesis, leading to conjugates with a thermolabile linker attached to superparamagnetic core-shell nanostructured particles. These nontoxic conjugates can be heated with release of the toxin in an external oscillating electromagnetic field. The conjugates can act simultaneously in hyperthermia and in chemotherapy.