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Combined Muta- and Semisynthesis: A Powerful Synthetic Hybrid Approach to Access Target Specific Antitumor Agents Based on Ansamitocin P3

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Abstract: Access of four new tumor specific folic acid/ansamitocin conjugates is reported that relies on a synthetic strategy based on the combination of mutasynthesis and semisynthesis. Two bromo-ansamitocin derivatives were prepared by mutasynthesis or by a modified fermentation protocol, respectively, that served as starting point for the semisynthetic introduction of an allyl amine linker under Stille conditions. A sequence of standard coupling steps introduced the pteroic acid/glutamic acid/cysteine unit to the modified ansamitocins. All new derivatives, including those that are expected to be generated after internalization of the folic acid/ansamitocin conjugates into the cancer cell and reductive cleavage of the disulfide linkage showed good to strong antiproliferative activity (IC_{50} <10 nM) for different cancer cell lines. Finally, the four conjugates were exposed to two cancer cell lines [cervix carcinoma, KB-3-1 (FR+) and lung carcinoma, A-459 (FR−)], the latter devoid of the membrane-bound folic acid receptor (FR−). All four conjugates showed strong antiproliferative activity for the FR+ cancer cell line but were inactive against the FR− cell line. The synthetic strategy pursued is based on the combination of mutasynthesis and semisynthesis and proved to be powerful for accessing new ansamitocin derivatives that are difficult to prepare by total synthesis.

Keywords: ansamitocins · antitumor agents · folate-drug conjugates · mutasynthesis · semisynthesis · Stille reaction

Introduction

Profiling natural products towards pharmaceutically useful drugs is commonly hampered either by their availability from natural sources or by the difficulty to create compound libraries for structure activity relationship studies. Often, the structural complexity precludes total synthesis approaches towards such libraries, while semisynthesis is limited to structural modifications of chemically accessible functional groups of the core. Mutational biosynthesis or in short mutasynthesis has emerged as a third concept to create compound libraries of complex natural products.[1] The technique requires the generation of mutants of a producer organism blocked in the formation of a biosynthetic building block of the end-product. Administration of chemically modified biosynthetic intermediates, so called mutasynthons, to the blocked mutant results in new metabolites.[2] Recently, we showed that mutasynthesis can be used to introduce chemically functionalities such as Br substituents that in the following allow to carry out semisynthetic modifications thereby broadening the opportunities of derivatizing complex natural products such as the clinically promising anticancer agents ansamitocins P-2–P-4 **1**–**3** (Figure 1).[3] They belong to the group of maytansinoids, which are of microbial origin (*Actinosynnema pretiosum*). Indeed, they are

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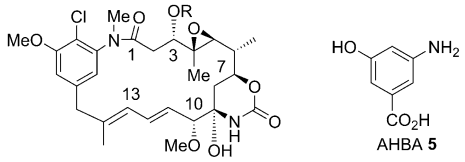


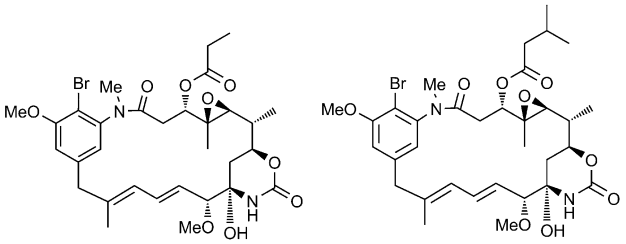
1: R= -C(O)CH_2CH_3 (ansamitocin P-2)
2: R= -C(O)$CH(CH_3)_2$ (ansamitocin P-3)
3: R= -C(O)$CH_2CH(CH_3)_2$ (ansamitocin P-4)
4: R= -C(O)$CH(CH_3)NHC(O)CH_3$ (maytansine)

Figure 1. Ansamitocins P-2 (**1**), P-3 (**2**) and P-4 (**3**), maytansine **4** and 3-amino-5-hydroxybenzoic acid **5** (AHBA), the starter unit of ansamitocin polyketide synthase (PKS).

highly potent antitumor active compounds that inhibit growth of different leukemia cell lines as well as human solid tumors at very low concentrations (10^{-3} to $10^{-7}\,\mu g\,mL^{-1}$) by disrupting microtubule assembly.[4–6] They bind microtubules in a manner similar to the vinca alkaloids but are 20- to 100-fold more potent at blocking mitosis. The antitumor activity of maytansine **4**, the plant derived ansamitocin derivative, was extensively evaluated in human clinical trials,[7] but although potent in vitro, maytansine displayed a poor therapeutic window in vivo. In conjunction with monoclonal antibodies,[8] vitamins such as folic acid[9], growth factors such as the epidermal growth factor and others[10] that bind to specific markers on the surface of tumor cells, anticancer compounds in general including the ansamitocins may become more tumor specific and thus less toxic. The high potency of maytansine makes it an attractive candidate for targeted delivery for the selective destruction of tumor cells.[11] Recently, a conjugate composed of the humanized anti-HER2 mAb antibody trastuzumab, which has been used in the treatment of breast cancer, and maytansine reached late clinical trials for the treatment of HER2+ metastatic breast cancer.[12] In 1997, Ladino and co-workers reported on the first successful preparation of a maytansine conjugate with folic acid. The folic acid was linked to the natural product via the ester group at C3.[13] Indeed, the vitamin folic acid has emerged as a promising targeting ligand for selective delivery of attached therapeutic agents to cancer tissues. Folic acid has a high affinity for the folate receptor (FR) ($K_d = 10^{-10}$ M), even when conjugated to a therapeutic drug. Importantly, the receptor is not widely distributed in normal tissue.[14] However, many different cancer cells have been found to overexpress FR.[15] In order to exploit these phenomena in targeted cancer therapy, folic acid-drug conjugates need to be constructed that are assembled of three components: a) the folic acid, b) the cytotoxic drug, and c) the linker connecting the drug to the tumor-specific ligand. The linker can be designed in such a way in that a release mechanism of the active drug is incorporated into the molecular architecture of the conjugate.[16] One possible mechanism could exploit the difference in reducing power between extra- and intracellular milieus to induce the selective release of a disulfide-linked drug inside its target cell for which evidence had been collected with mitomycin conjugates.[17,18] It was demonstrated that the folate–disulfide–drug conjugate reduction occurs following endocytosis. It begins in endosomes and does not significantly depend upon the redox machinery located on the cell surface.

Results and Discussion

As extension of a research program that is dedicated to show that mutasynthesis is a synthetic method and that in combination with other synthetic methods it can emerge as a very versatile synthetic tool in natural product synthesis,[19] we now disclose a new synthetic approach towards four folic acid/ansamitocin conjugates and describe their biological properties. One of our architectural key requirements is that the nature and position of the folic acid bearing group would not diminish the potency of the parent molecule. We chose to attach the tumor-specific ligand to the aromatic moiety following an established route and linker concept.[11–13] The aryl ring is not the pharmacophore and unlike existing approaches of conjugation we do not need to alter the key pharmacophore, the ester side chain at C3. The aromatic ring of the ansamitocins can be modified by mutasynthesis using a blocked mutant (strain HGF073) of the ansamitocin producer *Actinosynnema pretiosum* which has lost the ability to biosynthesize the polyketide synthase (PKS) starter unit, 3-amino-5-hydroxybenzoic acid AHBA (**5**).[20] Feeding AHBA-derivatives to this blocked mutant mutasynthesis yields new ansamitocins modified in the aromatic ring. In contrast to chloro-functionalized AP-3, ansamitocin derivatives that are brominated at C-19 are well suited for semisynthetic modifications. They do allow to link folic acid to ansamitocin. Brominated ansamitocin derivatives **6** and **8** were obtained by two different fermentation concepts. Supplementing *Actinosynnema pretiosum* (HGF073) with AHBA and sodium bromide ($c = 0.1\,mol\,L^{-1}$) the bromo derivative AP-3 (**6**) was isolated in very good yield ($132\,mg\,L^{-1}$; 13% with respect to AHBA employed. The corresponding 19-bromo AP-2 (**12**; $14\,mg\,L^{-1}$) and AP-4 (**13**; $4\,mg\,L^{-1}$) were also isolated as byproducts).[21c] Formation of chlorinated analogues was not detected which simplified HPLC purification. Most likely, the halogenase generates a reactive hypohalite species by a two-electron oxidation of the corresponding halide. The redox potential of the bromide anion favors its oxidation over the chloride anion.[21] While this bromination is not a mutasynthesis in a true sense, earlier we reported on the synthesis of AP-3 derivative (**6**) by a mutasynthetic approach using 3-amino-4-bromo-5-hydroxybenzoic acid as mutasynthon.[20d] Compared to the present strategy, the isolated yield of **6** was dramatically lower ($7.1\,mg\,L^{-1}$). In addition, mutasynthesis employing mutasynthon **7** yielded 19-bromo-20-desmethoxy derivative **8** ($15\,mg\,L^{-1}$).[22] In the following, the synthetic potential of these aryl bromides was exploited and allyl amine was attached to the ring by a Stille coupling. We found that this cross-coupling works best for 19-bromo ansamitocin analogues because of the neutral reaction conditions in con-



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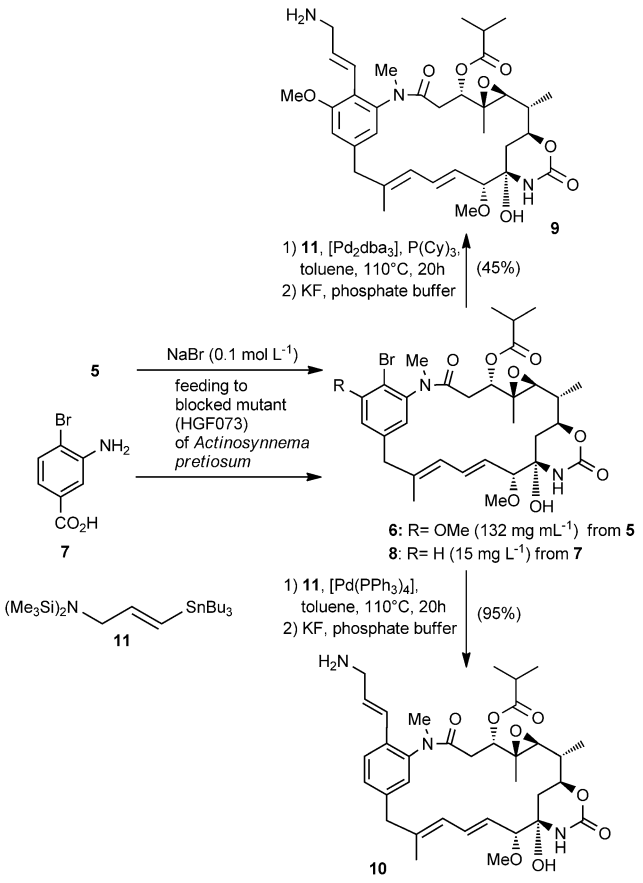


Scheme 1. Mutasynthetic preparation[21c] of 19-bromo ansamitocin derivatives **6** and **8** and Stille cross-coupling with stannane **11**.

trast to, for example, the Heck reaction. By employing stannane **11** as coupling partner[23] two new ansamitocin derivatives **9** and **10** were prepared after mild desilylation with KF. The 20-desmethoxy bromide **8** smoothly underwent the Stille coupling reaction using the standard catalyst [Pd(Ph_3)$_4$] (Scheme 1). In contrast, substantial optimization was necessary to achieve the same transformation with bromo AP-3 derivative (**6**). Most common catalysts failed which can be ascribed to the presence of two *ortho* substituents in the aromatic moiety. In our hands, only Fu's Pd catalytic system that contains bulky PCy_3 ligands yielded cross-coupling product **9** in moderate yield after 20 h in refluxing toluene. Noteworthy, this catalytic system had originally been developed for Suzuki–Miyaura cross-coupling reactions of sterically hindered substrates.[24,25] This protocol paved the way to modify AP-3 derivative **6** bearing two *ortho* substituents next to the bromide in satisfactory yield. The amino group was further elaborated for coupling to folic acid by treatment with doubly activated 3-(2-pyridyldithio)-propionic ester (SPDP) **14** and 4-[1-(2-pyridyldithio)-ethyl]-benzoic ester (SMPT) **15**, respectively (Scheme 2)[26] to yield four pyridyl disulfides **16**–**19**. Again the 20-methoxy functionalization in AP-3 led to slightly lower yields in these coupling reactions too. The free thiols **26**–**28** were quantitatively liberated after treatment with dithiothreitol (DTT) in PBS-buffered acetonitrile.[13] The biological activities were first tested for the new bromo ansamitocins **6**, **12** and **13** using a standard set of human cancer cell lines (Table 1). They all showed strong antiproliferative activity, most of them with IC_{50} values between 6 and 0.01 nmol L^{-1}. Finally, these activated disulfides **16**–**19** were reacted with pteroyl-L-glutamic acid extended by L-cysteine (Pte-Glu-Cys) **21** which had been prepared under typical solid-phase conditions utilizing the Fmoc strategy (see Supporting Information) to furnish the four conjugates **22**–**25**.[27] Next, wet tested the antiproliferative activities of synthetic precursors of folate–ansamitocin conjugates **26**–**28** (Table 2). These AP-3 derivatives are supposed to be liberated inside the cell after internalization and reductive cleavage from conjugates **22**–**24**.[28] Gratifyingly, these thiols also showed good to strong antiproliferative activities against selected cell lines including a lung carcinoma cell A-549 deficient of membrane-bound folic acid receptors (FR), although by a factor of about 10–100 lower compared to ansamitocin P3 (**2**). Because of the extreme cytotoxicity of the ansamitocins these reduced activities are still in the range to be suited for an antitumor drug. Finally, the validity of the folic acid–ansamitocin conjugates as anticancer agents was probed by exposing two human tumor cell lines to conjugates **22**–**25**. KB-3-1 (FR+) has the FR expressed while A-549 (FR−) is devoid of FR on the cell surface. These properties were confirmed by immunofluorescence staining of the specific folate receptor (Figure 2). The

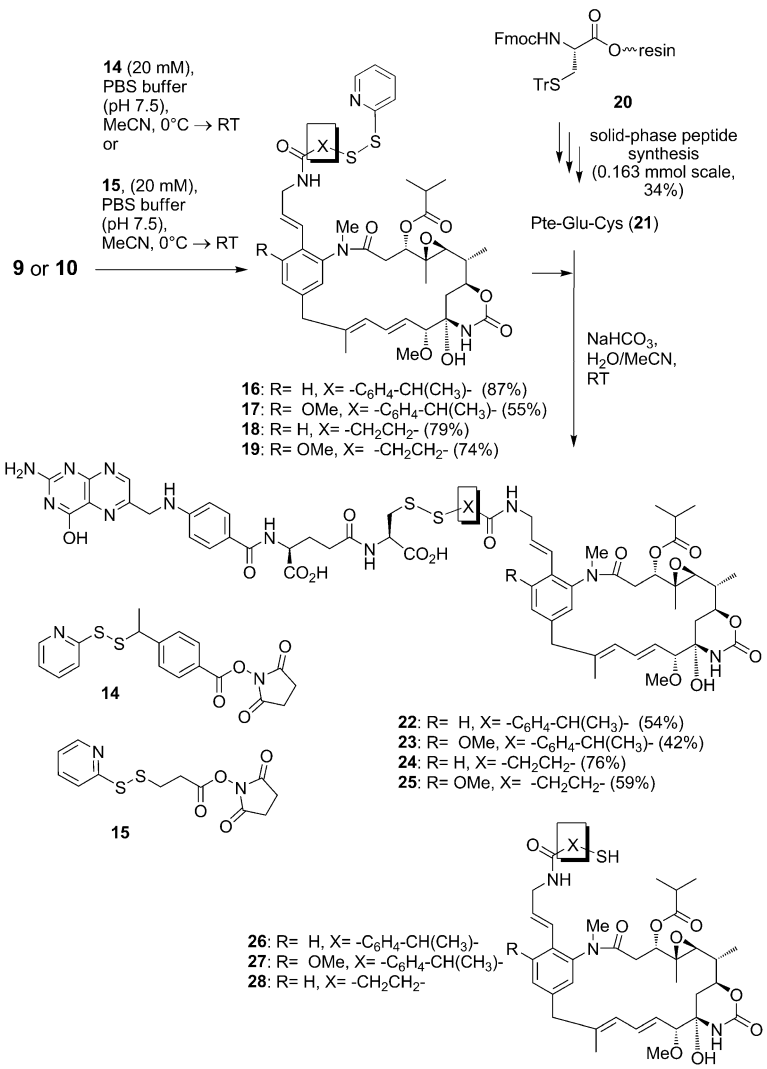
Table 1. Antiproliferative activity IC_{50} [nM] of bromo ansamitocin derivatives **6**, **12**, **13** and **8** for different cell lines (values shown are based on two determinations in parallel).[a]

Cell line	**2**	**6**	**12**	**13**	**8**
L-929	0.2	0.1	0.9	1.0	1.1
KB-3-1	0.17	0.19	n.d.	0.73	0.46
U-937	0.006	0.012	n.d.	0.046	0.046
A-431	0.08	0.06	0.16	0.23	0.15
A-498	1.7	1.3	4.2	3.7	6.2
A-549	0.16	0.07	n.d.	0.17	0.46
SK-OV-3	0.047	0.059	1.35	0.107	0.077
PC-3	0.055	0.147	0.06	0.114	0.154
MCF-7	n.d.	0.019	0.057	0.058	n.d.
HUVEC	0.024	n.d.	n.d.	n.d	0.231

[a] Human cell lines: L-929 (mouse fibroblasts), KB-3-1 (cervix cacinoma), U-937 (histiocytic lymphoma), A-431 (epidermoid carcinoma), A-498 (kidney carcinoma), A-549 (lung carcinoma), SK-OV-3 (ovary adenocarcinoma), PC-3 (prostate adenocarcinoma), MCF-7 (breast adenocarcinoma), HUVEC (umbilical vein endothelial cells); n.d. = not determined.

Table 2. Antiproliferative activity IC_{50} [nM] of ansamitocin derivatives **26**–**28** in SW-480, A-549 and HCT-116 cells (FR−: devoid of membrane-bound folic acid receptors).

Cell line	Origin	**26**	**27**	**28**
SW-480	colon carcinoma	5	6	42
KB-3-1	cervix carcinoma	4	4	14
A-549 (FR−)	lung carcinoma	6	24	420
HCT-116	colon carcinoma	8	10	49



Scheme 2. Preparation of folate-ansamitocin P-3 conjugates **22**–**25** (Fmoc = fluorenylmethoxycarbonyl, PBS = phosphate buffered saline, Tr = triphenylmethyl, Pte = pteroic acid ,Glu = glutamic acid, Cys = cysteine).

cell lines (see Table 3), none of them showed cytotoxic effects on the cell line A-549 (FaR−). In contrast when folate receptors are expressed such as in cell line KB-3-1 (FR+) strong antiproliferative activity is regained. The values favorably compare to those measured for thiols **26**–**28** (see Table 2) the supposed products from in vivo reductive cleavage of **22**–**25**. Supportive data were gained from fluorescence-activated cell sorting (FACS) analysis. We encountered an increase of the subG1 peak in KB-3-1 (FR+) cell lines compared to A-549 (FR−) cells in the presence of conjugate **24** (Figure 3). In fact, A-549 lung carcinoma cells (FR−) did not show altered behavior modifications in the subG1 phase. The solvent DMSO served as a control.

Conclusion

In this report we disclose new folate-coupled derivatives of strongly cytotoxic ansamycin antibiotics that show good in vitro antiproliferative selectivity between cells with cell surface expressed folate receptors (FR)

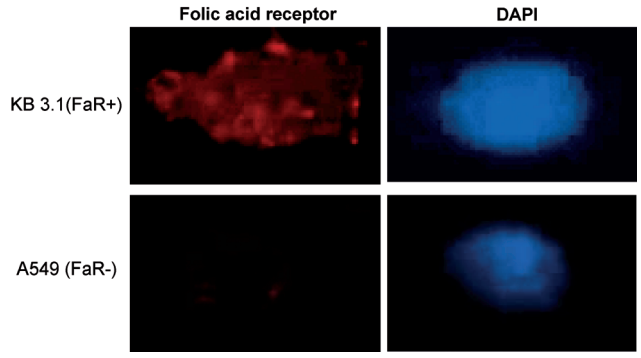


Figure 2. Folate receptor stains (red) of KB-3-1 (top) and A-549 (bottom). The folate receptor staining is shown on the left and DAPI (blue) stains of cells on the right (fluorescent staining of DNA in nucleus).

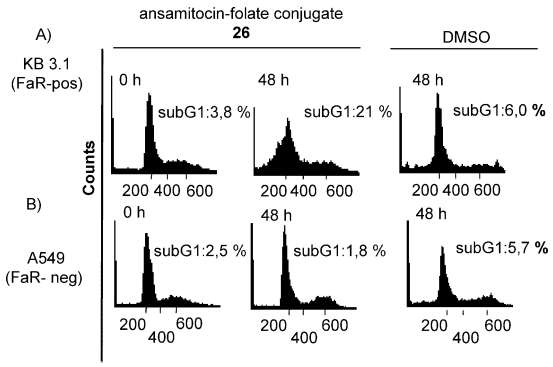


Figure 3. Influence of the folate-ansamitocin conjugate 24 on the subG1 phase of KB-3-1 and A-549 cells. FACS analysis reveals that more KB-3-1 (FR+) cells A) die after 48 h incubation compared to the A-549; B) folic receptor negative (FR−) cell line (DMSO as control).

KB-3-1 cells were generally more sensitive to the treatment compared to the A-549 cells. When all four new folate–ansamitocin conjugates **22**–**25** were administered to these two compared to those that are devoid of FR. Our synthetic approach relies on a combined muta-/semisynthetic strategy that has not been pursued in this context and which provides

Table 3. Antiproliferative activity IC_{50} [nM] of folate-AP-3 conjugates **22–25** (KB-3-1 cells: folate receptor positive and A-549 cells: folate receptor negative).

Cell line	Origin	**22**	**23**	**24**	**25**
KB-3-1 (FR+)	cervix carcinoma	13	8	21	7
A-549 (FR−)	lung carcinoma	$>10^4$	$>10^4$	$>10^4$	$>10^4$

new ansamitocin derivatives that would be difficult to access by total or semisynthesis. To the best of our knowledge, we disclosed the most complex and advanced example of such a combined muta-/semisynthetic approach thereby expanding synthetic strategies towards complex natural product derivatives.[29] These target-specific conjugates are promising candidates for further in in vivo evaluation that are currently pursued in our laboratories. Our combined muta-/semisynthetic strategy paves the way to principally label ansamitocins at the aromatic moiety for example, with other tumor-specific ligands such as antibodies or with fluorescence dyes for addressing the biological target without dramatically affecting the excellent antiproliferative potency of the ansamitocins.

Experimental Section

General methods: ^{1}H NMR spectra were recorded at 400 MHz with a Bruker AVS-400 and at 500 MHz with Bruker AM-500. ^{13}C NMR were recorded at 100 MHz with a Bruker AVS-400 and at 125 MHz with a Bruker AM-500. If not otherwise noted, $CDCl_3$ was the solvent for all NMR experiments using the signal of residual undeuterated solvent as the internal standard.[S1] Multiplicities are described using the following abbreviations: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, b = broad. Chemical shift values of ^{13}C NMR spectra are commonly reported as values in ppm relative to the deuterated solvent as internal standard. The multiplicities refer to the resonances in the off-resonance decoupled spectra and were elucidated using the distortionless enhancement by polarisation transfer (DEPT) spectral editing technique, with secondary pulses at 90° and 135°. Multiplicities are reported using the following abbreviations: s = singlet (due to quaternary carbon), d = doublet (methine), t = triplet (methylene), q = quartet (methyl). Mass spectra (EI) were obtained at 70 eV with a type VG Autospec apparatus (Micromass), with a type LCT (ESI) (Micromass) with an Acquity-UPLC (Waters) coupled with a Q-Tof Premier mass spectrometer (Waters). Ion mass (m/z) signals are reported as values in atomic mass units. Analytical thin-layer chromatography was performed using precoated silica gel 60 F_{254} plates (Merck, Darmstadt), and the spots were visualised with UV light at 254 nm or by staining with H_2SO_4/4-methoxybenzaldehyde in ethanol. Flash column chromatography was performed on Merck silica gel 60 (230–400 mesh). Reversed phase high performance liquid chromatography was performed on a Merck Hitachi LaChrome system (pump L-7150, diode array detector L-7455). Operating conditions and used stationary phases are noted in the respective experimental part. Melting points were measured on a SRS OptiMelt apparatus and are uncorrected. Commercially available reagents and dry solvents (toluene, dimethylformamide) were used as received or purified by standard techniques according to the literature (see Supporting Information ref. [S2].

Feeding experiments

General parameters: *A. pretiosum* mutant HGF073, a reconstruction of HGF056 by application of the suicide vector pHGF9029, was stored as stock cultures at −80 °C in 40 % glycerol/water. The strain was grown on a YMG agar plate at 30 °C for 3 d. A single colony of the 3 d old agar plate with HGF073 mutant was used to inoculate the YMG precultures which were incubated at 30 °C for 2 d. Precultures were prepared in YMG-medium (50 mL per flask, 4 g L^{-1} yeast extract, 10 g L^{-1} malt extract, 4 g L^{-1} glucose, distilled water, pH 7.3). Main cultures were prepared in medium K (60 g L^{-1} dextrin from potato starch, 5.25 g L^{-1} Proflo cotton seed flour, 0.3 g L^{-1} K_2HPO_4, 30 g L^{-1} maltose, 4.5 g L^{-1} yeast extract, 2 mg L^{-1} $FeSO_4 \times 7\ H_2O$, 5 g L^{-1} $CaCO_3$, distilled water, pH 7.2, 3 mL filter-sterilized 3 % L-valine solution (per 25 mL culture broth) added after sterilization). Main cultures were inoculated with 2 mL preculture per 25 mL culture broth. Liquid culture fermentations were incubated at 30 °C under vigorous shaking (225 rpm) in 500 mL Erlenmeyer flasks with a steel spring. Mutasynthons (dissolved in DMSO/water 1:1 or pure water, sterile) were fed continuously via syringe pump after 48 h for a period of 96 h. The production cultures were harvested after 7 d of fermentation and extracted twice with EtOAc. The EtOAc extracts were dried over $MgSO_4$ and concentrated in vacuo. The residue was filtered through a short silica gel column (EtOAc) and purified by reversed phase HPLC.

Folic acid-ansamitocin conjugate 22: Pte-L-Glu-L-Cys **21** (1.15 mg, 2.11 μmol, 1.0 equiv) was suspended in water (600 μL), the mixture was deaerated by passing nitrogen through and adjusted to pH 7 by adding a solution of $NaHCO_3$ [0.1 M]. After addition of disulfide **16** (1.9 mg, 2.11 μmol, 1.0 equiv), dissolved in 600 μL MeCN, stirring was continued until complete conversion was detected by UPLC-MS. The reaction mixture was directly purified by reversed phase HPLC (Trentec Reprosil-Pur 120 C18 AQ 5 μm, column: 250 mm × 8 mm, guard 40 mm × 8 mm; gradient 0.1 % in water TFA/MeCN 99:1 → 100 % MeCN in 55 min, then 5 min 100 % MeCN, flow rate 2.25 → 4 mL min^{-1}; t_r = 37.9 min) to yield the title compound **22** (1.5 mg, 1.13 μmol, 54 %) as a yellow solid. ^{1}H NMR (500 MHz, [D$_6$]DMSO, [D$_5$]DMSO = 2.50 ppm): δ = 8.73 (m, 1H, 24-NH), 8.65 (s, 1H, 48-H), 8.32 (m, 1H, 32-NH), 8.22 (m, 1H, 36-NH), 7.81 (d, J = 7.3 Hz, 2H, 27-H), 7.69 (d, J = 8.0 Hz, 1H, H-20), 7.66 (d, J = 8.8 Hz, 2H, 39-H), 7.45 (d, J = 7.3 Hz, 2H, 28-H), 7.34 (dd, J = 8.0, 1.4 Hz, 1H, 21-H), 7.21 (m, 1H, 43-NH$_a$), 7.11 (s, 1H, 43-NH$_b$), 7.06 (d, J = 1.4 Hz, 1H, 17-H), 7.00 (s, 1H, 38-NH), 6.91 (s, 1H, 9-NH), 6.64 (d, J = 8.8 Hz, 2H, 40-H), 6.58 (dd, J = 15.3, 11.1 Hz, 1H, 12-H), 6.45 (d, J = 15.9 Hz, 1H, 22-H), 6.37 (ddd, J = 15.9, 5.6, 5.2 Hz, 1H, 23-H), 6.21 (d, J = 11.1 Hz, 1H, 13-H), 5.40 (dd, J = 15.3, 8.9 Hz, 1H, 11-H), 4.56 (dd, J = 11.9, 2.8 Hz, 1H, 3-H), 4.48 (s, 2H, 42-H), 4.44 (m, 1H, 32-H), 4.29 (m, 1H, 36-H), 4.25 (d, J = 6.9 Hz, 1H, 30-H), 4.07 (m, 3H, 7-H, 24-H), 3.49 (d, J = 8.9 Hz, 1H, 10-H), 3.48 (d, J = 12.5 Hz, 1H, 15-H$_a$), 3.23 (d, J = 12.8 Hz, 1H, 15-H$_b$), 3.22 (s, 3H, 10-OMe), 3.00 (d, J = 2.5 Hz, 1H, OH), 2.98 (s, 3H, NMe), 2.90 (m, 1H, 31-H$_a$), 2.75 (m, 1H, 31-H$_b$), 2.67 (d, J = 9.8 Hz, 1H, 5-H), 2.64 (sep, J = 7.0 Hz, 1H, 2′-H), 2.48 (m, 1H, 2-H$_a$), 2.25 (m, 2H, 34-H), 2.02 (m, 2H, 2-H$_b$, 35-H$_a$), 1.91 (m, 1H, 35-H$_b$), 1.60 (d, J = 6.9 Hz, 3H, 30-Me), 1.59 (s, 3H, 14-Me), 1.43 (m, 1H, 6-H, 8-H$_a$), 1.31 (m, 1H, 8-H$_b$), 1.18 (d, J = 7.0 Hz, 3H, 2′-Me$_a$), 1.12 (d, J = 6.3 Hz, 3H, 6-Me), 1.11 (d, J = 7.0 Hz, 3H, 2′-Me$_b$), 0.75 ppm (s, 3H, 4-Me); ^{13}C NMR (125 MHz, [D$_6$]DMSO, [D$_6$]DMSO = 39.52 ppm): δ = 175.5 (s, C-1′), 173.8 (s, 36-CO_2H), 171.94 (s, 32-CO_2H), 171.88 (s, C-33), 168.3 (s, C-1), 166.4 (s, C-37), 165.9 (s, C-25), 160.8 (s, C-45), 153.6 (s, C-46), 151.3 (s, NHCOO), 150.8 (s, C-41), 148.9 (s, C-43), 148.6 (d, C-48), 144.8 (s, C-29), 143.0 (s, C-47), 141.0 (s, C-18), 140.9 (s, C-16), 139.5 (s, C-14), 133.7 (s, C-26), 132.4 (s, C-19), 132.2 (d, C-12), 129.9 (d, C-23), 129.7 (d, C-21), 129.0 (d, C-39), 128.9 (d, C-17), 128.2 (d, C-11), 128.0 (s, C-44), 127.5 (d, C-28), 127.4 (d, C-27), 126.5 (d, C-20), 124.4 (2x d, C-13, C-22), 121.3 (s, C-38), 111.2 (d, C-40), 88.3 (d, C-10), 80.1 (s, C-9), 76.0 (d, C-3), 73.4 (d, C-7), 65.8 (d, C-5), 60.5 (s, C-4), 56.2 (q, 10-OMe), 52.3 (d, C-36), 51.2 (d, C-32), 48.4 (d, C-30), 45.9 (t, C-42), 45.5 (t, C-15), 41.0 (t, C-24), 39.9 (t, C-31), 37.7 (d, C-6), 36.3 (q, NMe), 36.1 (t, C-8), 33.1 (d, C-2′), 32.4 (t, C-2), 31.9 (t, C-34), 26.6 (t, C-35), 20.2 (q, 2′-Me$_a$), 19.9 (q, 30-Me), 17.8 (q, 2′-Me$_b$), 15.3 (q, 14-Me), 14.6 (q, 6-Me), 11.5 ppm (q, 4-Me); HRMS (ESI): m/z: calcd for $C_{65}H_{76}N_{11}O_{16}S_2$ $[M-H]^-$: 1330.4913, found: 1330.4918.

Folic acid–ansamitocin conjugate 23: Pte-L-Glu-L-Cys **21** (2.5 mg, 4.52 μmol, 1.0 equiv) was suspended in water (1.2 mL), the mixture was deaerated by passing nitrogen through and adjusted to pH 7 by adding a solution of 0.1 M $NaHCO_3$. After addition of disulfide **17** (4.2 mg, 4.52 μmol, 1.0 equiv), dissolved in MeCN (1.2 mL), stirring was continued until complete conversion was detected by UPLC-MS. The reaction mix-

ture was directly purified by reversed phase HPLC (Trentec Reprosil-Pur 120 C18 AQ 5 µm, column: 250 mm × 8 mm, guard 40 mm × 8 mm; gradient 0.1 % in water TFA/MeCN 99:1 → 100 % MeCN in 55 min, then 5 min 100 % MeCN, flow rate 2.25 → 4 mL min^{-1}; t_r = 42.4 min) to yield the title compound **23** (2.6 mg, 1.91 µmol, 42 %) as a yellow solid. ^{1}H NMR (500 MHz, [D$_6$]DMSO, [D$_5$]DMSO = 2.50 ppm): δ = 8.70 (m, 1H, 24-NH), 8.66 (s, 1H, 48-H), 8.32 (m, 1H, 32-NH), 8.22 (m, 1H, 36-NH), 7.80 (d, J = 7.3 Hz, 2H, 27-H), 7.65 (d, J = 8.8 Hz, 2H, 39-H), 7.44 (m, 2H, 28-H), 7.30 (br s, 1H, 45-OH), 7.20 (s, 1H, 43-NH$_a$), 7.10 (s, 1H, 43-NH$_b$), 7.07 (s, 1H, 17-H), 6.99 (s, 1H, 38-NH), 6.90 (s, 1H, 9-NH), 6.70 (s, 1H, 21-H), 6.63 (d, J = 8.8 Hz, 2H, 40-H), 6.58 (dd, J = 15.3, 11.1 Hz, 1H, 12-H), 6.36 (m, 2H, 22-H, 23-H), 6.19 (d, J = 11.1 Hz, 1H, 13-H), 5.40 (dd, J = 15.3, 8.9 Hz, 1H, 11-H), 4.56 (dd, J = 11.9, 2.8 Hz, 1H, 3-H), 4.48 (s, 2H, 42-H), 4.44 (m, 1H, 32-H), 4.29 (m, 1H, 36-H), 4.25 (m, 1H, 30-H), 4.07 (m, 3H, 7-H, 24-H), 3.88 (s, 3H, 20-OMe), 3.49 (d, J = 8.9 Hz, 1H, 10-H), 3.48 (d, J = 12.5 Hz, 1H, 15-H$_a$), 3.23 (d, J = 12.8 Hz, 1H, 15-H$_b$), 3.22 (s, 3H, 10-OMe), 2.92 (s, 3H, NMe), 2.90 (m, 1H, 31-H$_a$), 2.75 (m, 1H, 31-H$_b$), 2.67 (d, J = 9.8 Hz, 1H, 5-H), 2.64 (sep, J = 7.0 Hz, 1H, 2′-H), 2.48 (m, 1H, 2-H$_a$), 2.25 (m, 2H, 34-H), 2.02 (m, 2H, 2-H$_b$, 35-H$_a$), 1.91 (m, 1H, 35-H$_b$), 1.62 (s, 3H, 14-Me), 1.60 (m, 3H, 30-Me), 1.43 (m, 1H, 6-H, 8-H$_a$), 1.31 (m, 1H, 8-H$_b$), 1.16 (d, J = 7.0 Hz, 3H, 2′-Me$_a$), 1.12 (d, J = 6.3 Hz, 3H, 6-Me), 1.10 (d, J = 7.0 Hz, 3H, 2′-Me$_b$), 0.81 ppm (s, 3H, 4-Me); ^{13}C NMR (125 MHz, [D$_6$]DMSO, [D$_6$]DMSO = 39.52 ppm): δ = 175.4 (s, C-1′), 173.8 (s, 36-CO$_2$H), 171.9 (s, 32-CO$_2$H), 171.8 (s, C-33), 168.2 (s, C-1), 166.3 (s, C-37), 165.8 (s, C-25), 160.7 (s, C-45), 157.9 (s, C-20), 153.5 (s, C-46), 151.2 (s, NHCOO), 150.7 (s, C-41), 148.9 (s, C-43), 148.4 (d, C-48), 144.9 (s, C-29), 142.2 (s, C-47), 142.2 (s, C-18), 140.9 (s, C-16), 139.2 (s, C-14), 133.7 (s, C-26), 132.2 (d, C-12), 132.1 (d, C-23), 129.0 (d, C-39), 128.1 (d, C-11), 127.9 (s, C-44), 127.4 (d, C-28), 127.3 (d, C-27), 124.4 (d, C-13), 121.3 (s, C-38), 121.2 (d, C-22), 120.9 (s, C-19), 120.1 (d, C-21), 112.6 (d, C-17), 111.1 (d, C-40), 88.2 (d, C-10), 80.0 (s, C-9), 76.1 (d, C-3), 73.3 (d, C-7), 65.8 (d, C-5), 60.5 (s, C-4), 56.1 (q, 10-OMe), 55.9 (q, 20-OMe), 52.1 (d, C-36), 51.2 (d, C-32), 48.6 (d, C-30), 45.9 (t, C-42), 45.8 (t, C-15), 42.2 (t, C-24), 39.9 (t, C-31), 37.7 (d, C-6), 36.0 (t, C-8), 35.8 (q, NMe), 33.0 (d, C-2′), 32.3 (t, C-2), 31.9 (t, C-34), 26.6 (t, C-35), 20.1 (q, 2′-Me$_a$), 19.8 (q, 30-Me), 17.7 (q, 2′-Me$_b$), 15.3 (q, 14-Me), 14.5 (q, 6-Me), 11.6 ppm (q, 4-Me); HRMS (ESI): m/z: calcd for $C_{66}H_{78}N_{11}O_{17}S_2$ $[M-H]^-$: 1360.5019, found: 1360.4988.

Folic acid–ansamitocin conjugate 24: Pte-L-Glu-L-Cys **21** (1.6 mg, 2.94 µmol, 1.0 equiv) was suspended in water (600 µL), the mixture was deaerated by passing nitrogen through and adjusted to pH 7 by adding a solution of 0.1 M NaHCO$_3$. After addition of disulfide **18** (2.4 mg, 2.94 µmol, 1.0 equiv), dissolved in MeCN (600 µL), stirring was continued until complete conversion was detected by UPLC-MS. The reaction mixture was directly purified by reversed phase HPLC (Trentec Reprosil-Pur 120 C18 AQ 5 µm, column: 250 mm × 8 mm, guard 40 mm × 8 mm; gradient 0.1 % in water TFA/MeCN 99:1 → 100 % MeCN in 55 min, then 5 min 100 % MeCN, flow rate 2.25 → 4 mL min^{-1}; t_r = 36.2 min) to yield the title compound **24** (2.8 mg, 2.23 µmol, 76 %) as a yellow solid. ^{1}H NMR (500 MHz, [D$_6$]DMSO, [D$_5$]DMSO = 2.50 ppm): δ = 8.68 (s, 1H, 45-H), 8.34 (d, J = 8.1 Hz, 1H, 33-NH), 8.21 (m, 2H, 24-NH, 29-NH), 7.67 (d, J = 9.0 Hz, 2H, 36-H), 7.63 (m, 1H, 20-H), 7.36 (br s, 1H, 42-OH), 7.32 (d, J = 8.1 Hz, 1H, 21-H), 7.19 (s, 1H, 43-NH$_a$), 7.09 (s, 1H, 43-NH$_b$), 7.05 (s, 1H, 17-H), 6.99 (s, 1H, 38-NH), 6.91 (s, 1H, 9-NH), 6.65 (d, J = 9.0 Hz, 2H, 37-H), 6.57 (dd, J = 15.9, 11.7 Hz, 1H, 12-H), 6.35 (d, J = 15.9 Hz, 1H, 22-H), 6.24 (ddd, J = 15.9, 6.4, 5.8 Hz, 1H, 23-H), 6.19 (d, J = 11.7 Hz, 1H, 13-H), 5.39 (dd, J = 15.9, 9.0 Hz, 1H, 11-H), 4.54 (dd, J = 12.3, 2.8 Hz, 1H, 3-H), 4.51 (s, 2H, 39-H), 4.46 (m, 1H, 33-H), 4.29 (m, 1H, 29-H), 4.08 (ddd, J = 12.0, 11.2, 1.4 Hz, 1H, 7-H), 3.85 (m, 2H, 24-H), 3.48 (d, J = 9.0 Hz, 1H, 10-H), 3.47 (d, J = 14.5 Hz, 1H, 15-H$_a$), 3.25 (d, J = 14.5 Hz, 1H, 15-H$_b$), 3.23 (s, 3H, 10-OMe), 3.10 (dd, J = 13.7, 4.6 Hz, 1H, 28-H$_a$), 2.98 (s, 3H, NMe), 2.96 (br s, 1H, OH), 2.89 (m, 3H, 27-H, 28-H$_b$), 2.66 (d, J = 9.9 Hz, 1H, 5-H), 2.63 (sep, J = 7.0 Hz, 1H, 2′-H), 2.47 (m, 3H, 31-H, 2-H$_a$), 2.24 (m, 2H, 26-H), 2.05 (m, 1H, 32-H$_a$), 2.02 (dd, J = 13.9, 2.8 Hz, 1H, 2-H$_b$), 1.91 (m, 1H, 32-H$_b$), 1.59 (s, 3H, 14-Me), 1.43 (m, 2H, 6-H, 8-H$_a$), 1.31 (m, 1H, 8-H$_b$), 1.17 (d, J = 7.0 Hz, 3H, 2′-Me$_a$), 1.10 (2x d, J = 7.0 Hz, 6H, 6-Me, 2′-Me$_b$), 0.75 ppm (s, 3H, 4-Me); ^{13}C NMR (125 MHz, [D$_6$]DMSO, [D$_6$]DMSO = 39.52 ppm): δ = 175.4 (s, C-1′), 173.8 (s, 33-CO$_2$H), 172.0 (s, 29-CO$_2$H), 171.9 (s, C-30), 169.8 (s, C-25), 168.2 (s, C-1), 166.3 (s, C-34), 160.5 (s, C-42), 153.3 (s, C-43), 151.2 (s, NHCOO), 150.7 (s, C-38), 149.4 (s, C-40), 148.4 (d, C-45), 142.0 (s, C-44), 141.0 (s, C-18), 140.8 (s, C-16), 139.4 (s, C-14), 132.4 (s, C-19), 132.2 (d, C-12), 129.9 (d, C-23), 129.7 (d, C-21), 129.0 (d, C-36), 128.9 (d, C-17), 128.1 (d, C-11), 128.0 (s, C-41), 126.6 (d, C-20), 124.4 (d, C-13), 124.2 (d, C-22), 121.4 (s, C-35), 111.2 (d, C-37), 88.3 (d, C-10), 80.1 (s, C-9), 75.9 (d, C-3), 73.4 (d, C-7), 65.8 (d, C-5), 60.5 (s, C-4), 56.1 (q, 10-OMe), 52.2 (d, C-33), 51.3 (d, C-29), 45.9 (t, C-39), 45.5 (t, C-15), 40.7 (t, C-24), 39.8 (t, C-28), 37.7 (d, C-6), 36.2 (q, NMe), 36.1 (t, C-8), 35.0 (t, C-26), 33.7 (t, C-27), 33.0 (d, C-2′), 32.3 (t, C-2), 31.9 (t, C-31), 26.6 (t, C-32), 19.8 (q, 2′-Me$_a$), 17.8 (q, 2′-Me$_b$), 15.3 (q, 14-Me), 14.5 (q, 6-Me), 11.5 ppm (q, 4-Me); HRMS (ESI): m/z: calcd for $C_{59}H_{72}N_{11}O_{16}S_2$ $[M-H]^-$: 1254.4600, found: 1254.4594.

Folic acid–ansamitocin conjugate 25: Pte-L-Glu-L-Cys **21** (2.5 mg, 4.59 µmol, 1.0 equiv) was suspended in water (900 µL), the mixture was deaerated by passing nitrogen through and adjusted to pH 7 by adding a solution of 0.1 M NaHCO$_3$. After addition of disulfide **19** (3.9 mg, 4.59 µmol, 1.0 equiv), dissolved in MeCN (900 µL), stirring was continued until complete conversion was detected by UPLC-MS. The reaction mixture was directly purified by reversed phase HPLC (Trentec Reprosil-Pur 120 C18 AQ 5 µm, column: 250 mm × 8 mm, guard 40 mm × 8 mm; gradient 0.1 % in water TFA/MeCN 99:1 → 100 % MeCN in 55 min, then 5 min 100 % MeCN, flow rate 2.25 → 4 mL min^{-1}; t_r = 32.9 min) to yield the title compound **25** (3.5 mg, 2.72 µmol, 59 %) as a yellow solid. ^{1}H NMR (500 MHz, [D$_6$]DMSO, [D$_5$]DMSO = 2.50 ppm): δ = 8.69 (s, 1H, 45-H), 8.33 (d, J = 8.1 Hz, 1H, 33-NH), 8.22 (m, 2H, 24-NH, 29-NH), 7.67 (d, J = 9.0 Hz, 2H, 36-H), 7.36 (br s, 1H, 42-OH), 7.20 (s, 1H, 43-NH$_a$), 7.10 (s, 1H, 43-NH$_b$), 7.06 (s, 1H, 17-H), 6.99 (s, 1H, 38-NH), 6.90 (s, 1H, 9-NH), 6.69 (s, 1H, 21-H), 6.64 (d, J = 9.0 Hz, 2H, 37-H), 6.58 (dd, J = 15.9, 11.7 Hz, 1H, 12-H), 6.26 (m, 2H, 22-H, 23-H), 6.19 (d, J = 11.7 Hz, 1H, 13-H), 5.39 (dd, J = 15.9, 9.0 Hz, 1H, 11-H), 4.57 (dd, J = 12.3, 2.8 Hz, 1H, 3-H), 4.51 (s, 2H, 39-H), 4.46 (m, 1H, 33-H), 4.29 (m, 1H, 29-H), 4.08 (ddd, J = 12.0, 11.2, 1.4 Hz, 1H, 7-H), 3.89 (s, 3H, 20-OMe), 3.80 (m, 2H, 24-H), 3.48 (d, J = 9.0 Hz, 1H, 10-H), 3.47 (d, J = 14.5 Hz, 1H, 15-H$_a$), 3.25 (d, J = 14.5 Hz, 1H, 15-H$_b$), 3.23 (s, 3H, 10-OMe), 3.10 (dd, J = 13.7, 4.6 Hz, 1H, 28-H$_a$), 2.92 (s, 3H, NMe), 2.89 (m, 3H, 27-H, 28-H$_b$), 2.68 (d, J = 9.9 Hz, 1H, 5-H), 2.62 (sep, J = 7.0 Hz, 1H, 2′-H), 2.47 (m, 3H, 31-H, 2-H$_a$), 2.24 (m, 2H, 26-H), 2.04 (m, 1H, 32-H$_a$), 2.02 (dd, J = 13.9, 2.8 Hz, 1H, 2-H$_b$), 1.91 (m, 1H, 32-H$_b$), 1.62 (s, 3H, 14-Me), 1.43 (m, 2H, 6-H, 8-H$_a$), 1.34 (m, 1H, 8-H$_b$), 1.16 (d, J = 7.0 Hz, 3H, 2′-Me$_a$), 1.10 (2x d, J = 7.0 Hz, 6H, 6-Me, 2′-Me$_b$), 0.80 ppm (s, 3H, 4-Me); ^{13}C NMR (125 MHz, [D$_6$]DMSO, [D$_6$]DMSO = 39.52 ppm): δ = 175.4 (s, C-1′), 173.8 (s, 33-CO$_2$H), 172.0 (s, 29-CO$_2$H), 171.7 (s, C-30), 171.4 (s, C-25), 169.7 (s, C-1), 168.2 (s, C-34), 160.4 (s, C-42), 157.9 (s, C-20), 153.3 (s, C-43), 151.2 (s, NHCOO), 150.6 (s, C-38), 149.5 (s, C-40), 148.3 (d, C-45), 142.2 (s, C-44), 142.2 (s, C-18), 140.9 (s, C-16), 139.2 (s, C-14), 132.2 (d, C-12), 132.0 (d, C-23), 129.0 (d, C-36), 128.1 (d, C-11), 128.0 (s, C-41), 124.4 (d, C-13), 121.4 (s, C-35), 120.90 (s, C-22), 120.88 (d, C-19), 120.1 (d, C-21), 112.6 (d, C-17), 111.1 (d, C-37), 88.2 (d, C-10), 80.0 (s, C-9), 76.1 (d, C-3), 73.3 (d, C-7), 65.8 (d, C-5), 60.5 (s, C-4), 56.1 (q, 10-OMe), 55.8 (q, 20-OMe), 52.1 (d, C-33), 51.2 (d, C-29), 45.9 (t, C-39), 45.8 (t, C-15), 41.5 (t, C-24), 39.9 (t, C-28), 37.7 (d, C-6), 36.0 (q, NMe), 35.8 (t, C-8), 34.9 (t, C-26), 33.6 (t, C-27), 33.0 (d, C-2′), 32.3 (t, C-2), 31.8 (t, C-31), 26.5 (t, C-32), 19.8 (q, 2′-Me$_a$), 17.7 (q, 2′-Me$_b$), 15.3 (q, 14-Me), 14.5 (q, 6-Me), 11.5 ppm (q, 4-Me); HRMS (ESI): m/z: calcd for $C_{60}H_{74}N_{11}O_{17}S_2$ $[M>M->H]^-$: 1284.4706, found: 1284.4694.

Descriptions of all experimental procedures and analytical characterization can be found in the Supporting information.

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