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Supporting Information

Genetic Engineering in Combination with Semi-Synthesis Leads to a New Route for Gram-Scale Production of the Immunosuppressive Natural Product Brasilicardin A

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1 Bacterial strains and plasmids

Table S1. Actinomycete strains used for heterologous expression of the brasilicardin BGC. The results are semi-quantitative and shown as a heat map: + (<100 mg/L); ++ (100-200 mg/L); +++ (<200 mg/L).

Host	**Integrated Construct**	**pRHAMO**	**Detected production of compounds**						
			1	**2**	**3**	**4**	**5**	**6**	**7**
Streptomyces argillaceus GLGC-AFTA[1]	bcaAB01		-	-	-	-	-	-	-
Streptomyces griseus 65[2]	bcaAB01		-	-	-	-	+++	+	-
Streptomyces lydicus	bcaAB01		-	-	-	-	++	-	-
Streptomyces olivaceus	bcaAB01		-	-	-	-	-	-	-
Streptomyces steffisburgensis	bcaAB01		-	-	-	-	-	-	-
Streptomyces sp. CS030	bcaAB01		-	-	-	-	++	-	-
Streptomyces sp. CS040[3]	bcaAB01		-	-	-	-	-	-	-
Streptomyces sp. CS044a	bcaAB01		-	-	-	-	++	-	-
Streptomyces sp. CS047	bcaAB01		-	-	-	-	-	-	-
Streptomyces sp. CS048	bcaAB01		-	-	-	-	-	-	-
Streptomyces sp. CS052a	bcaAB01		-	-	-	-	-	-	-
Streptomyces sp. CS052b	bcaAB01		-	-	-	-	-	-	-
Streptomyces sp. CS057[2]	bcaAB01		-	-	-	-	-	-	-
Streptomyces sp. CS074a	bcaAB01		-	-	-	-	-	-	-
Streptomyces sp. CS081a	bcaAB01		-	-	-	-	-	-	-
Streptomyces sp. CS084	bcaAB01		-	-	-	-	-	-	-
Streptomyces sp. CS088c	bcaAB01		-	-	-	-	-	-	-
Streptomyces sp. CS090a[2]	bcaAB01		-	-	-	-	-	-	-
Streptomyces sp. CS092[2]	bcaAB01		-	-	-	-	+++	-	-
Streptomyces sp. CS111	bcaAB01		-	-	-	-	-	-	-
Streptomyces sp. CS113[2]	bcaAB01		-	-	-	-	+++	-	-
Streptomyces sp. CS123[2]	bcaAB01		-	-	-	-	+	-	-
Streptomyces sp. CS131	bcaAB01		-	-	-	-	++	-	-
Streptomyces sp. CS134	bcaAB01		-	-	-	-	-	-	-
Streptomyces sp. CS147[2]	bcaAB01		-	-	-	-	-	-	-
Streptomyces sp. CS149[2]	bcaAB01		-	-	-	-	-	-	-
Streptomyces sp. CS170	bcaAB01		-	-	-	-	-	-	-

Table S1. continued.

Host	Integrated Construct	pRHAMO	Detected production of compounds						
			1	2	3	4	5	6	7
Streptomyces sp. CS174	bcaAB01		-	-	-	-	+	-	-
Streptomyces sp. CS180	bcaAB01		-	-	-	-	-	-	-
Streptomyces sp. CS190	bcaAB01		-	-	-	-	-	-	-
Streptomyces sp. CS215	bcaAB01		-	-	-	-	-	-	-
Streptomyces sp. CS217	bcaAB01		-	-	-	-	-	-	-
Streptomyces sp. CS227[2]	bcaAB01		-	-	-	-	-	-	-
Streptomyces coelicolor M1146	bcaAB01		-	-	-	-	+	+	-
Streptomyces coelicolor M1154	bcaAB01		-	-	-	-	+	+	-
Streptomyces lividans TK24	bcaAB01		-	-	-	-	+	+	-
Streptomyces lividans 1326	bcaAB01		-	-	-	-	+	+	-
Streptomyces albus J1074	bcaAB01		-	-	-	-	+	+	-
Streptomyces avermitilis SUKA5	bcaAB01		-	-	-	-	+	+	-
Streptomyces avermitilis SUKA22	bcaAB01		-	-	-	-	+	+	-
Streptomyces venezuelae ATCC 10712	bcaAB01		-	-	-	-	+	+	-
Streptomyces griseus 65[2]	bcaAB01	✓	-	-	+++	+	+++	+	+
Streptomyces. lydicus	bcaAB01	✓	-	-	+	-	++	-	-
Streptomyces sp. CS030	bcaAB01	✓	-	-	-	-	++	-	-
Streptomyces sp. CS044a	bcaAB01	✓	-	-	-	-	++	-	-
Streptomyces sp. CS092	bcaAB01	✓	-	-	-	-	+++	-	-
Streptomyces sp. CS113	bcaAB01	✓	-	-	-	-	+++	-	-
Streptomyces sp. CS123	bcaAB01	✓	-	-	-	-	+	-	-
Streptomyces sp. CS131	bcaAB01	✓	-	-	++	+	++	-	-
Streptomyces sp. CS174	bcaAB01	✓	-	-	-	-	+	-	-
Streptomyces griseus 65[2]	2G3	✓	-	-	++	-	+++	-	-
Streptomyces sp. CS131	2G3	✓	-	-	++	-	+++	-	-
Streptomyces sp. CS113	2G3	✓	-	-	+	-	+	-	-
Amycolatopsis tolypomicina NRRL B-24205	bcaAB01		-	-	-	-	-	-	-
Amycolatopsis tolypomicina NRRL B-12585	bcaAB01		-	-	-	-	-	-	-
Amycolatopsis mediterranea NRRL B-3240	2G3		-	-	-	-	-	-	-

Table S1. continued.

Host	Integrated Construct	pRHAMO	Detected production of compounds						
			1	2	3	4	5	6	7
Micromonospora megalomicea	bcaAB01		-	-	-	-	-	-	-
Micromonospora sp. DSM 46117	2G3		-	-	-	-	-	-	-
Arthrobacter crystallopoietes DSM 10117	2G3		-	-	-	-	-	-	-
Prauseria sp. CS074	bcaAB01		-	-	-	-	-	-	-
Saccharothrix aerocolonigenes ATCC39243	bcaAB01		-	-	-	-	-	-	-
Streptomyces coelicolor M1146	bcaAB01	✓	-	-	+	+	+	+	-
Streptomyces coelicolor M1154	bcaAB01	✓	-	-	+	+	+	+	-
Streptomyces lividans TK24	bcaAB01	✓	-	-	+	+	+	+	-
Streptomyces lividans 1326	bcaAB01	✓	-	-	+	+	+	+	-
Streptomyces albus J1074	bcaAB01	✓	-	-	+	+	+	+	-
Streptomyces avermitilis SUKA5	bcaAB01	✓	-	-	+	+	+	+	-
Streptomyces avermitilis SUKA22	bcaAB01	✓	-	-	+	+	+	+	-
Streptomyces venezuelae ATCC 10712	bcaAB01	✓	-	-	+	+	+	+	-
Streptomyces griseus 40236	bcaAB01		-	-	-	-	+	+	-
Amycolatopsis japonicum MG417-CF17	bcaAB01		-	-	+	+	+	+	-
Micromonospora sp. DSM46116	bcaAB01		-	-	+	-	-	-	-
Prauseria sp. CS172	bcaAB01		-	-	-	-	++	-	-
Actinosynnema mirum DSM438277	bcaAB01		-	-	+	-	+	-	-
Saccharopolyspora erythraea NRRL2338	2G3		-	-	-	-	-	-	-
Saccharothrix espanaensis DSM44229	2G3		-	-	+	+	-	+	-
Pseudonocardia autotrophica DSM43095	2G3		-	-	+	+	-	-	-
Rhodococcus rhodochrous IFO15564	2G3		-	-	+++	++	++	+	++
Rhodococcus erythropolis DSM43066	2G3		-	-	+++	++	+++	+	++
Rhodococcus erythropolis IFO12539	2G3		-	-	+++	++	++	++	+++
Rhodococcus jostii RHA1	2G3		-	-	++	+	+	+	+
Mycobacterium smegmatis mc2 155	2G3		-	-	-	-	-	-	-
Nocardia acidovorans DSM45049	2G3		-	-	-	-	-	-	-
Streptomyces sp. Tü2755	2G3		-	-	-	-	-	-	-

Table S2. Plasmids used in this study.

Plasmids	Characteristics	Reference
pCC1FOS™	CopyControl™ fosmid vector; Cm^R	Epicentre (USA)
pESAC13A	*E. coli-Streptomyces* Phage P1-derived Artificial Chromosome (PAC) vector, Am^R	BIO S&T (Canada)[4]
2F21	pESAC13A based PAC containing the *bra* BGC, Am^R	this study
2G3	pESAC13A based PAC containing the *bra* BGC, Am^R	this study
4G7	pCC1FOS based fosmid containing the *bra* BGC from *N. terpenica* IFM 0406, Cm^R	this study
epnLK01	Source of *int_neo*; Km^R	Schorn *et al.* [5]
bcaAB01	4G7 with Δ*cat::int_neo* (from epnLK01), Km^R, fosmid containing the *bra* BGC	this study and Schwarz *et al*.[6]
pUB307	Plasmid for triparental conjugation, *tra, oriT*; Km^R, Cm^R	Flett *et al*.[7]
pUZ8002	Plasmid for biparental conjugation Kan^R*; oriT*-; RK2 derivative	Paget *et al*.[8]
pIJ10257	*oriT,* ΦBT1 *attB*-int, Hyg^R, *ermEp** / pMS81-derived integrative overexpression vector	Hong *et al.* [9]
pRHAMO	Constructed by cloning a 1 kb XbaI-SpeI DNA fragment containing the *oriT* from pEFBAoriT (Horna *et al*., 2011)[10] into the XbaI site of pRHAM (Rodriguez *et al*., 2000)[11].	this study
pPSbra12	Heterologous Overexpression of *mva* genes, idi_{St} , *bra12, ggpps, fpps*	this study
pPSmva+idi+bra12 +ggpps_fpps	Heterologous Overexpression of *mva* genes, idi_{St} , *bra12, ggpps, fpps*	this study

2 Construction and screening of gene libraries

2.1 Construction and screening of the fosmid library

Mycelia for the fosmid library construction were prepared by growing *N. terpenica* IFM 0406 (obtained from MMRC, Japan) in 100 mL GPM medium (2% glycerol, 0.5% meat extract, 0.1% polypeptone, pH 7.0) with shaking at 200 rpm for 72 h at 30 °C. The culture was centrifuged to remove the medium (10 min; 4400 *g*; 4°C; Heraeus Multifuge 4KR) and washed twice using sterile Type 1 H_2O. High molecular-weight DNA of *N. terpenica* was isolated using the QIAGEN® Genomic DNA Isolation Kit according to the manufacturer's instructions (QIAGEN® midi-prep lysis protocol for bacteria, USA) and sheared by aspirating and expelling the DNA suspension. The DNA sample containing approximately 40 kb DNA fragments (as estimated by running the samples on a 0.7% (w/v) agarose gel in 1× TAE) was used for the preparation of the fosmid library, which was constructed using the pCC1FOS™ CopyControl™ Fosmid Library Production Kit (Epicentre, USA) following the manufacturer's instructions. Specific primer pairs binding at the upstream (F_303_bra1: 5'-GAACACCGTGGCATTTCGCC-3' and R_950_bra1: 5'-GCGAAGAACATGCGCAGGAC-3'), center (F_ggpps: 5'-CATGCATGCAC CCGCTACGCCGTGCAGCC-3' and R_ggpps: 5'-CACAAGCTTTCACCACGTGCGCCCGAT CA-3'), and downstream (F_1528_bra11: 5'-CACGCAGTGTTCCGGATAGC-3' and R_2648_bra11: 5'-CGGCCATTTCACCGATACCC-3') region of brasilicardin BGC, were designed and tested for the PCR amplification of a single specific band using genomic DNA of *N. terpenica* IFM 0406 as DNA template. The PCR was performed using the ThermoPol® PCR system (NEB, USA) with the following cycle conditions: initial denaturation 95 °C for 2 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 45-68 °C for 30 s, extension at 68 °C (1 min/kb), and final extension at 68 °C for 10 min. Each PCR product was sequenced to verify that the intended regions of the BGC were amplified (Eurofins, Germany). 960 single colonies of the transfected EPI300-T1^R cells (Epicentre, USA) were screened by PCR for clones containing the entire *bra* BGC according to the manufacturer's protocol. The fosmid DNA of the positive clone 4G7 was isolated by alkaline lysis, verified by restriction analysis, and end-sequenced (F_pCC1FOS: 5'-GGATGTGCTGCAAGGCGATTAAGTTGG-3' and R_pCC1FOS: 5'- CTCGTATGTTGTGTGGAATTGTGAGC-3'), respectively. The end-sequences were aligned with the genome of *N. terpenica* IFM 0406[12] using BLAST[13] (NCBI), which revealed an insert size of approximately 40 kb and that the fosmid 4G7 contained the entire *bra* BGC.

2.2 Construction and screening of the phage P1 artificial chromosome library

Mycelia for the phage P1 artificial chromosome (PAC) library construction were prepared by growing *N. terpenica* IFM 0406 in GPM medium (100 mL) for 72 h at 30 °C with shaking at 200 rpm. The mycelial pellets were washed twice (200 mM NaCl, 10 mM Tris-Cl (pH 8.0), 100 mM EDTA) and flash frozen in liquid nitrogen before being stored at -80 °C. The PAC library construction was performed by Bio S&T (Canada) by using a modified *E. coli* – *Streptomyces* Artificial Chromosome ESAC vector (pESAC13A)[4]. Vector DNA was digested with BamHI, dephosphorylated and purified using standard procedures. High molecular weight DNA was isolated by embedding the mycelium in 1 % low-melting-point agarose plugs that were treated with proteinase K overnight, and then partially-digested with Sau3AI. The partially digested high molecular weight DNA was size-fractionated on a 1 % (w/v) pulsed field agarose gel in 0.5× TBE using a CHEF DRIII (Bio-Rad, Canada). The 100-250 kb DNA fragments were eluted from the gel by PFGE and dialyzed against 1× TE (10 mM Tris-Cl (pH 8.0), 1 mM EDTA,) prior to ligation with the vector. The ligation mix was used to transform *E. coli* DH10B cells (Invitrogen). Transformants were selected on LB agar containing Am incubated at 37 °C. For quality control, the insert size was determined by DraI digestion of mini-prepped PAC-DNA and PFGE gel separation.

Specific primer pairs binding at the upstream (F_54_bca: 5’-TGGATCTGGCCGCCGACAGCATCC-3’ and R_413_bca: 5’-GGTGCAGTTCGGTCATGGCGGGAC-3’), center (F_10276_bca: 5’-GACCCCACGATCCGTCCGAGTGCT-3’ and R_10694_bca: 5’-GGAACTGCTGTCGGCG CTGGCGAA-3’) and downstream (F_21361_bca: 5’-GTGTTCTCCCAGGCCGGAATCGGC-3’ and R_21871_bca: 5’-CGTGACCGAGGGTGTCGTTGACGG-3’) region of the brasilicardin BGC, respectively, were designed and tested as described above. Two PAC clones, 2F21 and 2G3, identified by PCR screening as likely to contain the entire *bra* BGC, were end-sequenced (F_pESAC13A: 5’-CGCTAATACGACTCACTATAGGGAGA-3’ and R_pESAC13A: 5’-GCCGT CGACATTTAGGTGACACTATA-3’), respectively. The end-sequences were aligned with the genome of *N. terpenica* IFM 0406[12] using BLAST[13] (NCBI), which revealed insert sizes of 81,168 bp and 81,611 bp, respectively.

3 Heterologous expression

3.1 Media and culture conditions

E. coli strains were grown at 37°C in Luria broth medium (LB)[14]. To detect brasilicardin production, the heterologous hosts were inoculated in 50 mL tryptic soy broth medium (TSB) preculture for 48 h, followed by a 50 mL main culture in different media depending on the strain.

Non-optimized *Nocardia* medium (NM): glycerol, 2 g/L; polypeptone, 1 g/L; meat extract, 0.5 g/L; distilled water; pH 7.

Liquid cultures of *A. japonicum* MG417-CF17, *S. griseus* 65 and *R. erythropolis* IFO12539 were carried out in an orbital shaker (220 rpm) in 500-mL baffled Erlenmeyer flasks at 27°C. When required, antibiotics were added to media at the following final concentrations: ampicillin (100 μg/mL), kanamycin (50 μg/mL), nalidixic acid (25 μg/mL), apramycin (25 μg/mL), and thiostrepton (50 μg/mL).

For *S. griseus* 65 we used optimized medium 1 (OM1): Soy protein, 10 g/L; glucose, 50 g/L; peptone, 4 g/L; meat extract, 4 g/L; yeast extract, 1 g/L; NaCl, 2.5 g/L; $CaCO_3$, 5 g/L; tap water; pH 7.6.

For *A. japonicum* MG417-CF17 we used OM2: glucose, 2 g/L; glycerol, 40 g/L; starch, 2 g/L; soy protein, 5 g/L; bactopeptone 5 g/L; yeast extract, 5 g/L; NaCl, 5 g/L; $CaCO_3$, 2 g/L; tap water; pH 6.4.

For *R. erythropolis* IFO12539 we used OM3: bactopeptone, 10 g/L; malt extract, 21 g/L; glycerol, 40 g/L; distilled water; pH 6.4.

3.2 Nucleotide sequence accession number

The GenBank accession number of the genome sequence of *N. terpenica* IFM 0406 is LWGR00000000.1,[12] of *A. japonicum* is CP008953,[15] and of *S. griseus* 65 is QBHW01000026.1. The brasilicardin BGC sequence GenBank accession number is MT247069. Furthermore, the brasilicardin BGC has been deposited in the Minimum Information about a Biosynthetic Gene Cluster (MIBiG) repository under accession number BGC0000632.

3.3 Construction of bcaAB01

A 6,646 bp XbaI restriction fragment from epnLK01[5] was generated representing an integration cassette (int_neo) for stable chromosomal integration in *Streptomyces*. int_neo was

used to replace *cat* in fosmid 4G7 using *E. coli* BW25113/pKD20[16] as described elsewhere.[17] The resulting fosmid bcaAB01 was verified by restriction and sequence analysis.

3.4 Construction of pRHAMO

Plasmid pRHAMO was constructed by cloning a 1 kb XbaI-SpeI DNA fragment containing the *oriT* from pEFBAoriT[10] into the XbaI site of pRHAM.[11]

3.5 Construction of the integrative expression vectors derived from pIJ10257

The construction of pPSbra12 and pPSmva+idi+bra12+ggpps_fpps was performed as described in Schwarz *et al*.[6]

3.6 Intergeneric conjugation procedure

For conjugation of *Streptomyces griseus* 65, *Rhodococcus erythropolis* IFO12539 and all other actinomycetes listed in this study, the *Streptomyces* conjugation protocol described by Kieser *et. al*[18] was used.

For conjugation of *A. japonicum*, the intergeneric conjugation procedure with *A. japonicum* spores and *E. coli* ET12567_pUZ8002[19] was modified. *E. coli* ET12567_pUZ8002 is a methylation deficient strain carrying the genes required for plasmid transfer (*tra* genes) on the plasmid pUZ8002.[20] *E. coli* ET12567_pUZ8002 was transformed with the plasmid that was intended to be transferred into *A. japonicum*. 500 µL overnight culture of the transformed *E. coli* ET12567_pUZ8002 was used to inoculate 50 mL LB without antibiotics. The culture was grown to an OD600 of 0.6 and used for conjugation as described by Stegmann *et al*.[19] Successful integration of bcaAB01 and PAC clones into the genome of actinomycetes was confirmed by PCR using 2 µL pre-culture as PCR template.

3.7 Detection and quantification of brasilicardin congeners by HPLC/MS

Culture samples (1 mL) were extracted with 1 volume of n-butanol. Organic extracts were dried under vacuum, and residues were dissolved in 100 µl of DMSO: methanol (50:50). HPLC/MS analysis was performed with an Agilent 1200 HPLC series coupled with an Ultra Trap System XCT 6330 (Agilent, Waldbronn, Germany). Samples (2.5 µL) were injected on a 3 µm Nucleosil 100 C18 column (100 x 2 mm, fitted with a precolumn 10 x 2 mm, Dr. Maisch HPLC GmbH, Ammerbuch, Germany) and separated with eluent A 0.1% formic acid in water and eluent B 0.06% formic acid in acetonitrile by gradient elution (20%-50% B over 10 min followed by 50%-100% B over 5 min) at a flow rate of 400 µL/min. Detection was carried out

at 220, 240, 300, 360 and 435 nm. Electrospray ionization in ultra scan mode (positive and negative, alternating) was done with a capillary voltage of 3.5 kV and a drying gas temperature of 350°C.[6b] Detection of m/z values was conducted with Agilent DataAnalysis for 6300 Series Ion Trap LC/MS Version 3.4 (Bruker Daltonik GmbH, Billerica, USA). MS^2 and MS^3 analysis were performed in positive mode under the same conditions. To determine the concentration of brasilicardin congeners produced isolated brasilicardin C (BraC) and brasilicardin E (BraE) ranging from 1 to 1000 mg/mL in 10 dilution steps were used to generate a standard curve in HPLC/MS. The concentration of brasilicardin congeners was then determined by analysing the peak height of the MS spectrum in regards to the standard curve.

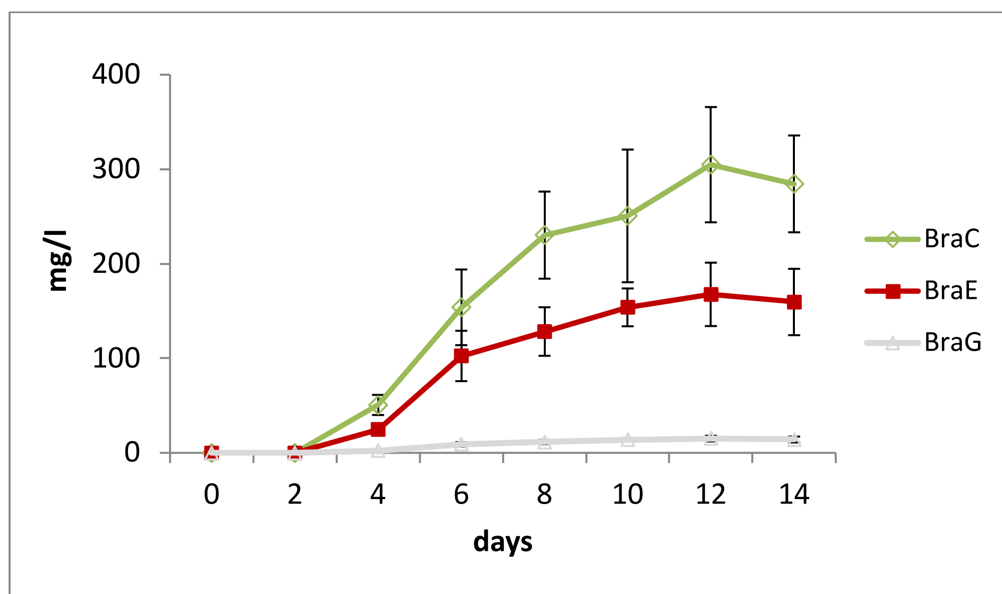


Figure S1. Quantification of brasilicardin BraC, BraE and BraG production in *S. griseus*::bcaAB01.

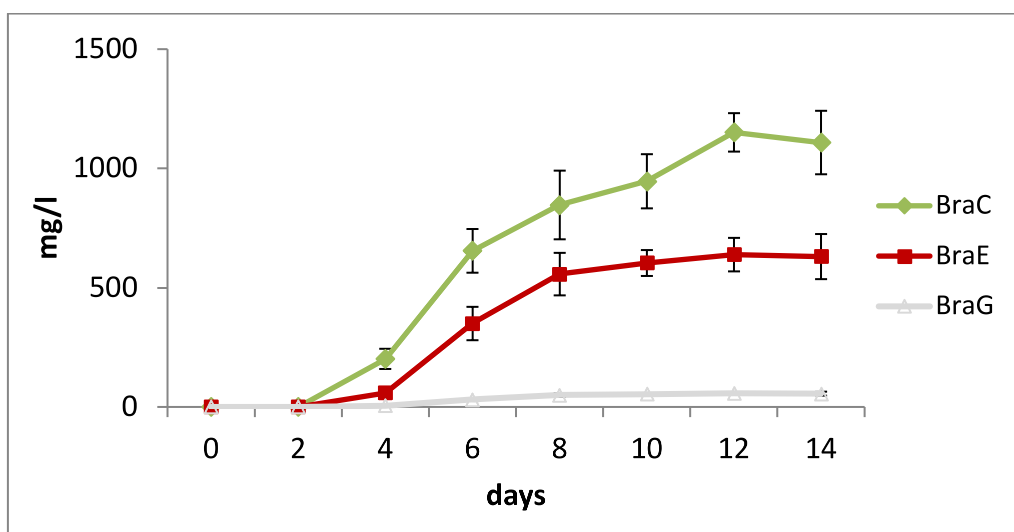


Figure S2. Quantification of *S. griseus*::bcaAB01 production yields during 14 days of cultivation in the optimized medium.

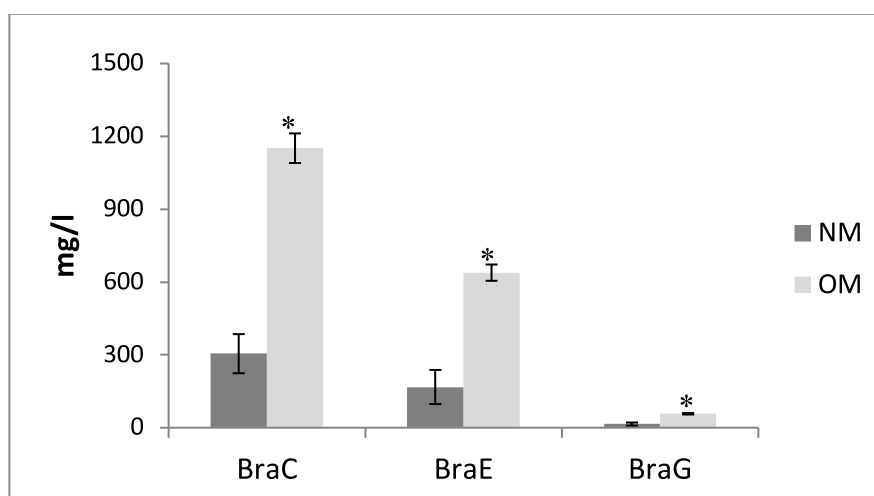
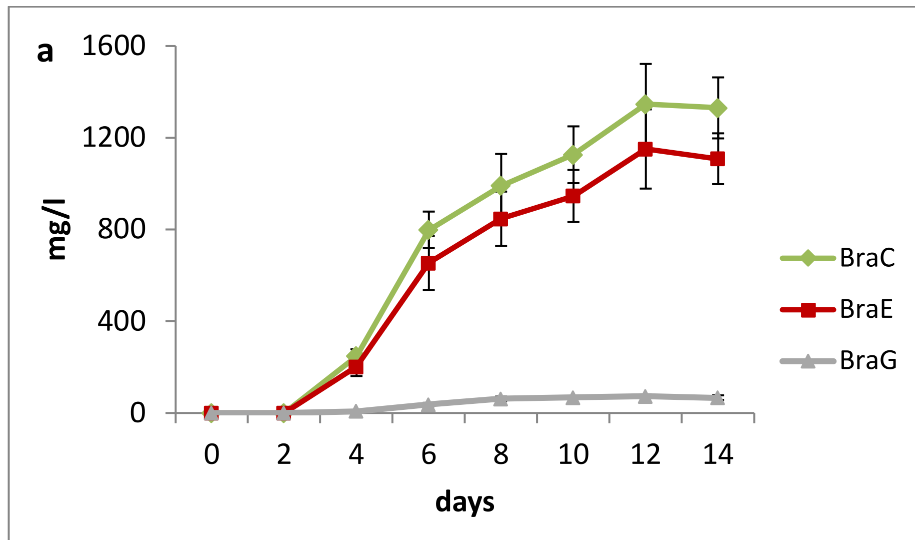


Figure S3. Quantification of brasilicardin congener's production of *S. griseus*::bcaAB01 after 12 days of cultivation in the optimized media (OM) and the non-optimized medium (NM). Significance was calculated in comparison to NM media production yields. Mann-Whitney test; (**: $P \leq 0.01$, *: $P \leq 0.05$). n=6.



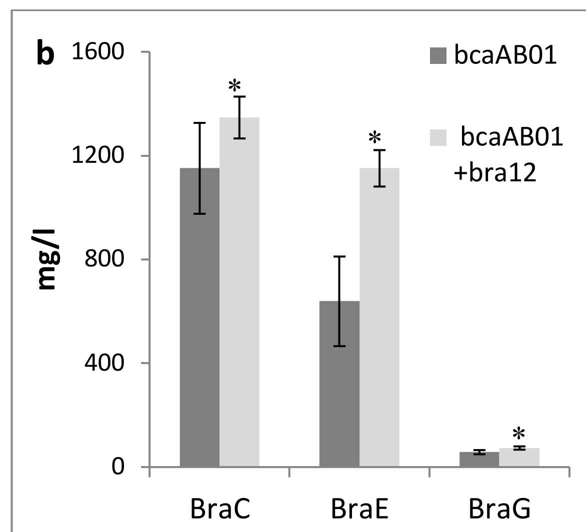
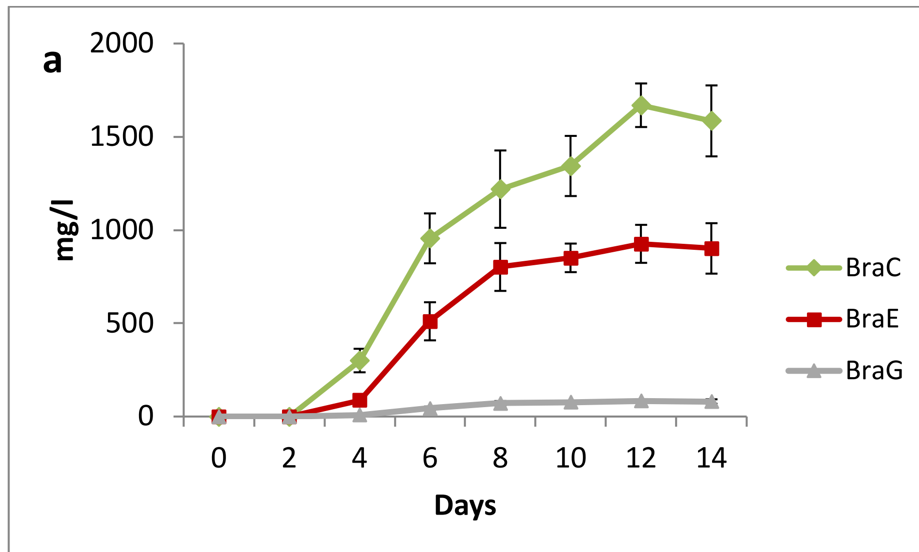


Figure S4. Quantification of the *bra12* influence on brasilicardin congener's production a) *S. griseus*::bcaAB01_*bra12*. and b) *S. griseus*::bcaAB01 / *S. griseus*::bcaAB01_*bra12* after 12 days of cultivation. Significance was calculated in comparison to *S. griseus*::bcaAB01. Mann-Whitney test; (**: $P \leq 0.01$, *: $P \leq 0.05$). n=6.



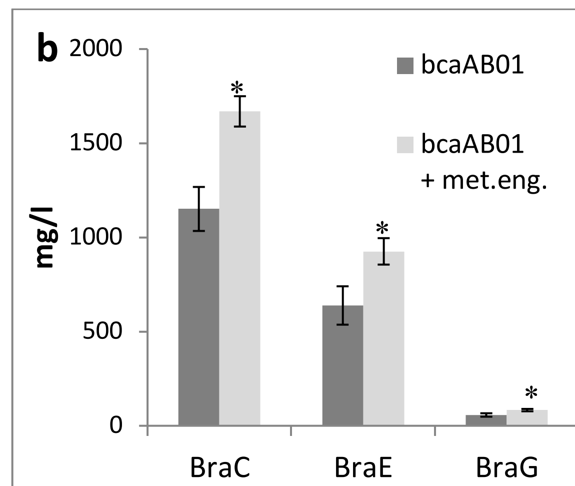


Figure S5. Quantification of metabolic engineering on brasilicardin congener's production in a) *S. griseus*::bcaAB01_met.eng. and b) *S. griseus*::bcaAB01 / *S. griseus*::bcaAB01_met.eng. after 12 days of cultivation. Significance was calculated in comparison to *S. griseus*::bcaAB01. Mann-Whitney test; (**: $P \leq 0.01$, *: $P \leq 0.05$). n=6.

4 Chemical characterization of brasilicardins C, E and G

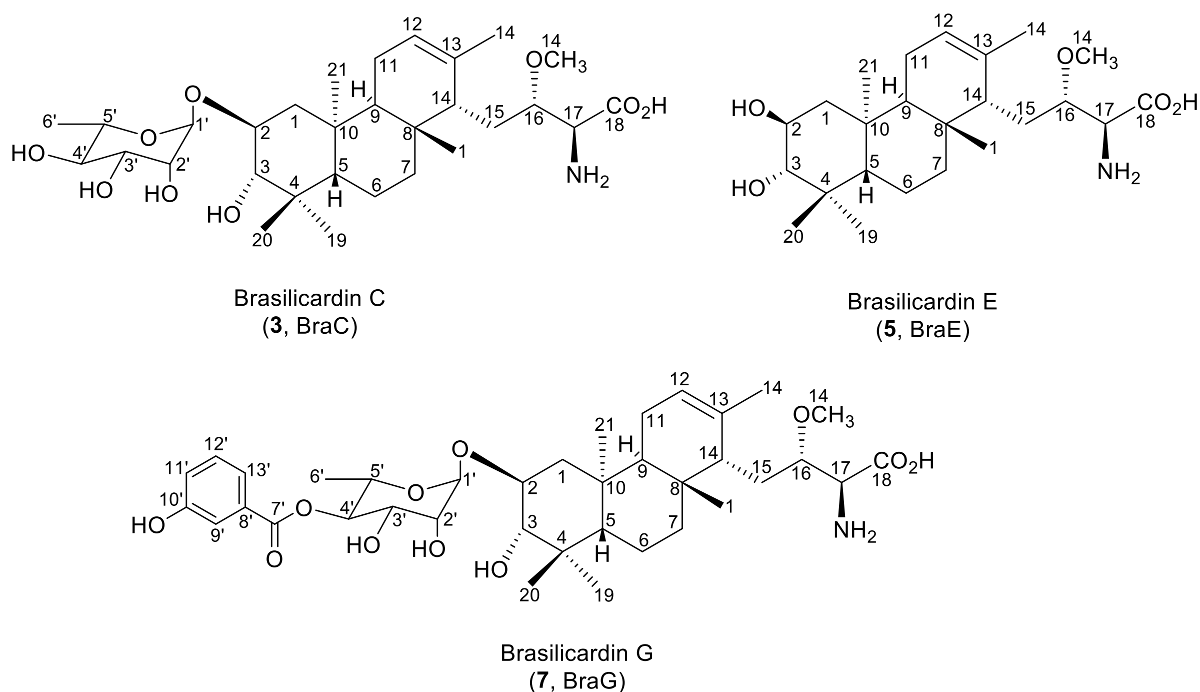


Figure S6: Structures of brasilicardin C (**3**), E (**5**) and G (**7**).

4.1 Purification of brasilicardin C, brasilicardin E and brasilicardin G

For purification purposes of brasilicardin aglycon (BraE) and brasilicardin C (BraC), *S. griseus* bcaAB01 (pRHAMO) was grown by a two-step culture method, as previously described[21] and under non-optimized conditions. In the production step, five 2-liter Erlenmeyer flasks, each containing medium (400 mL), were incubated for 15 days. The cultures were centrifuged and filtered, and applied to a solid-phase extraction cartridge (Sep-Pak Vac C18, 10 g, Waters). The retained material was eluted with a mixture of methanol and 0.1% TFA in water. A linear gradient from 0 to 100% methanol in 55 min, at 5 mL/min, was used. Fractions were taken every 5 min, and analyzed by HPLC-MS. Fractions containing the desired compounds were evaporated in vacuum, and resolved in a small volume of a mixture of DMSO and methanol (50:50). Products were purified by preparative HPLC using a SunFire C18 column (10 µm, 10x150 mm, Waters). A second gradient from 30 to 100% acetonitrile in 5.5 min, at 5 mL/min was used. Fractions containing the desired compounds were evaporated *in vacuo*, and dissolved in a small volume of a mixture of DMSO and methanol (50:50). Compounds were chromatographed with mixtures of acetonitrile and 0.1% TFA in water, in isocratic conditions optimized for each compound, at 5 mL/min. 27 mg of BraE and 54 mg of BraC were obtained.

For the isolation of brasilicardin G (BraG) seed cultures of *R. erythropolis* IFO12539::2G3 were grown in 250 mL Erlenmeyer flasks containing 50 mL of TSB for 2 days at 30°C and 250 rpm. These cultures were used to inoculate two 2L Erlenmeyer flasks containing 500 mL of SM25 medium (10 g/L peptone; 21 g/L malt extract; 40 g/L glycerol, pH 6.5) using 2% inoculum and were grown for 8 days at 30°C. The full cultures were extracted twice with equal volumes of n-butanol and dried down. The extract was resuspended in water and loaded into a Waters-C18 Sep-Pak Column (10 g) cartridge and eluted with a 10% step gradient of methanol. Fractions corresponding to 70% and 80% methanol were pooled, dried down and resuspended in water. The extract was loaded into a semi-preparative Agilent Technologies HPLC 1200 System using an Eclipse XDB-C18, RR 9.4 x 250 mm, 5 µm Agilent column. The sample was resolved using an isocratic program with 55% of A (0.1% TFA in water) and 45% of B (Acetonitrile) and 40°C temperature column. The fractions were collected in 0.1M potassium phosphate buffer and analyzed by HPLC, pooled and lyophilized. The extract was resuspended in water and desalted using a Waters-C18 Sep-Pak Column (1g). BraG was eluted with 100% methanol and lyophilized. The obtained amount of BraG was 14 mg and was subsequently used for NMR analysis and activity studies.

4.2 Analysis of brasilicardin C, brasilicardin E and brasilicardin G

LC-DAD-HRMS analyses were carried out on an Agilent 1200 Rapid Resolution HPLC system coupled to a Bruker maXis mass spectrometer. For the NMR analyses samples were dissolved in CD_3OD/DMSO-d_6 (ca. 4:1) and transferred to a 1.7 mm tube. Acquisitions were carried out on a Bruker AVANCE III 500 MHz spectrometer equipped with a 1.7 mm TCI Microcryoprobe. All spectra were registered at 24 °C.

Brasilicardin C (3). The HRMS information rendered a molecular formula of $C_{30}H_{51}NO_9$ based on the observed ion $[M+H]^+$ at 570.3643 (calcd. for $C_{30}H_{52}NO_9^+$ = 570.3637). The ^{1}H NMR and the HSQC spectra (Figures S7 and S8) revealed the identity of the compound which corresponds to brasilicardin C. The NMR data perfectly matched those reported for this compound by Komatsu *et al*.[22]

Brasilicardin E (5). The HRMS information rendered a molecular formula of $C_{24}H_{41}NO_5$ based on the observed ion $[M+H]^+$ at 424.3065 (calcd. for $C_{24}H_{42}NO_5^+$ = 424.3058). The ^{1}H NMR and the HSQC spectra (Figures S9 and S10) revealed the identity of the compound. Comparison with the spectra obtained for brasilicardin C (Figure S11) clearly shows that the compound lacks the deoxysugar moiety and it corresponds to the aglycon of brasilicardin.

Brasilicardin G (7). The HRMS information rendered a molecular formula of $C_{37}H_{55}NO_{11}$ based on the observed ion $[M+H]^+$ at 690.3847 (calcd. for $C_{37}H_{56}NO_{11}^+$ = 690.3848). The ^{1}H NMR, and the HSQC spectra (Figures S12 and S13) revealed the identity of the compound.

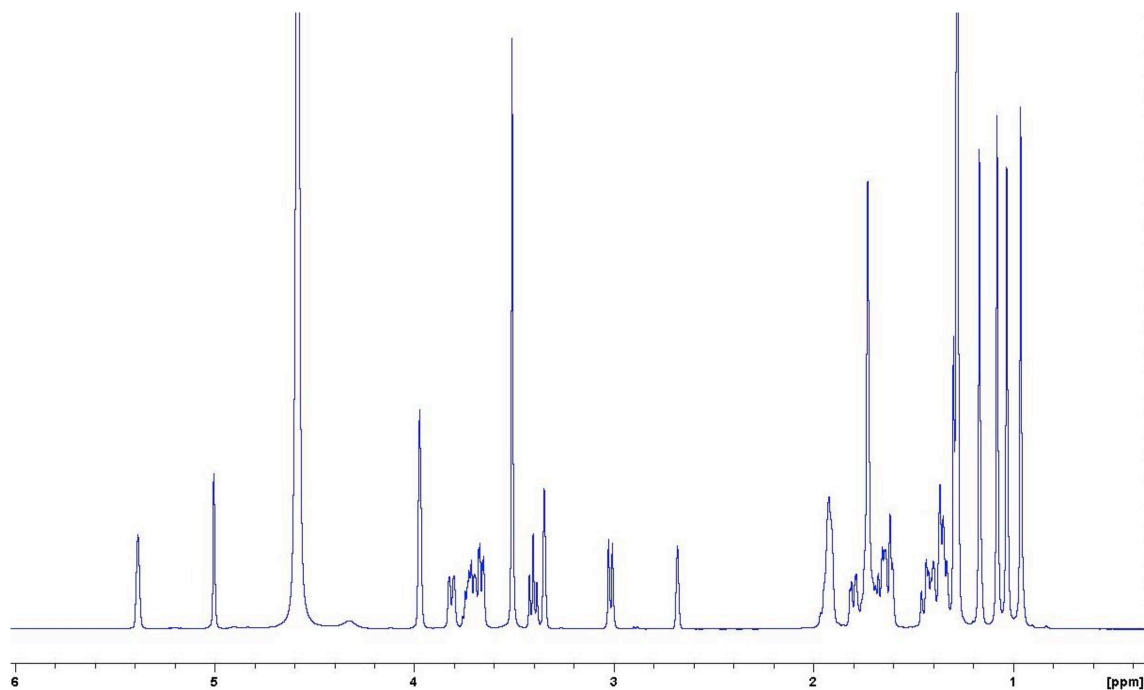


Figure S7. ^{1}H NMR spectrum of brasilicardin C (CD$_3$OD/DMSO-d_6, 500 MHz).

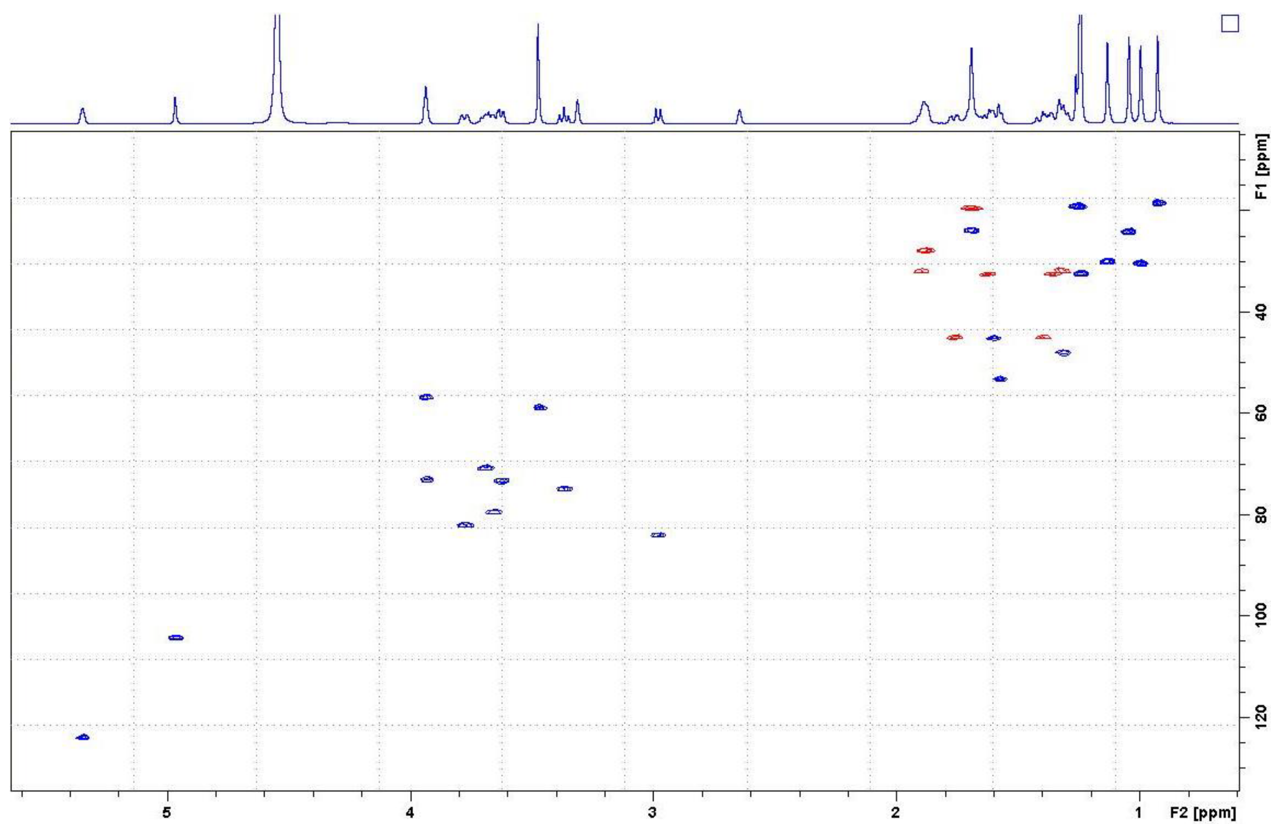


Figure S8. ^{1}H-^{13}C-HSQC NMR spectrum of brasilicardin C (CD_3OD/DMSO-d_6, 500 MHz).

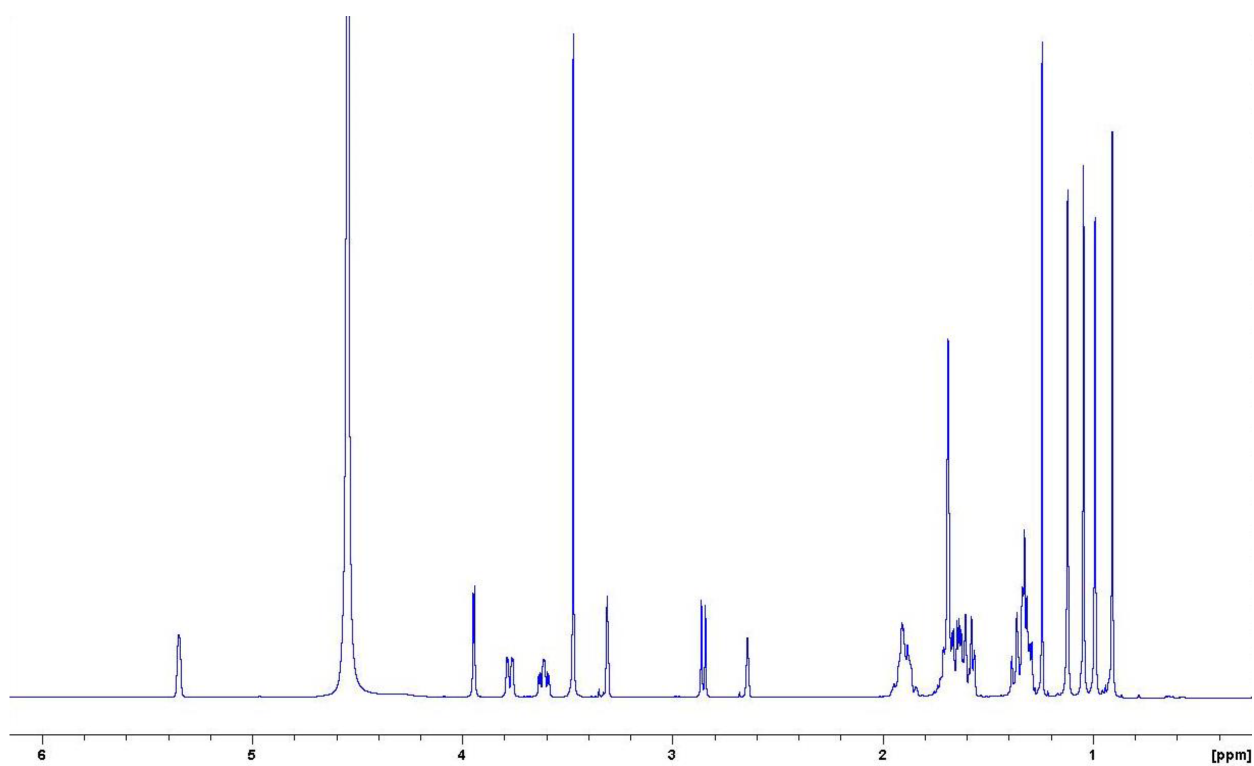


Figure S9. ^{1}H NMR spectrum of brasilicardin E (CD_3OD/DMSO-d_6, 500 MHz).

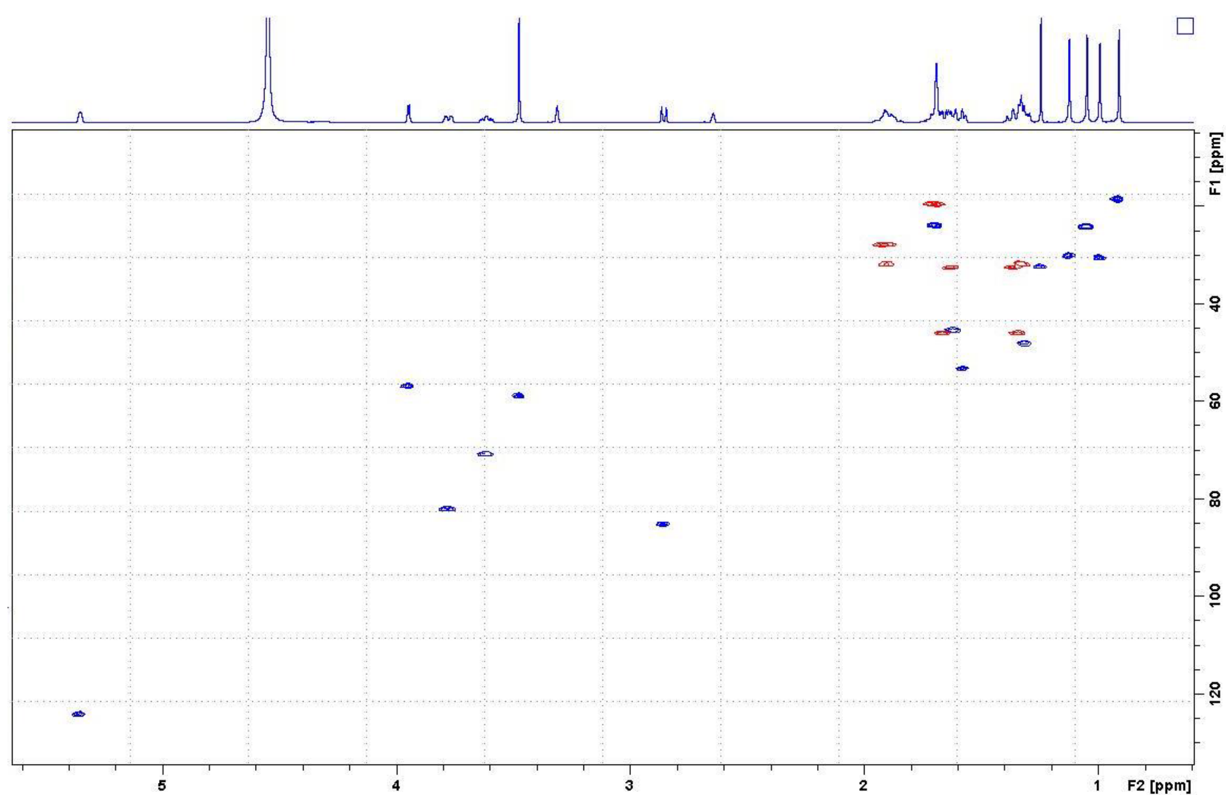


Figure S10. ^{1}H-^{13}C-HSQC NMR spectrum of brasilicardin E (CD_3OD/DMSO-d_6, 500 MHz).

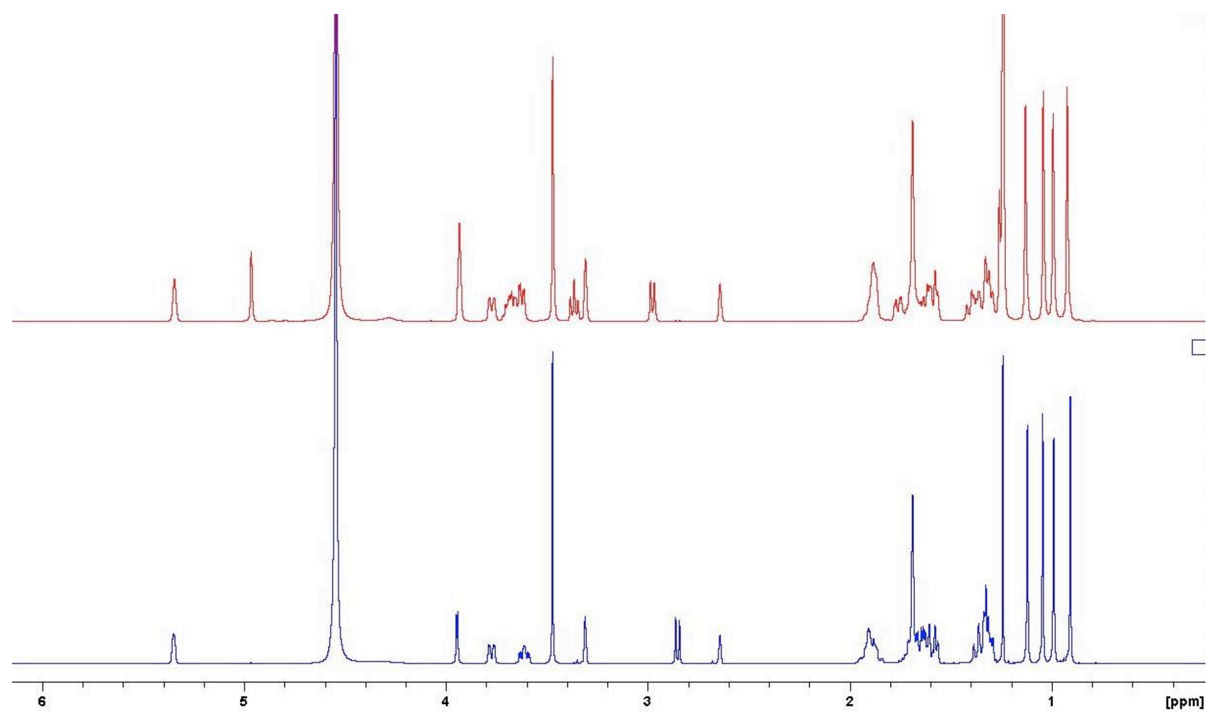


Figure S11. Comparison of the ^{1}H NMR spectra of brasilicardin C (red) and brasilicardin E (blue).

Table S3. NMR data of brasilicardin C (**3**) and brasilicardin E (**5**).

Position	Brasilicardin C		Brasilicardin E	
	^{1}H NMR	^{13}C NMR	^{1}H NMR	^{13}C NMR
	δ_H, m (*J* in Hz)	δ_C, type	δ_H, m (*J* in Hz)	δ_C, type
1	a 1.76, dd (12.5, 3.6)	45.1, CH_2	a 1.66, dd (12.0, 3.5)	45.9, CH_2
	b 1.40, t (12.3)		b 1.34, m	
2	3.65, m	79.5, CH	3.61, ddd (11.1, 10.4, 4.2)	70.8, CH
3	2.98, d (9.5)	84.0, CH	2.85, d (9.5)	85.2, CH
4	-	42.1, C	-	41.8, C
5	1.60, m	45.2, CH	1.61, m	45.3, CH
6	1.69, m	19.6, CH_2	1.69, m	19.6, CH_2
7	a 1.90, m	32.0, CH_2	a 1.90, m	31.9, CH_2
	b 1.31, m		b 1.31, m	
8	-	39.3, C	-	39.3, C
9	1.31, m	48.0, CH	1.31, m	48.0, CH
10	-	38.4, C	-	38.4, C
11	1.88, m	27.9, CH_2	1.90, m	27.9, CH_2
12	5.35, m	124.0, CH	5.35, m	124.0, CH
13	-	139.9, C	-	139.9, C
14	1.57, m	53.2, CH	1.57, m	53.2, CH
15	A 1.63, m	32.6, CH_2	A 1.63, m	32.6, CH_2
	b 1.36, m		b 1.36, m	
16	3.77, dd (11.2, 2.3)	82.1, CH	3.77, dd (11.2, 2.3)	82.1, CH
17	3.94, m	56.9, CH	3.95, m	56.9, CH
18	-	171.9, C	-	171.9, C
19	0.92, s	18.5, CH_3	0.91, s	18.5, CH_3
20	0.99, s	30.4, CH_3	0.99, s	30.5, CH_3
21	1.13, s	30.0, CH_3	1.12, s	30.1, CH_3
22	1.04, s	24.2, CH_3	1.04, s	24.2, CH_3
23	1.69, s	23.9, CH_3	1.69, s	23.9, CH_3
OCH_3	3.47, s	58.9, CH_3	3.47, s	58.9, CH_3
1’	4.97, br s	104.3, CH		
2’	3.93, m	73.0, CH		
3’	3.63, dd (9.5, 3.1)	73.3, CH		
4’	3.37, t (9.3)	74.9, CH		
5’	3.69, m	70.7, CH		
6’	1.25, d (6.4)	19.2, CH_3		

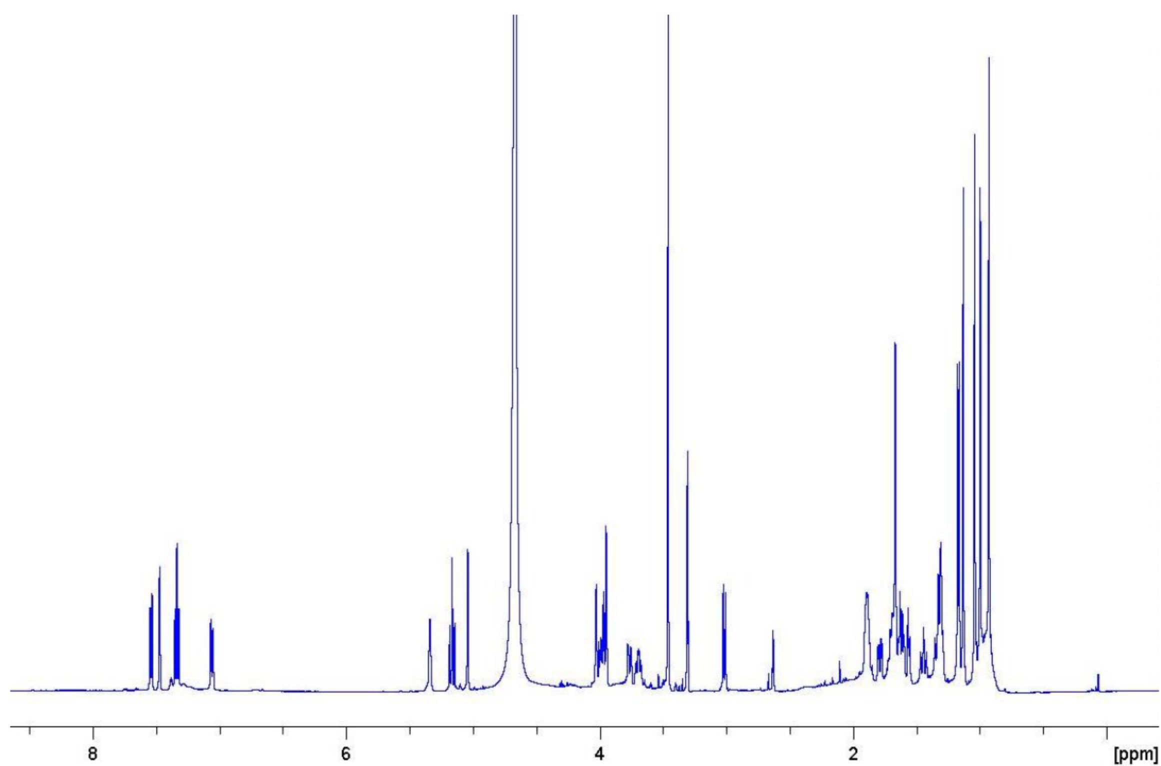


Figure S12. ^{1}H NMR spectrum of brasilicardin G (CD_3OD/DMSO-d_6, 500 MHz).

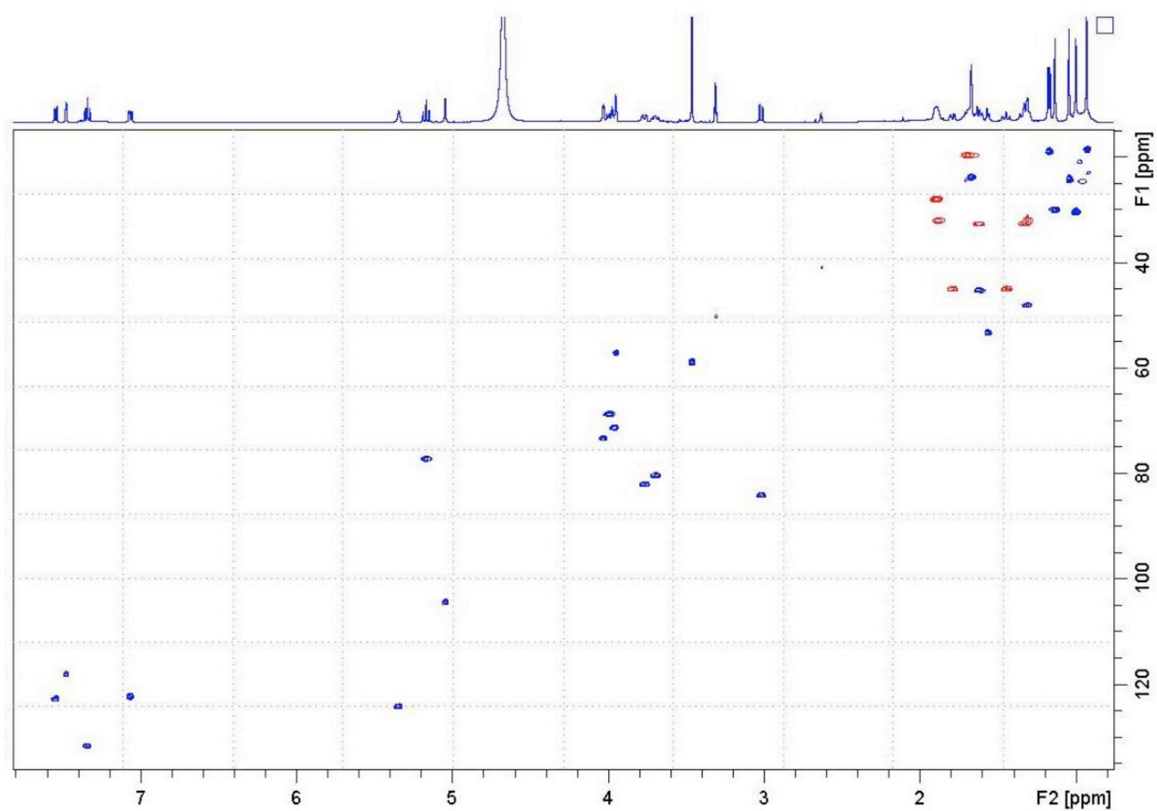


Figure S13. ^{1}H-^{13}C-HSQC NMR spectrum of brasilicardin G (CD_3OD/DMSO-d_6, 500 MHz).

Table S4. NMR data of brasilicardin G (**7**).

Position	^{1}H NMR δ$_H$, m (*J* in Hz)	^{13}C NMR δ$_C$, type	Position	^{1}H NMR δ$_H$, m (*J* in Hz)	^{13}C NMR δ$_C$, type
1	a 1.79, dd (12.1, 4.2) b 1.45, t (12.0)	45.1, CH_2	20	1.00, s	30.4, CH_3
2	3.70, ddd (11.3, 10.0, 4.3)	80.4, CH	21	1.13, s	30.0, CH_3
3	3.02, d (9.6)	84.2, CH	22	1.04, s	24.2, CH_3
4	-	42.2, C	23	1.67, br s	23.8, CH_3
5	1.62, m	45.3, CH	OCH_3	3.46, s	58.9, CH_3
6	1.69, m	19.6, CH_2	1’	5.04, d (1.3)	104.4, CH
7	a 1.89, m b 1.31, m	32.1, CH_2	2’	4.03, dd (3.2, 1.7)	73.4, CH
8	-	39.4, C	3’	3.96, m	71.4, CH
9	1.31, m	48.1, CH	4’	5.17, t (9.7)	77.2, CH
10	-	38.5, C	5’	3.69, dq, (9.7, 6.3)	68.8, CH
11	1.89, m	28.0, CH_2	6’	1.17, d (6.3)	19.0, CH_3
12	5.34, m	124.2, CH	7’	-	168.6, C
13	-	139.9, C	8’	-	133.8, C
14	1.56, (br d, 6.8)	53.3, CH	9’	7.47, dd (2.4, 1.5)	118.2, CH
15	A 1.62, m b 1.33, m	32.7, CH_2	10’	-	159.8, C
16	3.77, dd (11.2, 2.9)	82.1, CH	11’	7.06, ddd (8.1, 2.6, 0.9)	122.4, CH
17	3.95, m	57.1, CH	12’	7.34, dd (7.9, 7.9)	131.7, CH
18	-	172.2, C	13’	7.54, dt (7.8, 1.2)	122.7, CH
19	0.93, s	18.4, CH_3			

4.3 Structure determination of brasilicardin E (5).

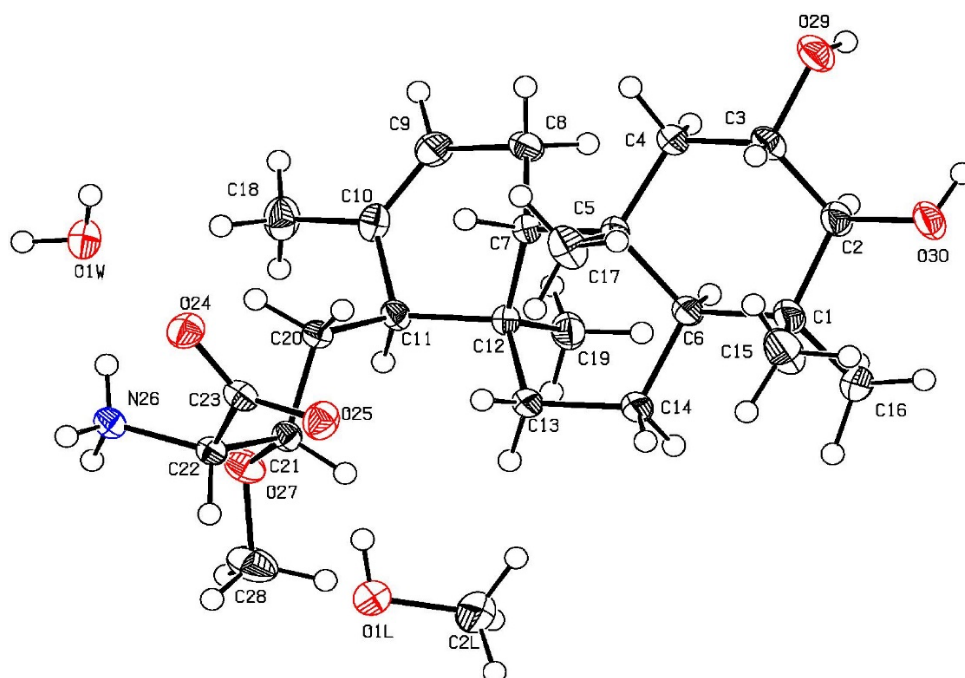


Figure S14. Crystal structure of compound **5**. For data for atomic coordinates, thermal parameters and reflections, see Eitel *et al*.[23]

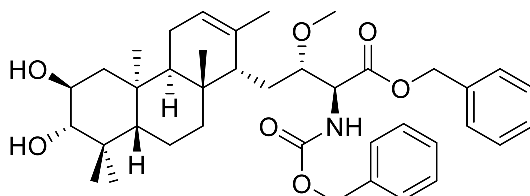
5 Semi-synthesis

5.1 General

All reagents and solvents were of commercial quality and utilized without further purification. Thin layer chromatography (TLC) reaction controls were performed for all reactions using fluorescent silica gel 60 F_{254} plates (Merck) and visualized using a 254 nm UV lamp and/or by treatment with H_2SO_4 / EtOH and/or ninhydrin / EtOH followed by heating. High performance liquid chromatography (HPLC) was performed at an Agilent 1100 Series HPLC system equipped with a UV DAD (detection at 218 nm, 254 nm and 280 nm) using a XBridge™ C18 column (150 mm x 4.6 mm, 5 µm) at 30 °C oven temperature. The injection volume was 10 µL and the flow 1.5 mL/min using the following gradients: 0.01 M KH_2PO_4, pH = 2.3 (solvent A), MeOH (solvent B), Method 1: 45 % B to 85 % B in 9 min; 85 % B for 6 min, Method 2: 55 % B to 95 % B in 10 min; 95 % B for 5 min. Column chromatography was performed on Davisil LC60A 20 – 45 µm silica from Grace Davison and Geduran Si60 63 – 200 µm silica from Merck for the pre-column using an Interchim PuriFlash 430 automated flash chromatography system. Preparative HPLC was performed at an Interchim PuriFlash 4250 equipped with an iQuad HPLC pump using a ReproSil®-XR 120 C18 column (250 mm x 30 mm, 5 µm) from Dr. Maisch-GmbH. Reversed phase chromatography (RPLC) was performed using Polygoprep™ C18 from Macherey-Nagel™. Nuclear magnetic resonance (NMR) spectra were measured on a Bruker Avance III HD NMR spectrometer at 400 MHz. Chemical shifts are reported in parts per million (ppm) relative to tetramethylsilane. All spectra were calibrated against the (residual proton) peak of the deuterated solvent used ($CDCl_3$ at 7.26 ppm for ^1H and 77.06 ppm for ^{13}C, CD_3OD at 3.31 ppm for ^1H and 49.03 ppm for ^{13}C). Mass spectra were performed on an Advion Expression S electrospray ionization mass spectrometer (ESI-MS) with TLC interface. High resolution mass spectra (HRMS) were recorded on a Bruker Daltonics Maxis 4G electrospray time of flight mass spectrometer (ESI-TOF-MS).

5.2 Experimental procedures

Benzyl (2*S*,3*S*)-2-(((benzyloxy)carbonyl)amino)-4-((1*S*,4a*S*,4b*S*,6*S*,7*S*,8a*S*,10a*S*)-6,7-dihydroxy-2,4b,8,8,10a-pentamethyl-1,4,4a,4b,5,6,7,8,8a,9,10,10a-dodecahydrophenanthren-1-yl)-3-methoxybutanoate (8)

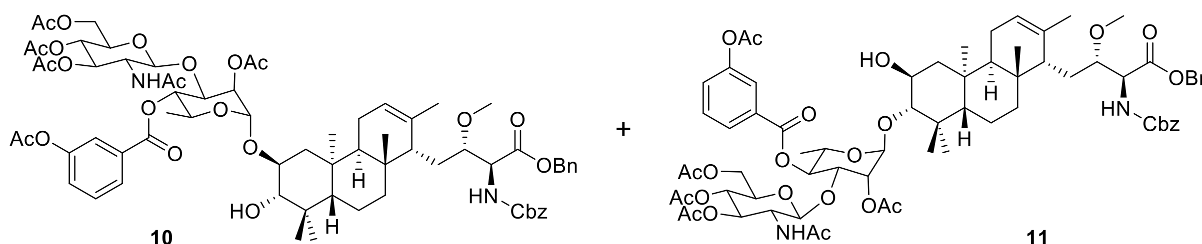


To an ice cooled suspension of aglycone **5** (85 mg, 201 µmol) in THF (3.5 mL) a solution of NaOH (16 mg, 402 µmol) in water (2.5 mL) and subsequently a solution of CbzCl (34.4 µL, 241 µmol) in THF (0.95 mL) was added slowly and the reaction mixture was stirred at rt for 3 h. The reaction mixture was acidified by 0.05 M aqueous HCl solution to pH = 2 and the crude product was extracted 5 times with EtOAc (50 mL), dried over Na_2SO_4 and concentrated by evaporation *in vacuo* to give the Cbz-protected aglycone as a colorless solid (100 mg). This compound and Cs_2CO_3 (69 mg, 211 µmol) was dissolved in DMF (5 mL) and stirred at rt. After 30 min benzyl bromide (28.5 µL, 241 µmol) was added slowly and the reaction mixture was stirred for 15 h at rt. The solution was diluted with EtOAc (60 mL), washed with sat. aq. $NaHCO_3$ sol. (20 mL), water (20 mL) and brine (20 mL). After the organic layer was dried over anhydrous Na_2SO_4, the solvent was removed under reduced pressure and the residue was purified by flash chromatography (SiO_2, n-hexane / EtOH: 100 / 00 to 80 / 20) to give the protected aglycone **8** (76 mg, 58 % over 2 steps) as a white solid. TLC: R_f = 0.85 (nHex / EtOAc: 1 / 5). HPLC (method 1): 11.3 min. HRMS (ESI): calcd for $C_{39}H_{53}NO_7$ $(M+Na)^+$ 670.37142, found 670.37148. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.40 - 7.28 (m, 10H), 5.55 - 5.43 (m, 1H), 5.28 (br-s, 1H), 5.23 (d, *J* = 12.4 Hz, 1H), 5.12 (t, *J* = 12.4 Hz, 2H), 5.10 (d, *J* = 12.4 Hz, 1H), 4.79 (dd, *J* = 8.2, 2.7 Hz, 1H), 3.67 (ddd, *J* = 11.9, 10.7, 4.0 Hz, 1H), 3.55 (dd, *J* = 9.7, 2.0 Hz, 1H), 3.43 (s, 3H), 2.93 (d, *J* = 9.5 Hz, 1H), 1.91 - 1.72 (m, 2H), 1.68 (dd, *J* = 12.5, 4.2 Hz, 1H), 1.61 (s, 3H), 1.57 - 1.41 (m, 5H), 1.39 - 1.28 (m, 3H), 1.22 - 1.14 (m, 2H), 0.98 (s, 3H), 0.96 (s, 3H), 0.95 (s, 3H), 0.86 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.6, 156.2, 137.5, 136.2, 134.8, 128.7, 128.5, 128.2, 128.1, 128.0, 122.2, 83.7, 82.0, 69.4, 67.4, 67.1, 58.3, 55.5, 51.0, 46.0, 43.2, 43.0, 39.6, 37.2, 36.4, 32.0, 30.0, 29.7, 28.6, 28.3, 26.1, 22.5, 22.4, 17.5, 16.7.

(2*R*,3*S*,4*R*,5*R*,6*S*)-5-Acetamido-6-(((2*S*,3*R*,4*R*,5*S*,6*S*)-3-acetoxy-5-((3-acetoxybenzoyl)oxy)-2-(((2*S*,3*S*,4a*S*,4b*S*,8*S*,8a*S*,10a*S*)-8-((2*S*,3*S*)-4-(benzyloxy)-3-(((benzyloxy)carbonyl)amino)-2-methoxy-4-oxobutyl)-2-hydroxy-1,1,4a,7,8a-pentamethyl-1,2,3,4,4a,4b,5,8,8a,9,10,10a-dodecahydrophenanthren-3-yl)oxy)-6-methyltetrahydro-2*H*-pyran-4-yl)oxy)-2-(acetoxymethyl)tetrahydro-2*H*-pyran-3,4-diyl diacetate (10)

and

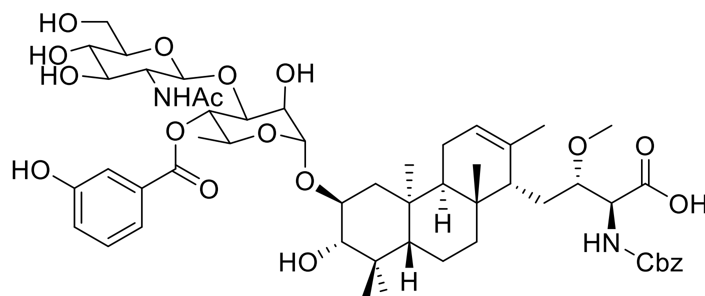
(2*R*,3*S*,4*R*,5*R*,6*S*)-5-Acetamido-6-(((2*S*,3*R*,4*R*,5*S*,6*S*)-3-acetoxy-5-((3-acetoxybenzoyl)oxy)-2-(((2S,3*S*,4a*S*,4b*S*,8*S*,8a*S*,10a*S*)-8-((2*S*,3*S*)-4-(benzyloxy)-3-(((benzyloxy)carbonyl)amino)-2-methoxy-4-oxobutyl)-3-hydroxy-1,1,4a,7,8a-pentamethyl-1,2,3,4,4a,4b,5,8,8a,9,10,10a-dodecahydrophenanthren-2-yl)oxy)-6-methyltetrahydro-2*H*-pyran-4-yl)oxy)-2-(acetoxymethyl)tetrahydro-2*H*-pyran-3,4-diyl diacetate (11)



Protected Aglycone **8** (33 mg, 51 µmol) was dissolved in dry DCM (2 mL) and poured into a stirred suspension of powdered 4 Å molecular sieves in dry DCM (1 mL) under argon atmosphere at rt. After cooling to 0 °C the carbohydrate trichloroacetimidate **9** (129 mg, 153 µmol) dissolved in dry DCM (2 mL), followed by 0.6 µL TMSOTf was added and stirred for 0.5 h at this temperature. The reaction mixture was warmed to rt, stirred for another 1.5 h, quenched by Et_3N, diluted with DCM (20 mL) and filtrated through a Celite pad. The solvents were removed under reduced pressure and the residue was purified by flash chromatography (SiO_2, n-hexane / EtOH: 100 / 00 to 80 / 20) to give the protected brasilicardin A **10** and its 3-isomer **11** in a 2:1 ratio as a white solid (29 mg) along with recovered protected aglycone **8** (18.2 mg, 55 %). Both isomers were separated by preparative HPLC (C18, H_2O (0.1 % TFA) / ACN 80 % in H_2O (0.1 % TFA): 07 / 93) to yield 17.2 mg (25 %, 57 % brsm) of protected brasilicardin A **10** and 8.8 mg (13 %, 29 % brsm) of 3-isomer **11**. Compound **10**: TLC: R_f = 0.61 (nHex / EtOAc: 1 / 5). HPLC (method 2): 10.7 min. HRMS (ESI): calcd for $C_{70}H_{90}N_2O_{23}$ $(M+Na)^+$ 1349.58266, found 1349.58237. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.97 (d, *J* = 7.8 Hz, 1H), 7.80 (br-s, 1H), 7.53 (t, *J* = 7.53 Hz, 1H), 7.40 - 7.28 (m, 11H), 5.77 (d, *J* = 9.3 Hz, 1H), 5.40 (d, *J* = 8.5 Hz, 1H), 5.30 - 5.19 (m, 4H), 5.19 (d, *J* = 12.4 Hz, 1H), 5.12 (t, *J* = 12.4 Hz, 2H), 5.08 (d, *J* = 12.4 Hz, 1H), 5.14 - 5.04 (m, 1H), 4.96 (s, 1H), 4.87 - 4.74 (m, 2H),

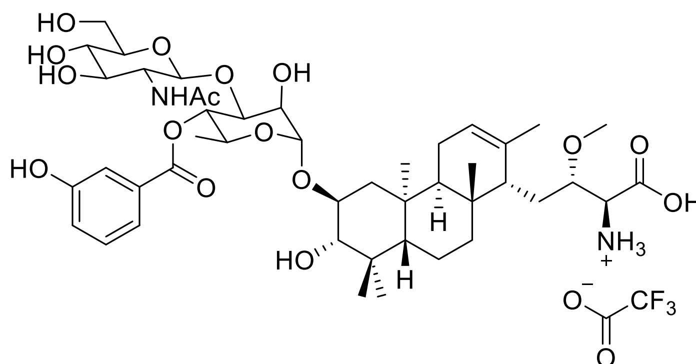
4.69 (d, J = 8.3 Hz, 1H), 4.18 (dd, J = 10.1, 3.2 Hz, 1H), 4.02 - 3.95 (m, 1H), 3.86 (q, J = 9.3 Hz, 1H), 3.79 - 3.57 (m, 3H), 3.54 (dd, J = 9.4, 2.3 Hz, 1H), 3.43 (s, 3H), 3.13 (d, J = 9.4 Hz, 1H), 2.39 (s, 3H), 2.16 (s, 3H), 2.12 (s, 3H), 2.09 (s, 3H), 2.03 (s, 3H), 1.99 - 1.96 (m, 1H), 1.87 - 1.72 (m, 3H), 1.60 (s, 3H), 1.57 - 1.33 (m, 9H), 1.28 (s, 3H), 1.24 (d, J = 6.4 Hz, 3H), 1.00 (s, 3H), 0.98 (s, 3H), 0.96 (s, 3H), 0.88 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.1, 171.8, 170.9, 170.6, 170.2, 170.1, 169.4, 164.4, 156.2, 150.3, 137.8, 136.1, 134.8, 130.9, 130.2, 128.7, 128.6, 128.5, 128.3, 128.2, 128.1, 127.7, 126.3, 123.1, 121.9, 101.4, 99.8, 82.0, 81.7, 80.9, 75.1, 73.6, 72.3, 72.2, 71.4, 68.3, 67.6, 67.3, 66.9, 59.7, 58.4, 55.4, 54.4, 51.0, 45.9, 43.0, 42.2, 39.7, 37.2, 36.4, 32.0, 30.0, 29.8, 28.8, 28.2, 26.1, 22.5, 22.4, 21.9, 21.3, 21.0, 20.9, 20.6, 17.6, 17.4, 16.8. HMBC NMR: H-2 (δ_H 3.67) correlates to C-1’ (δ_C 99.8), H-1’ (δ_H 4.96) correlates to C-2 (δ_C 71.4). INEPT NMR: C-1’ ($^1J_{C,H}$ = 171.1 Hz) indicates α attachment of rhamnose and C-1’’ ($^1J_{C,H}$ = 165.3 Hz) indicates β attachment of glucosamine. Compound **11**: TLC: R_f = 0.61 (nHex / EtOAc: 1 / 5), HPLC (method 2): 11.1 min, HRMS (ESI): calcd for $C_{70}H_{90}N_2O_{23}$ $(M+Na)^+$ 1349.58266, found 1349.58208. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.97 (d, J = 7.7 Hz, 1H), 7.79 (br-s, 1H), 7.52 (t, J = 8.1 Hz, 1H), 7.40 - 7.29 (m, 11H), 5.77 (d, J = 9.4 Hz, 1H), 5.44 - 5.37 (m, 2H), 5.28 (br-s, 1H), 5.25 - 5.15 (m, 3H), 5.14 - 5.06 (m, 4H), 5.01 (s, 1H), 4.86 - 4.76 (m, 2H), 4.73 (d, J = 8.2 Hz, 1H), 4.14 - 4.03 (m, 2H), 3.89 - 3.73 (m, 3H), 3.63 (dt, J = 9.9, 2.4 Hz, 1H), 3.55 (dd, J = 9.5, 2.4 Hz, 1H), 3.43 (s, 3H), 2.97 (d, J = 9.3 Hz, 1H), 2.39 (s, 3H), 2.16 (s, 3H), 2.10 (s, 3H), 2.08 (s, 3H), 2.03 (s, 3H),1.99 - 1.96 (m, 1H), 1.87 - 1.74 (m, 3H), 1.71 (dd, J = 12.6, 3.3 Hz, 2H), 1.60 (s, 3H), 1.54 - 1.42 (m, 5H), 1.25 (s, 3H), 1.22 (d, J = 6.2 Hz, 3H), 1.37 - 1.33 (m, 2H), 1.02 (s, 3H), 0.98 (s, 3H), 0.95 (s, 3H), 0.93 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.5, 171.7, 170.8, 170.6, 170.1, 170.0, 169.4, 164.4, 156.3, 150.3, 137.3, 136.1, 134.9, 130.9, 130.2, 128.7, 128.6, 128.5, 128.3, 128.2, 128.1, 127.6, 126.3, 123.1, 122.5, 101.4, 100.9, 94.8, 82.0, 78.9, 75.7, 73.6, 72.4, 72.3, 71.4, 69.2, 68.2, 67.5, 67.2, 59.8, 58.4, 55.5, 54.4, 51.0, 46.0, 43.7, 43.3, 40.0, 37.2, 36.3, 32.1, 30.1, 29.8, 28.8, 28.3, 29.9, 22.6, 22.4, 22.0, 21.1, 21.0, 20.8, 20.6, 17.9, 17.7, 17.4. HMBC NMR: H-3 (δ_H 2.97) correlates to C-1’ (δ_C 100.9), H-1’ (δ_H 5.01) correlates to C-2 (δ_C 94.8). INEPT NMR: C-1’ ($^1J_{C,H}$ = 170.7 Hz) indicates α attachment of rhamnose and C-1’’ ($^1J_{C,H}$ = 162.9 Hz) indicates β attachment of glucosamine.

(2*S*,3*S*)-4-((1*S*,4a*S*,4b*S*,6*S*,7*S*,8a*S*,10a*S*)-6-(((2*S*,3*R*,4*S*,5*S*,6*S*)-4-(((2*S*,3*R*,4*R*,5*S*,6*R*)-3-Acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2*H*-pyran-2-yl)oxy)-3-hydroxy-5-((3-hydroxybenzoyl)oxy)-6-methyltetrahydro-2*H*-pyran-2-yl)oxy)-7-hydroxy-2,4b,8,8,10a-pentamethyl-1,4,4a,4b,5,6,7,8,8a,9,10,10a-dodecahydrophenanthren-1-yl)-2-(((benzyloxy)carbonyl)amino)-3-methoxybutanoic acid (12)



Protected brasilicardin A **10** (20 mg, 15 µmol) was dissolved in a 0.1 M solution (1.5 mL) of tBuOK in H_2O / THF (1 / 1) and stirred for 5 h at rt. The THF was removed under reduced pressure and the residual aqueous solution was washed with EtOAc and acidified with 0.1 M HCl solution to pH = 2. The crude product was extracted 5 times with EtOAc (10 mL), dried over Na_2SO_4 and evaporated *in vacuo*. The yellowish residue was purified by preparative HPLC (C18, H_2O (0.1 % TFA) / ACN 80 % in H_2O (0.1 % TFA): 50 / 50 to 30 / 70) to give the N-Cbz protected brasilicardin A **12** (12.3 mg, 80 %) as a white solid. TLC: R_f = 0.64 (nHex / EtOH: 1 / 4). HPLC (method 1): 11.0 min. HPLC (method 2): 8.4 min. HRMS (ESI): calcd for $C_{53}H_{74}N_2O_{18}$ $(M+Na)^+$ 1049.48288, found 1049.48242. ^1H NMR (400 MHz, CD_3OD) δ 7.55 (dt, *J* = 7.9, 1.2 Hz, 1H), 7.47 (dd, 2.3, 1.7 Hz, 1H), 7.39 - 7.26 (m, 6H), 7.05 (ddd, *J* = 8.1, 2.6, 0.9 Hz, 1H), 5.30 (br-s, 1H), 5.27 (t, *J* = 9.8 Hz, 1H), 5.10 (s, 2H), 5.03 (d, *J* = 1.5 Hz, 1H), 4.71 (d, 3.9 Hz, 1H), 4.53 (d, *J* = 8.3 Hz, 1H), 4.34 (dd, *J* = 3.0, 1.8 Hz, 1H), 4.07 (dd, *J* = 9.7, 3.1 Hz, 1H), 4.0 (m, 1H), 3.89 (dd, *J* = 12.0, 1.8 Hz, 1H), 3.73 - 3.65 (m, 2H), 3.64 - 3.59 (m, 1H), 3.55 (dd, *J* = 9.8, 8.5 Hz, 1H), 3.42 (s, 3H), 3.40 - 3.25 (m, 3H), 3.00 (d, *J* = 9.7 Hz, 1H), 1.87 (br-d, *J* = 7.3 Hz, 2H), 1.81 - 1.71 (m, 3H), 1.63 (s, 3H), 1.61 - 1.52 (m, 2H), 1.49 (s, 3H), 1.46 - 1.30 (m, 6H), 1.13 (d, *J* = 6.4 Hz, 3H), 1.07 (s, 3H), 1.02 (s, 3H), 0.99 (s, 3H), 0.91 (s, 3H). NMR (101 MHz, CD_3OD) δ 174.1, 167.0, 159.0, 139.2, 138.3, 132.5, 130.9, 129.5, 129.0, 128.8, 123.0, 122.0, 121.6, 117.4, 104.1, 103.1, 83.4, 83.1, 80.0, 79.6, 77.8, 75.3, 74.1, 72.1, 71.8, 68.1, 67.7, 62.5, 58.4, 57.5, 52.5, 47.2, 44.4, 44.1, 41.2, 38.5, 37.6, 33.1, 31.4, 29.3, 28.9, 27.1, 23.0, 22.7, 22.6, 22.1, 18.8, 17.8, 17.4.

(1*S*,2*S*)-3-((1*S*,4a*S*,4b*S*,6*S*,7*S*,8a*S*,10a*S*)-6-(((2*S*,3*R*,4*S*,5*S*,6*S*)-4-(((2*S*,3*R*,4*R*,5*S*,6*R*)-3-Acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2*H*-pyran-2-yl)oxy)-3-hydroxy-5-((3-hydroxybenzoyl)oxy)-6-methyltetrahydro-2*H*-pyran-2-yl)oxy)-7-hydroxy-2,4b,8,8,10a-pentamethyl-1,4,4a,4b,5,6,7,8,8a,9,10,10a-dodecahydrophenanthren-1-yl)-1-carboxy-2-methoxypropan-1-aminium trifluoroacetate (1a)



Thioanisole (60 µL, 511 µmol) was added to a stirred solution of TFA (320 µL) at rt. This greenish blue solution was added at once to N-Cbz protected brasilicardin A **12** (10.5 mg, 10 µmol) and stirred for 3 h at rt to occur in a clear light yellow solution. The solvents were removed under reduced pressure and the residue was purified by preparative HPLC (C18, H_2O (0.1 % TFA) / ACN 80 % in H_2O (0.1 % TFA): 70 / 30 to 45 / 55) to give the brasilicardin A trifluoroacetate **1a** as a white solid (6.3 mg, 61 %). TLC: R_f = 0.23 (nHex / EtOH: 1 / 4). HPLC (method 1): 7.0 min. HRMS (ESI): calcd for $C_{45}H_{68}N_2O_{16}$ (**1**) $(M+Na)^+$ 915.44610, found 915.44521. ^1H NMR (400 MHz, CD_3OD) δ 7.55 (dt, *J* = 7.9, 1.2 Hz, 1H), 7.47 (dd, 2.3, 1.7 Hz, 1H), 7.34 (t, *J* = 7.9 Hz, 1H), 7.05 (ddd, *J* = 8.1, 2.6, 0.9 Hz, 1H), 5.34 (br-s, 1H), 5.27 (t, *J* = 9.8 Hz, 1H), 5.02 (d, *J* = 1.5 Hz, 1H), 4.53 (d, *J* = 8.3 Hz, 1H), 4.34 (dd, *J* = 3.0, 1.8 Hz, 1H), 4.32 (d, *J* = 3.5 Hz, 1H), 4.07 (dd, *J* = 9.7, 3.1 Hz, 1H), 4.0 (m, 1H), 3.89 (dd, *J* = 12.0, 1.8 Hz, 1H), 3.77 (dd, *J* = 11.4, 3.5 Hz, 1H), 3.73 - 3.65 (m, 2H), 3.54 (dd, *J* = 10.1, 8.4 Hz, 1H), 3.48 (s, 3H), 3.40 - 3.27 (m, 3H), 3.01 (d, *J* = 9.6 Hz, 1H), 1.88 (br-d, *J* = 6.7 Hz, 2H), 1.83 - 1.72 (m, 3H), 1.65 (s, 3H), 1.63 - 1.55 (m, 2H), 1.49 (s, 3H), 1.48 - 1.26 (m, 6H), 1.12 (d, *J* = 6.2 Hz, 3H), 1.10 (s, 3H), 1.05 (s, 3H), 1.00 (s, 3H), 0.92 (s, 3H). ^{13}C NMR (101 MHz, CD_3OD) δ 174.1, 170.3, 167.0, 159.0, 138.6, 132.5, 130.9, 123.6, 122.0, 121.5, 117.4, 104.1, 103.2, 83.4, 80.6, 79.9, 79.8, 77.8, 75.3, 74.1, 72.1, 71.8, 68.1, 62.6, 58.3, 57.5, 55.0, 52.3, 47.4, 44.3, 41.2, 38.6, 37.6, 31.9, 31.3, 29.3, 29.0, 27.1, 23.0, 22.7, 22.6, 18.8, 17.8, 17.4. TFA salt was verified by ^{19}F NMR (377 MHz, CD_3OD) δ – 76.96.

NMR spectra of compounds 8,10-12 and 1a

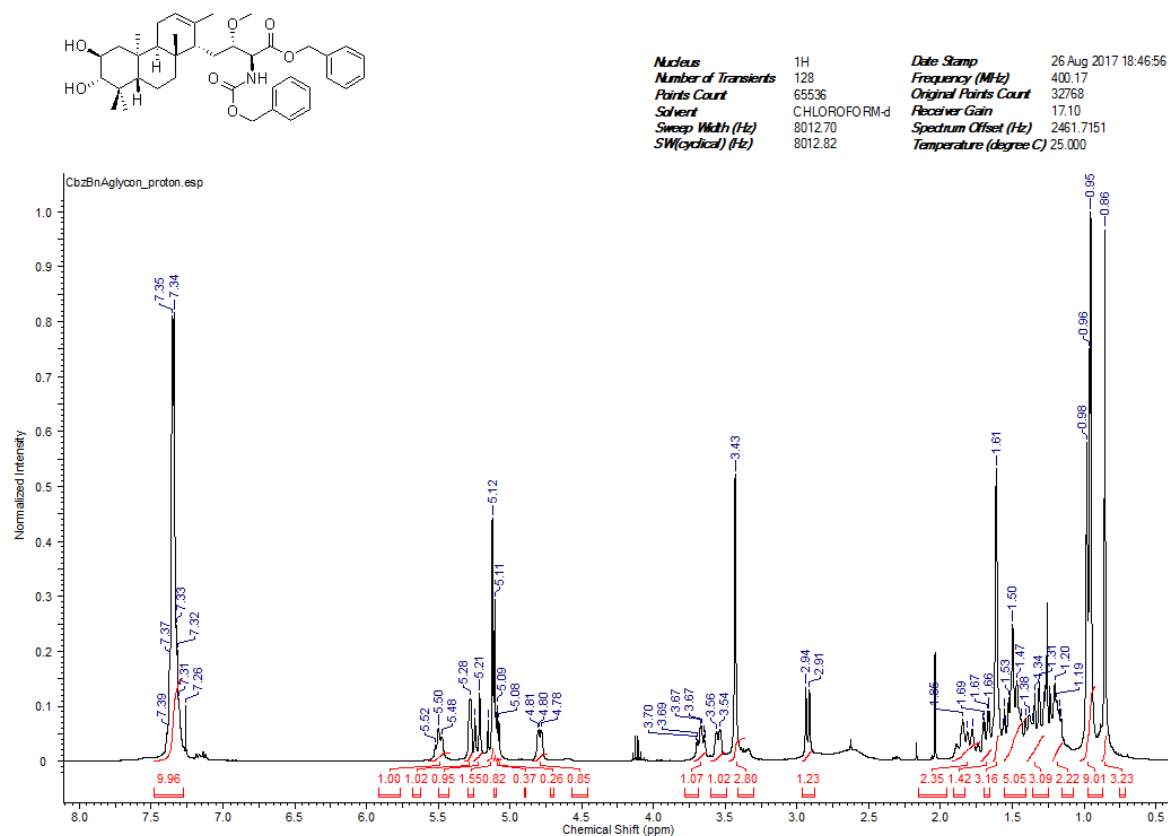


Figure S15. ^{1}H NMR spectrum of protected aglycone **8**.

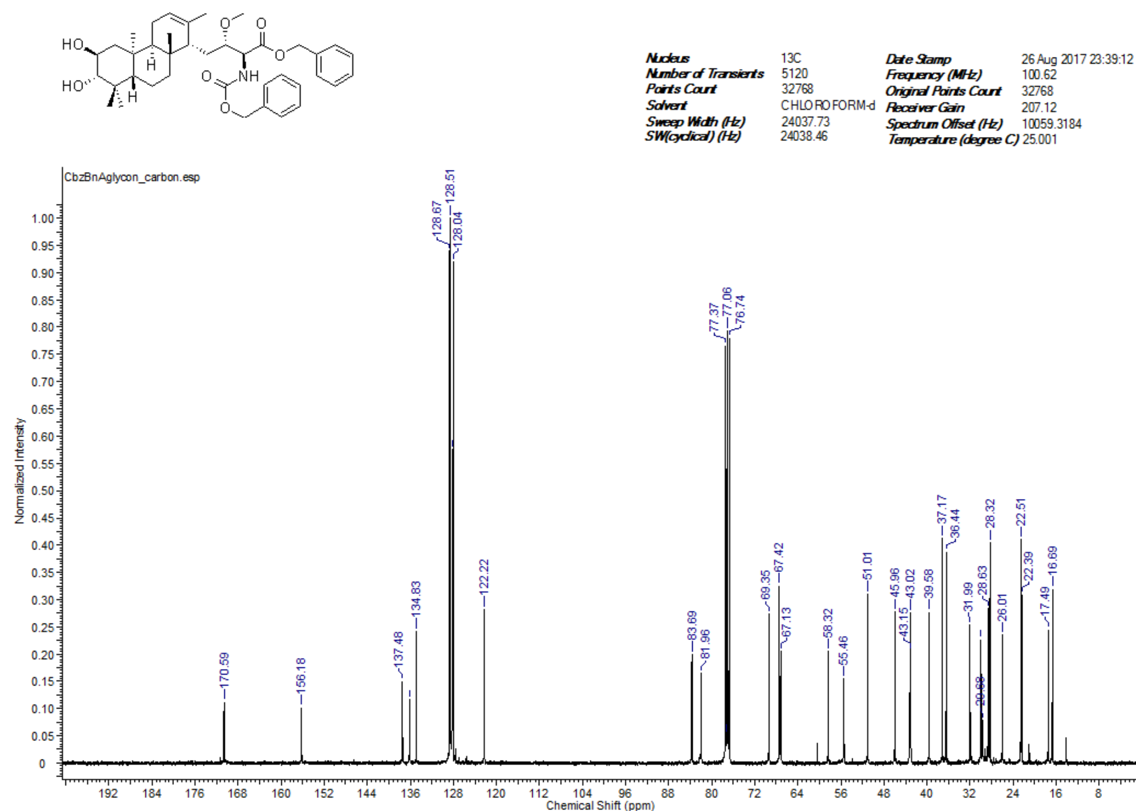


Figure S16. ^{13}C NMR spectrum of protected aglycone **8**.

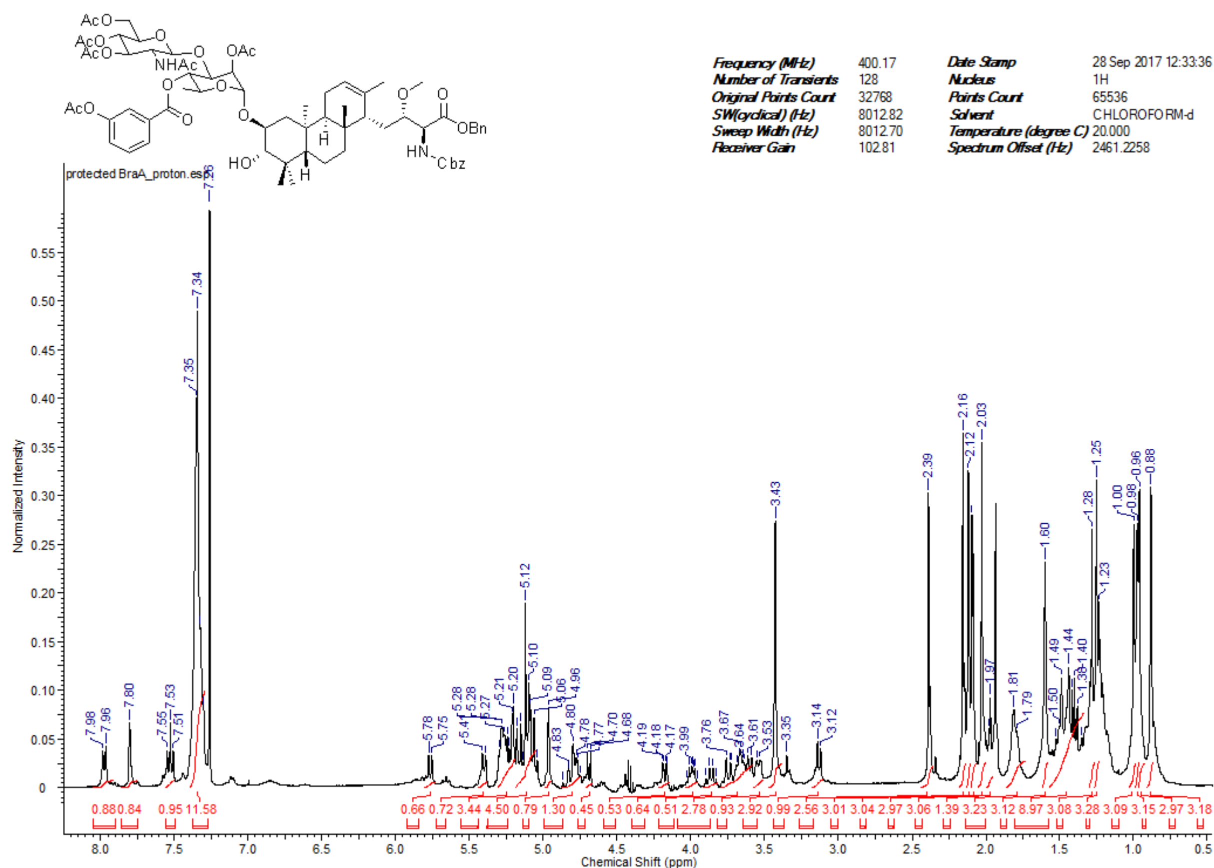


Figure S17. ^{1}H NMR spectrum of protected brasilicardin A **10**.

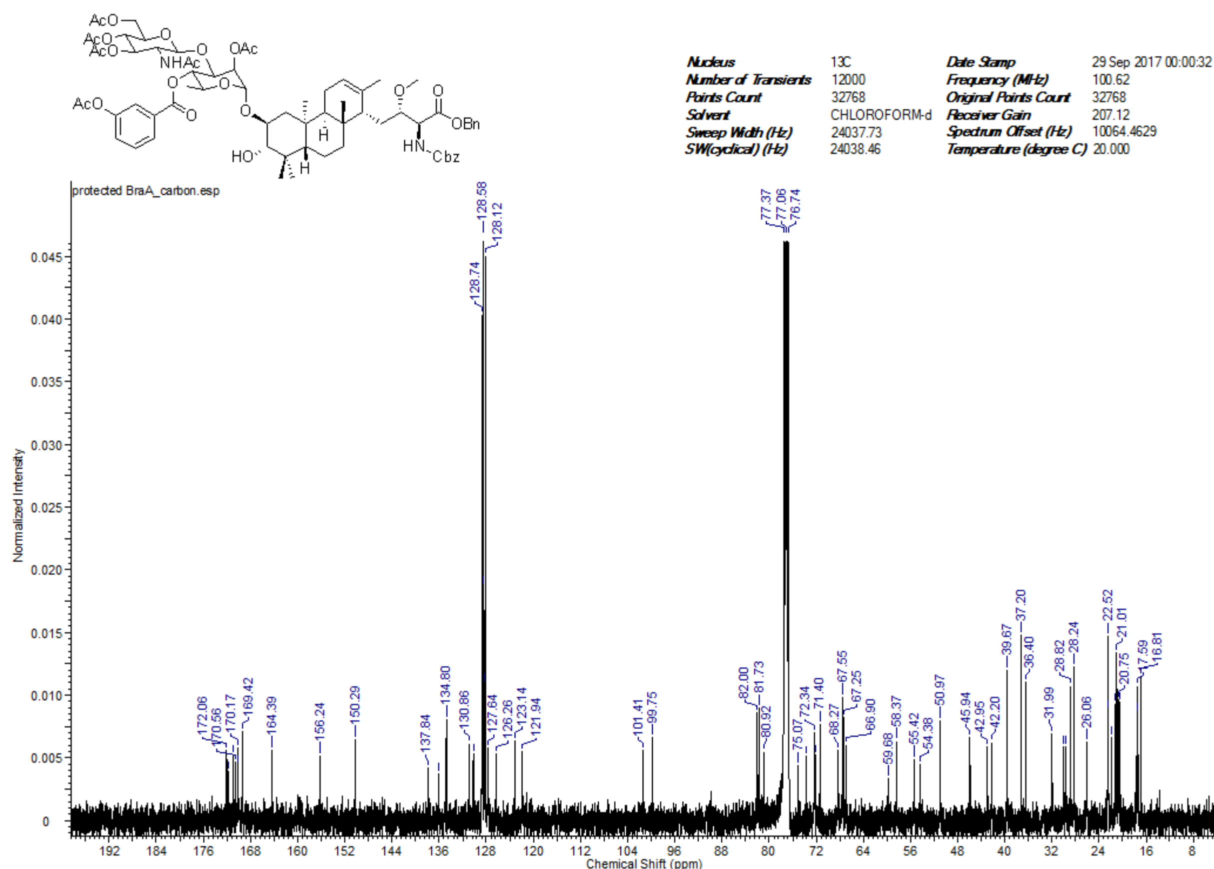


Figure S18. ^{13}C NMR spectrum of protected brasilicardin A **10**.

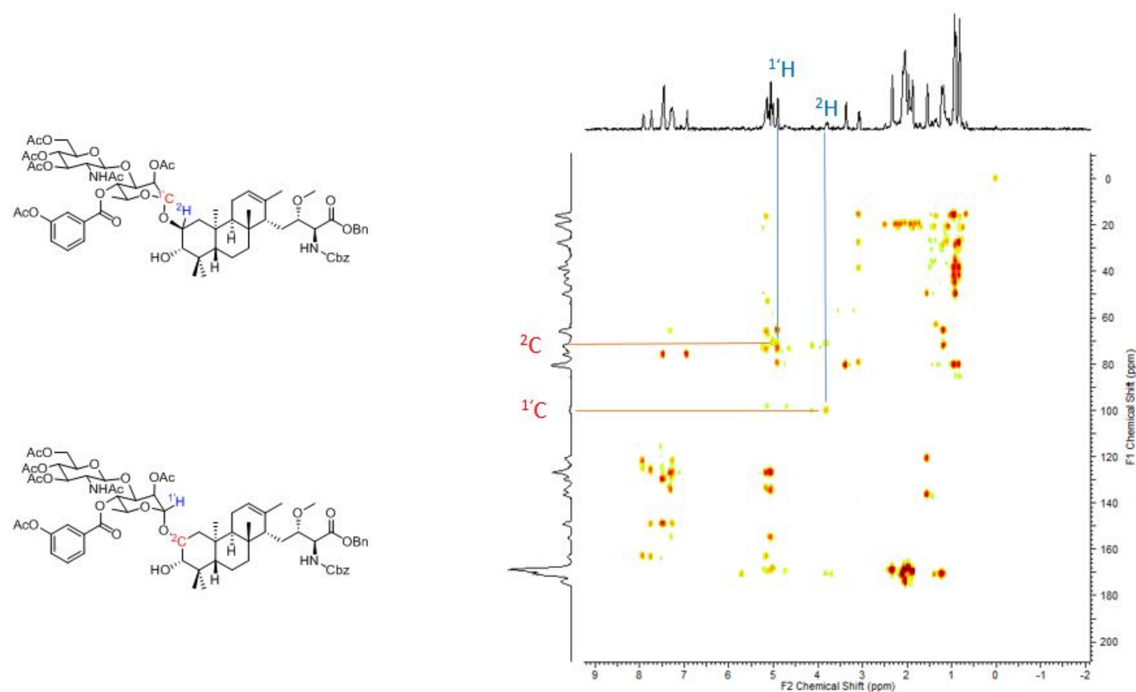


Figure S19. ^{1}H-^{13}C-HMBC NMR spectrum of protected brasilicardin A **10**.

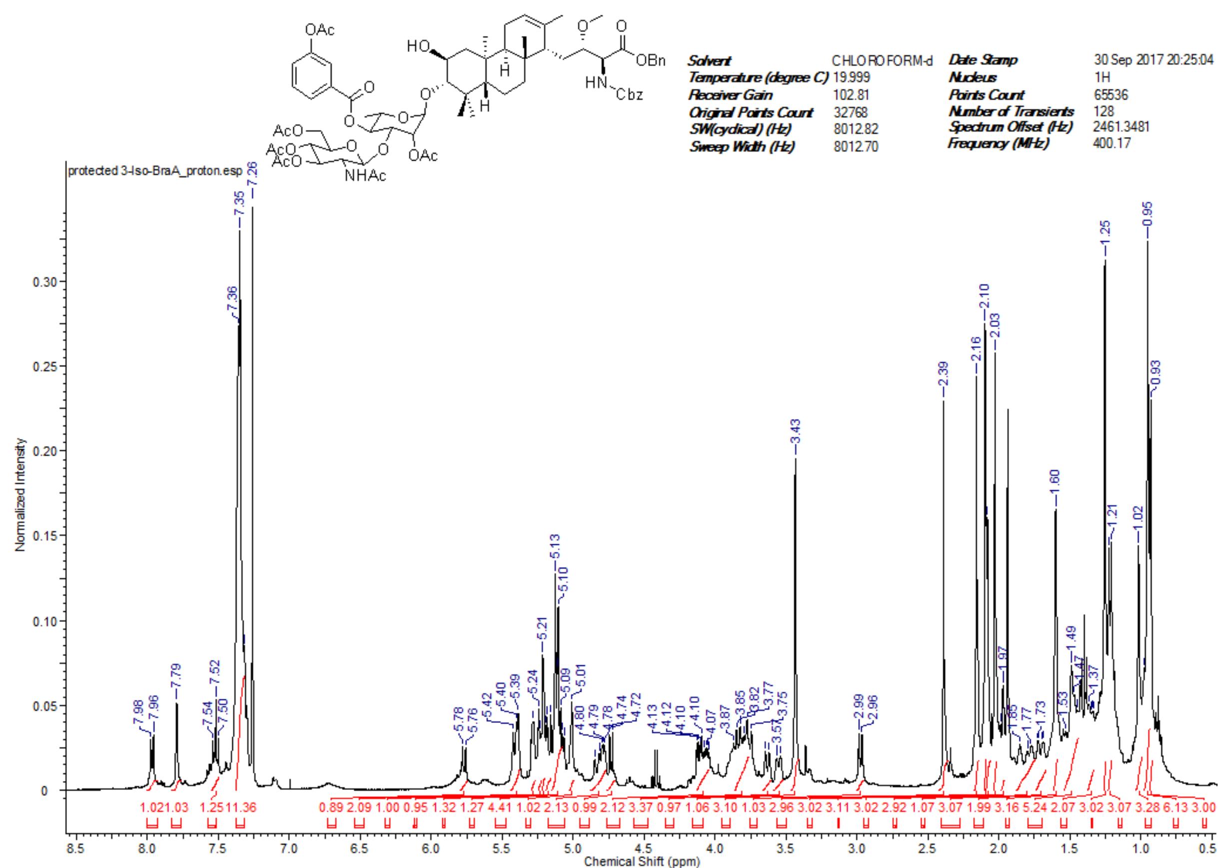


Figure S20. ^{1}H NMR spectrum of protected 3-iso-brasilicardin A **11**.

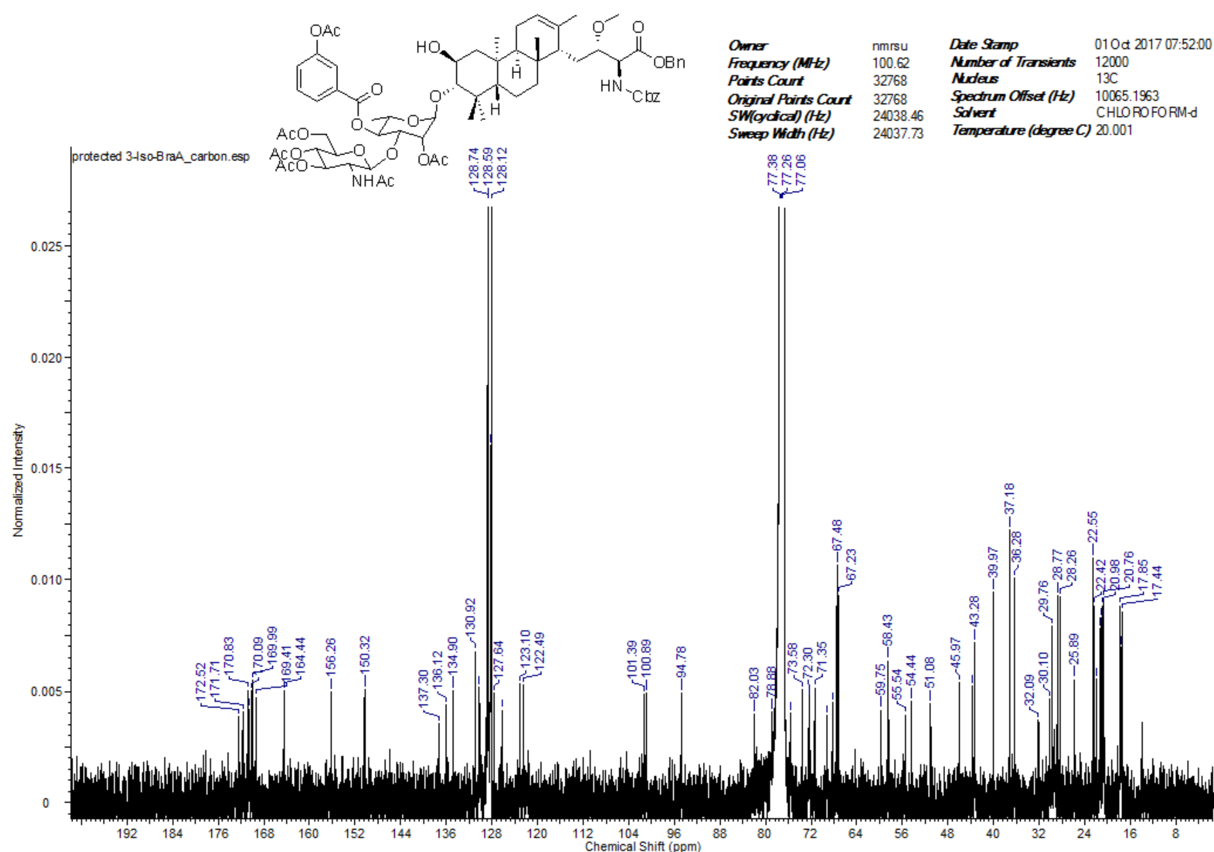


Figure S21. ^{13}C NMR spectrum of protected 3-iso-brasilicardin A **11**.

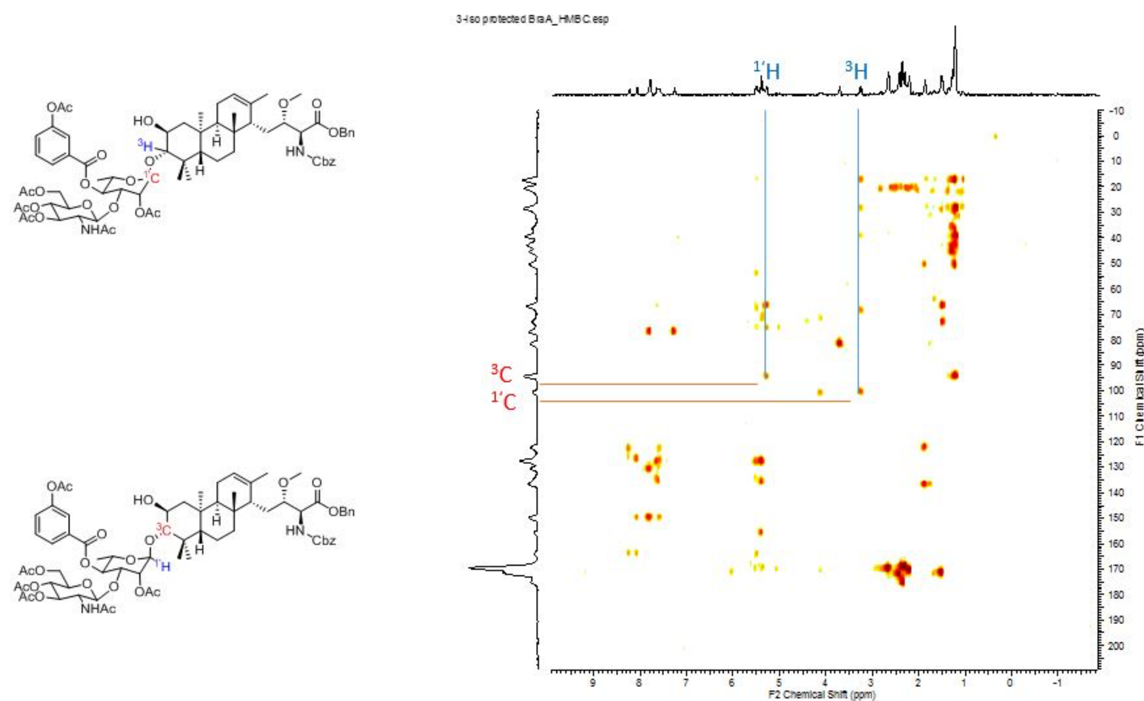


Figure S22. ^{1}H-^{13}C-HMBC NMR spectrum of protected 3-iso-brasilicardin A **11**.

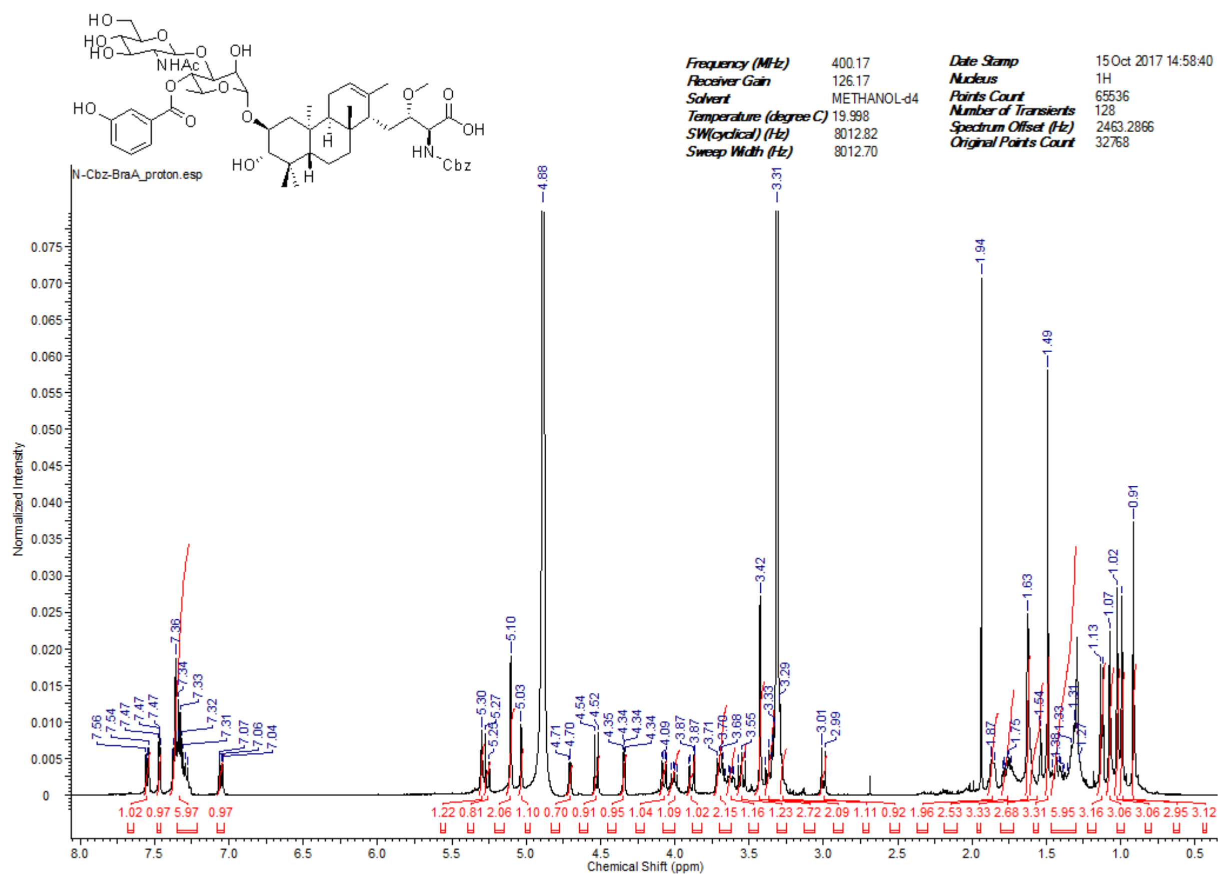


Figure S23. ^{1}H NMR spectrum of N-Cbz protected brasilicardin A **12**.

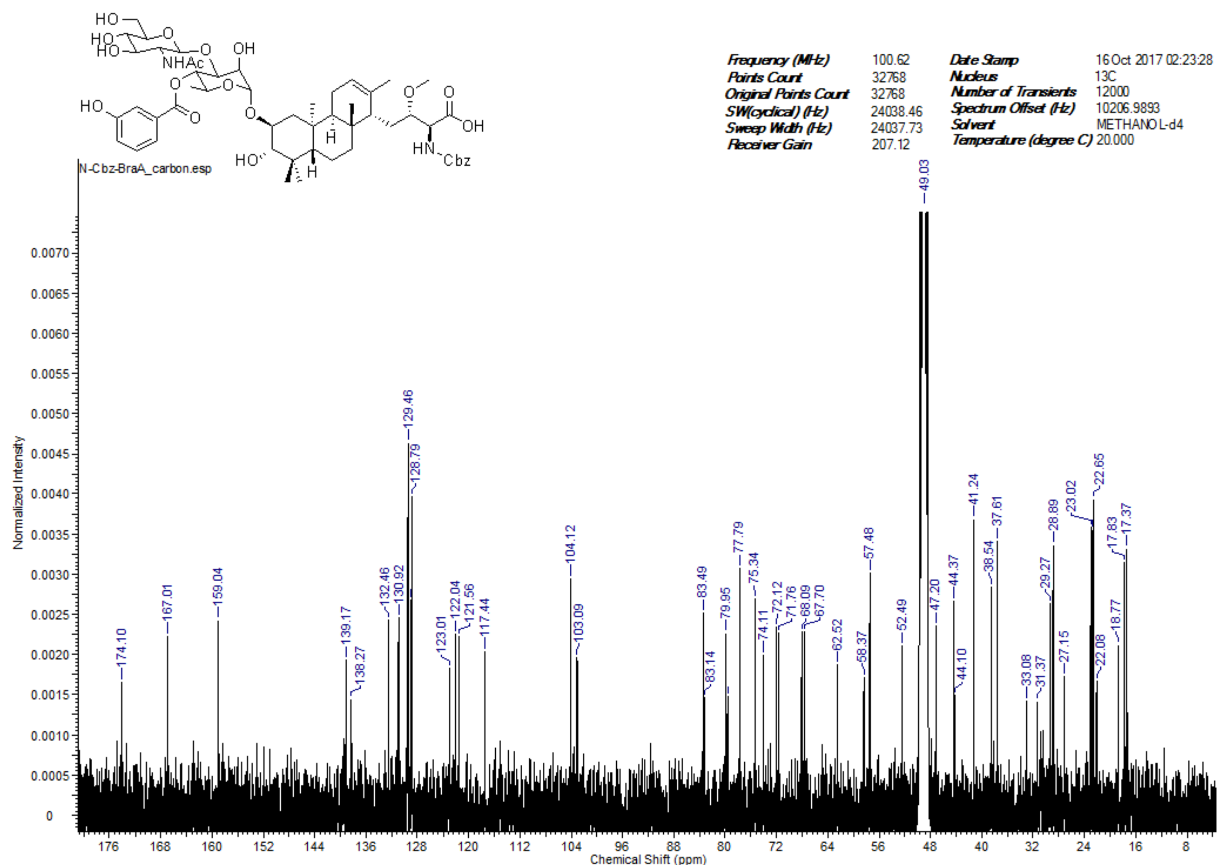


Figure S24. ^{13}C NMR spectrum of N-Cbz protected brasilicardin A **12**.

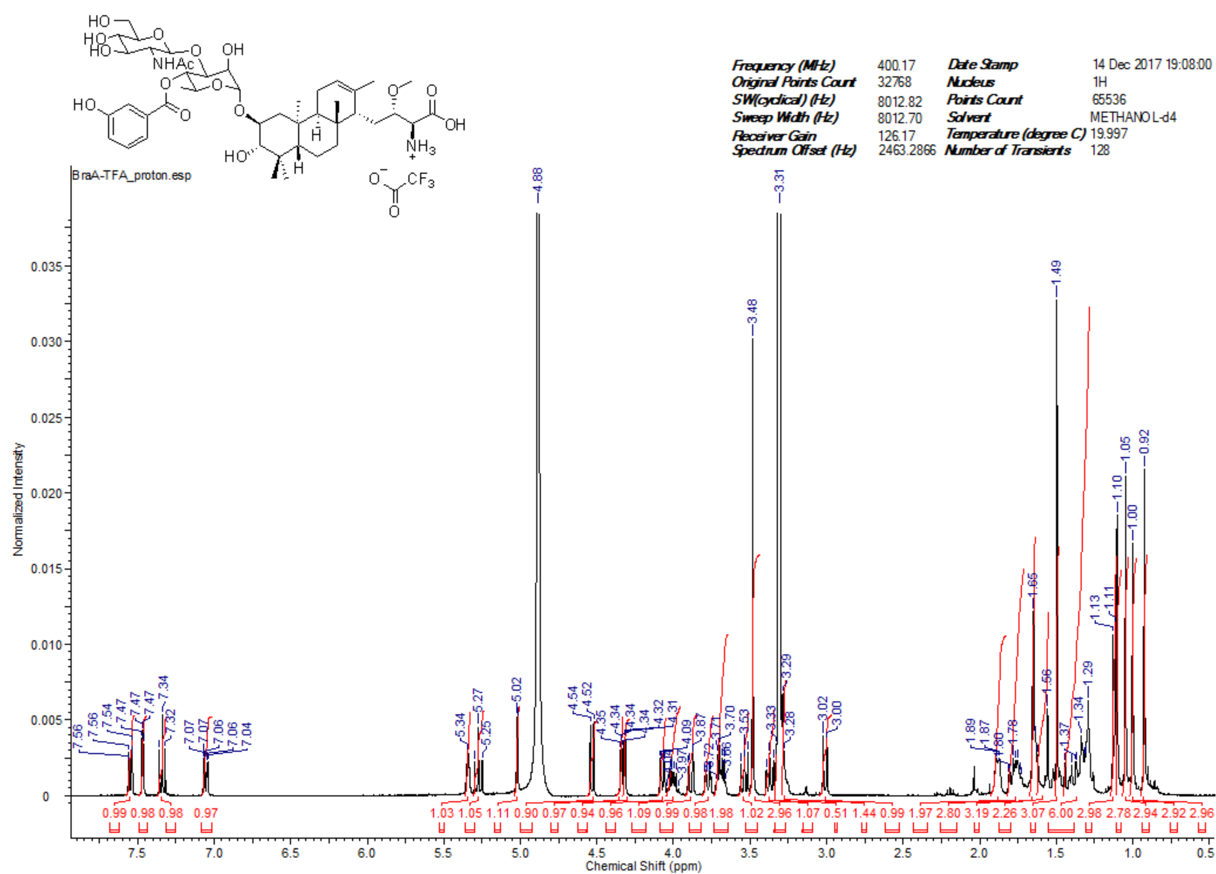


Figure S25. ^{1}H NMR spectrum of brasilicardin A trifluoroacetate (**1a**).

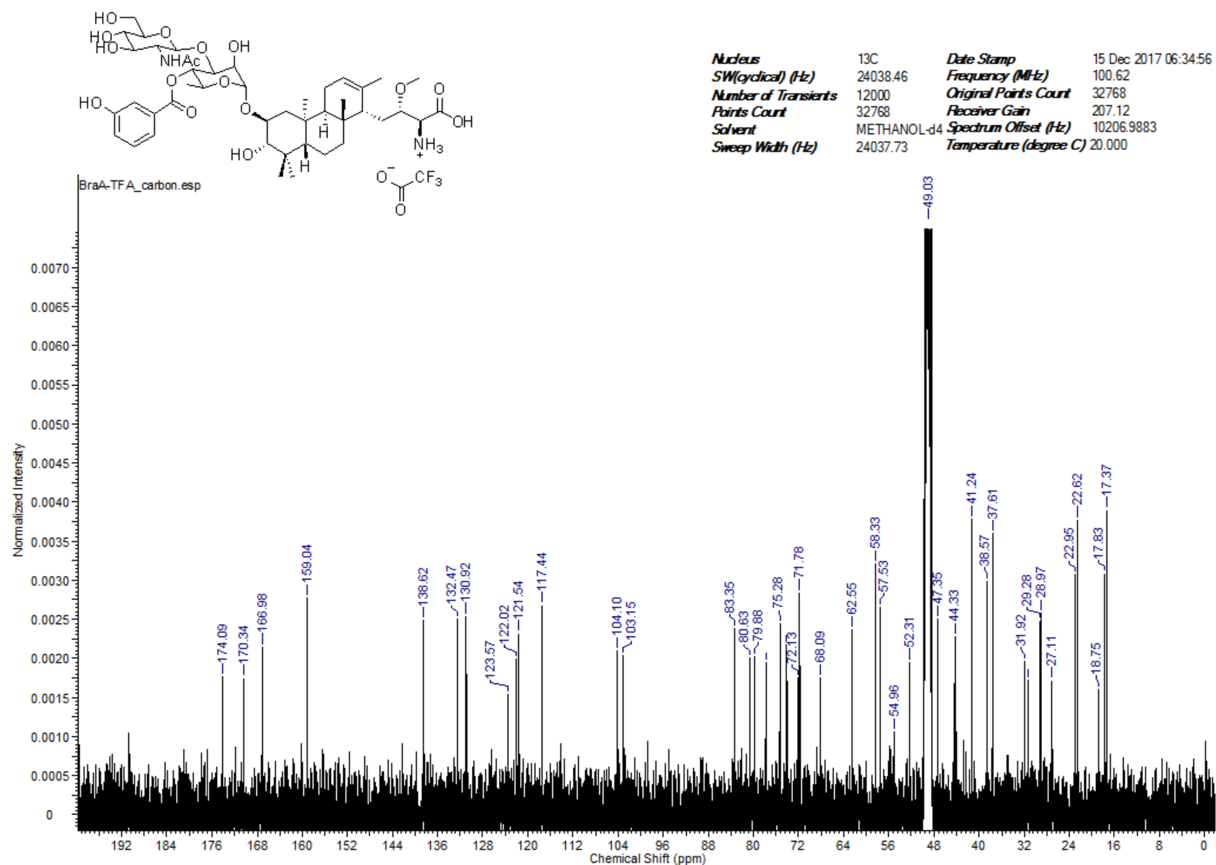


Figure S26. ^{13}C NMR spectrum of brasilicardin A trifluoroacetate (**1a**).

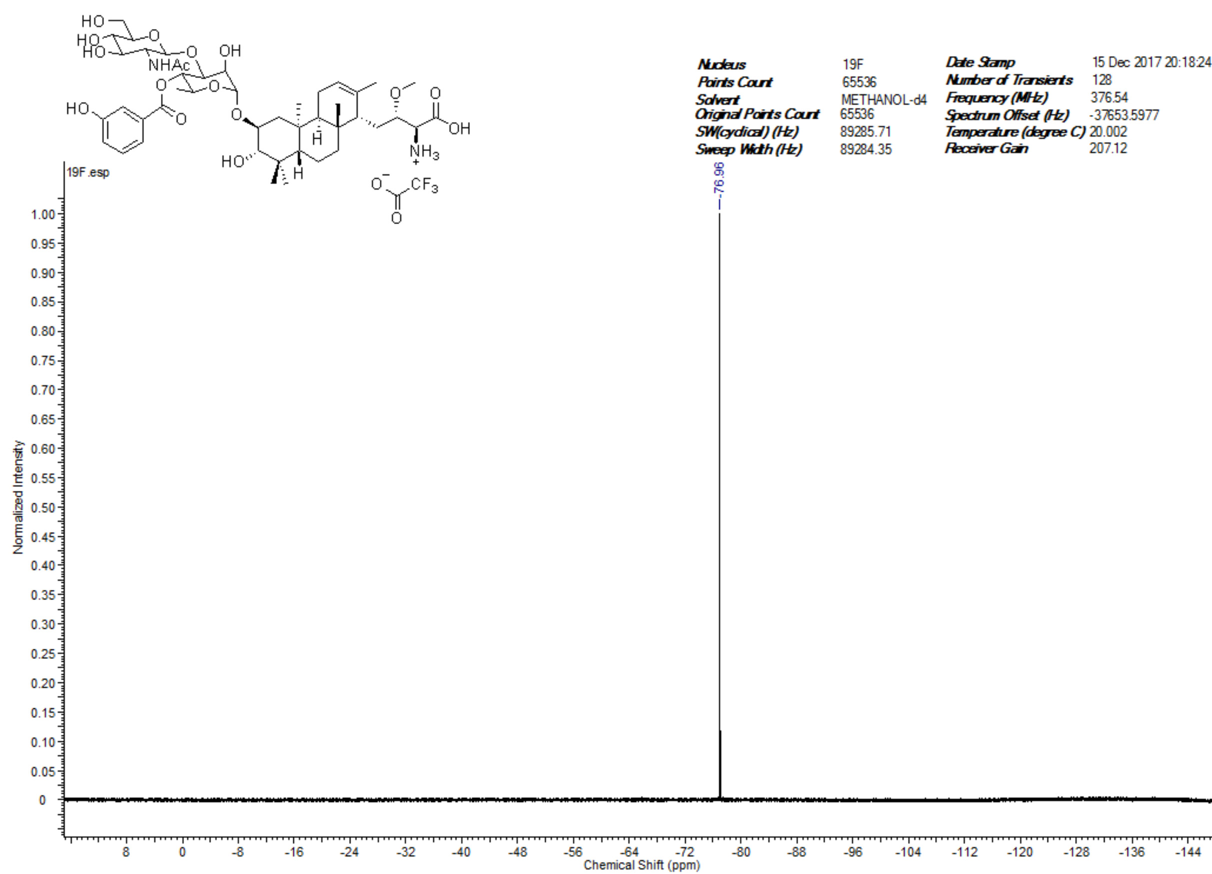
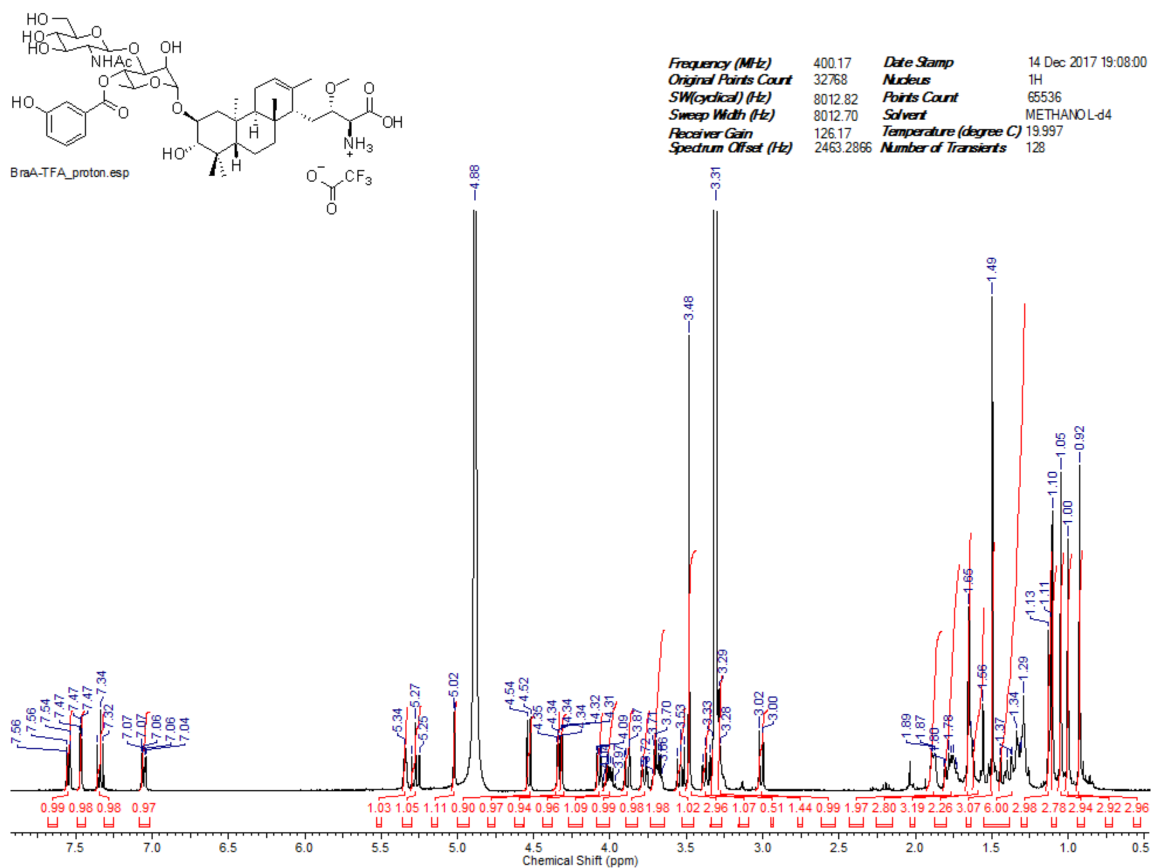


Figure S27. ^{19}F NMR spectrum of brasilicardin A trifluoroacetate (**1a**).



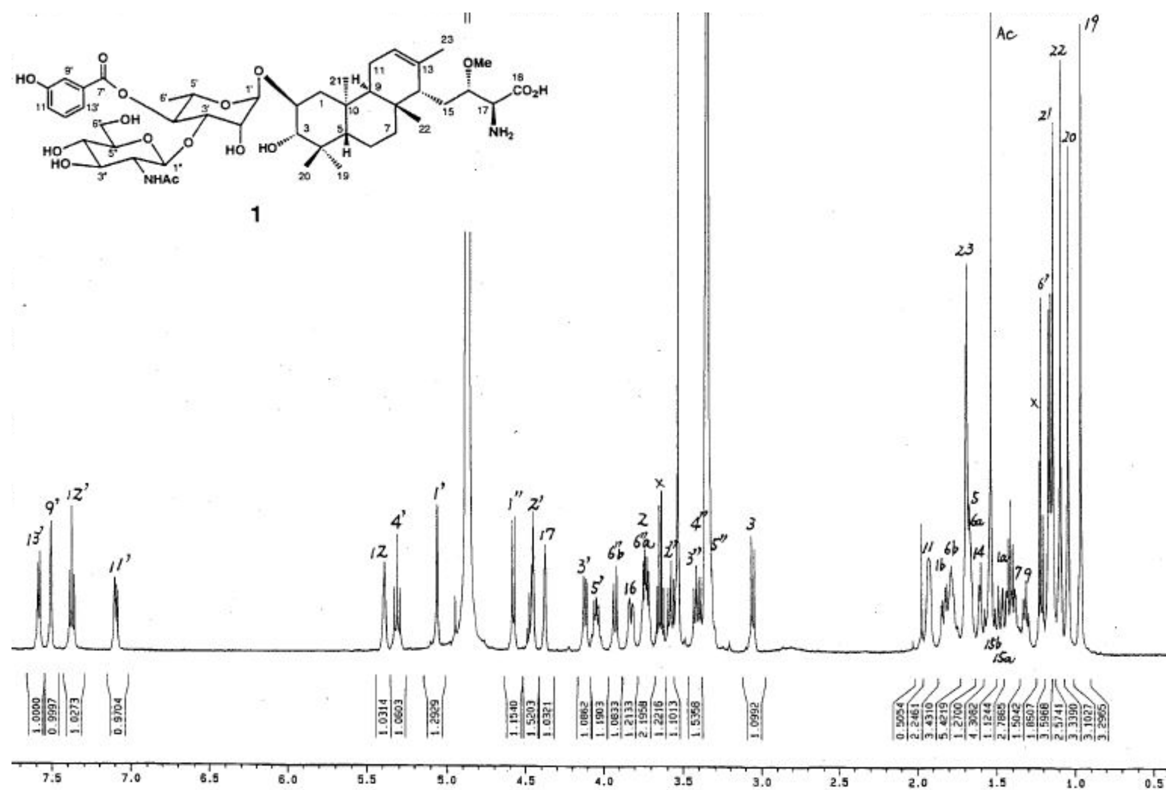
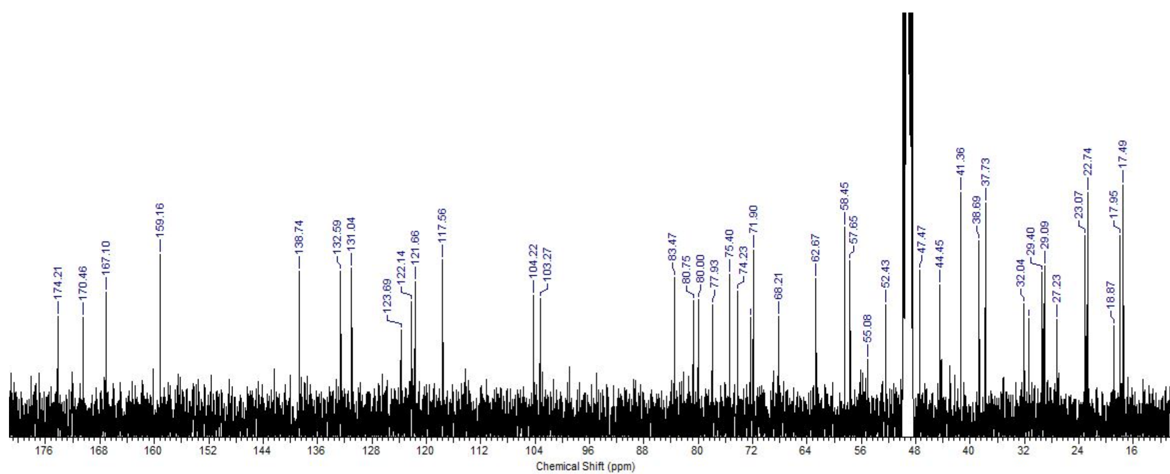


Figure S28. Comparison of ^{1}H NMR spectra of synthesized BraA trifluoroacetate (**1a**) (top) and isolated BraA (**1**) (bottom, taken from Ref. [24]); *differences regarding the signal of C17-H can be delineated to the protonation of the primary amino function in **1a**.



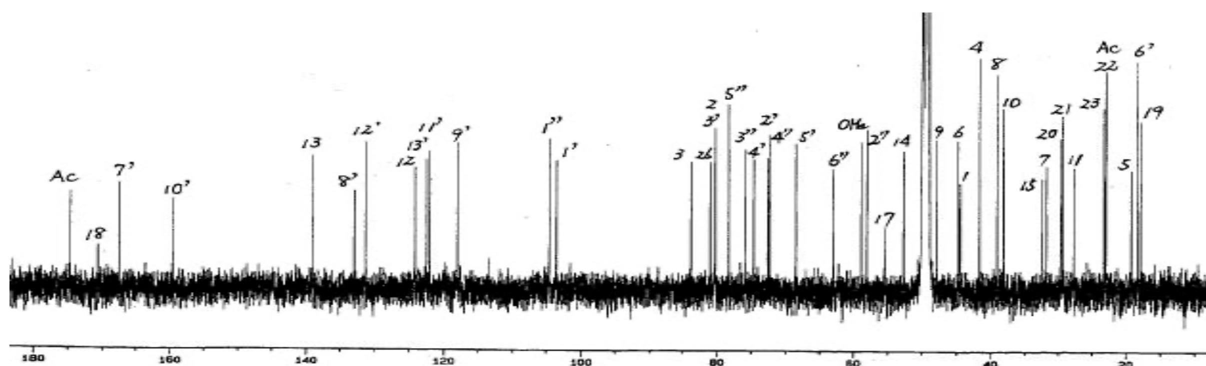


Figure S29. Comparison of ^{13}C NMR spectra of synthesized BraA trifluoroacetate (**1a**) (top) and isolated BraA (**1**) (bottom, taken from Ref. [24])

6 Testing

6.1 Purity of tested compounds

Table S5. Purity of the tested compounds determined by different analytical experiments. IS, internal standard, ES: external standard, N/A: not available.

compound	qNMR	qLC/MS	HPLC		
	(IS)	(ES)	230 nm	254 nm	TIC
3	97%	N/A	N/A	N/A	97%
5	96%	N/A	N/A	N/A	95%
7	N/A	94%	92%	92%	94%
1	N/A	87%	94%	96%	89%
1a	N/A	92%	93%	93%	90%

Quantitative NMR

Quantitative proton NMR analysis was measured on a Bruker Avance III HDX NMR spectrometer equipped with a Prodigy ($^{1}H/^{13}C/^{15}N$)-TCI Cryo Probe Head at 700 MHz. For all measurements the sample spinning was switched off, the puls angle was set to 90° and the delay time D1 was increased to 60s. The commercially available certified reference material (CRM) maleic acid TraceCERT® from Merck was applied as internal standard (IS). The brasilicardin congeners were spiked with an adequate amount of IS and then immediately dissolved in deuterated methanol. 512 scans were recorded with a ^{1}H relaxation time of 60 s. Quantification of the brasilicardins C and E were directly calculated from the ^{1}H-NMR peak areas and the initial weights of the compound and the IS. For increasing the accuracy of the calculation, the phase and baseline corrections were performed manually. The purity was calculated by the mean value of four ^{1}H-NMR integrals of the analyzed compound.

Brasilicardin C (**3**)

Component	Assay % (w/w)	Integral	Number of H	Mw (g/mol)	Component mass (mg)
BraC	97.113	1.1757	1	569.740	2.2720
BraC	96.593	1.1694	1	569.740	2.2720
BraC	98.058	3.5613	3	569.740	2.2720
BraC	96.693	3.5118	3	569.740	2.2720
IS	99.940	2.2271	2	116.070	0.4260

The purity of the Internal Standard (IS) is 99.94 % (w/w)
The purity of BraC is 97.114 % (w/w), SD= 0.67 % (w/w), RSD= 0.69 %

Brasilicardin E (**5**)

Component	Assay % (w/w)	Integral	Number of H	Mw (g/mol)	Component mass (mg)
BraE	96.820	0.2670	1	423.590	1.4880
BraE	94.796	0.2614	1	423.590	1.4880
BraE	96.652	0.2665	1	423.590	1.4880
BraE	95.868	0.2643	1	423.590	1.4880
IS	99.940	0.7786	2	116.070	0.5760

The purity of the Internal Standard (IS) is 99.94 % (w/w)
The purity of BraE is 96.034 % (w/w), SD= 0.92 % (w/w), RSD= 0.96 %

Quantitative HPLC/MS

HPLC/MS measurements were performed in positive mode under the same conditions described in 3.7. Brasilicardin C (**3**) with a purity of 97% determined by qNMR was dissolved in 1 mL MeOH and then diluted in 15 aliquots ranging from 0.1 to 1 mg/mL to generate a standard curve. The aliquots (m/V) were converted to molar concentrations (n/V) to get the values comparable. To determine the purity of compounds **1**, **1a** and **7** the peak area of the total ion current (TIC) for the extracted molecule ion peak was divided to the value got from the standard curve multiplied by the purity of the standard (97%).

compound	**c_m (mg/mL)**	**c_n (mmol/mL)**	**X (TIC area)**	**Y (mmol/mL)**	**purity [%]**
7	0.378	5.48E-04	1.89E+09	5.60E-04	93.9
1	0.264	2.96E-04	8.56E+07	3.25E-04	87.2
1a	0.302	3.00E-04	8.89E+07	3.13E-04	92.1

6.2 Immunosuppressive activity

Material and methods

T cells isolation and culture. Primary T cells were isolated from whole blood healthy donors on a Ficoll-Hypaque density gradient centrifugation, followed by negative selection enrichment using Pan T cell isolation kit (Miltenyi). Cell enrichment was performed according to the manufacturer´s protocol. CD3+ cells were cultured in RPMI 1640 with 10% heat inactivated FBS, 100 IU/mL penicillin G, and 100 µg/mL streptomycin.

CFSE proliferation assay. Purified CD3+ T cells were labeled with Cell Trace CFSE (Life Technologies) at final concentration of 1 µM for 10 min at 37°C. After labeling, cells were washed 3 times and resuspended at 106 cells/mL, 24 hours later, CD3+ cells were preincubated for one hour with test compound(s), and then incubated for 96 h with ImmunoCult Human CD3/CD28/CD2 T Cell Activator (Stem Cell). CFSE staining was quantified by flow cytometry. The proliferation index was calculated according to the number of cell divisions and the percentage of cells in each CFSE peak of the CD3+ population.

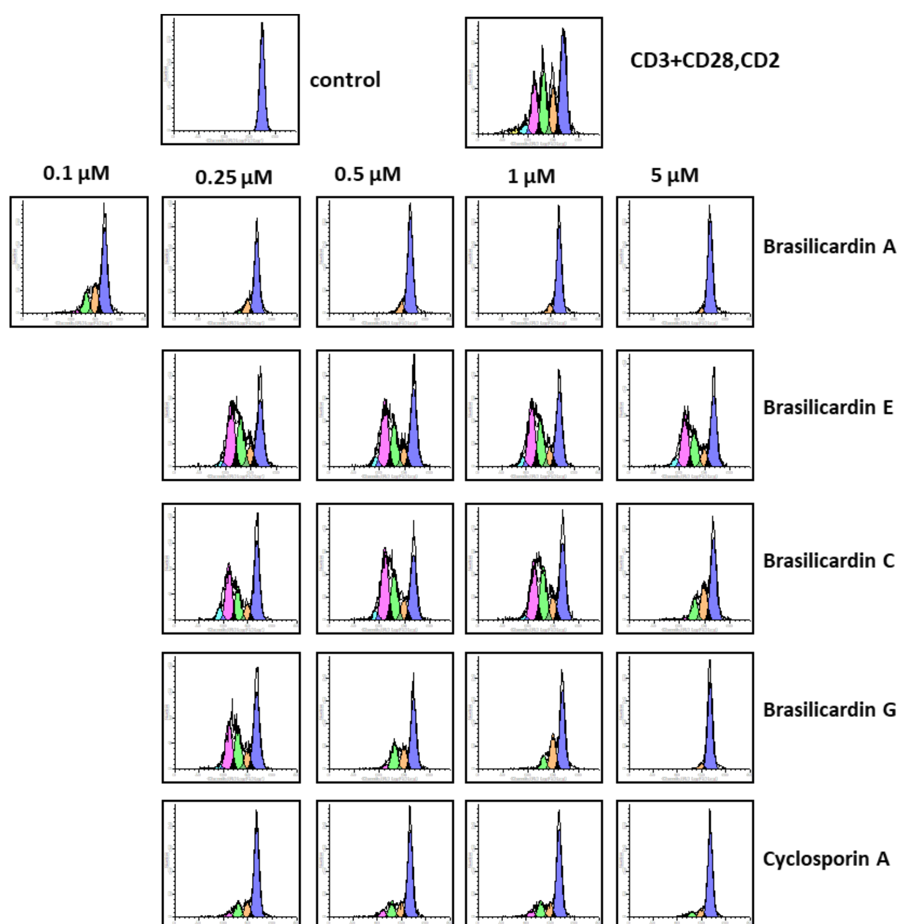


Figure S30. CD3+ Proliferation Assay

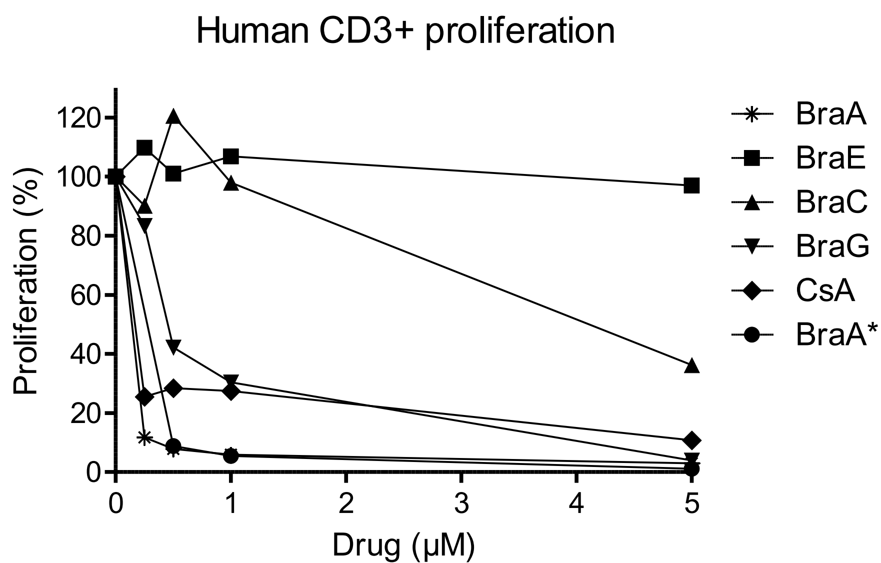


Figure S31. Effect of the brasilicardin analogs on human CD3+ cell proliferation. Data represent the relative proliferation referred to as the stimulated samples in the absence of drugs as 100%. The experiment was repeated three times.
*Synthetic brasilicardin A **1a**. (CsA, cyclosporin A)

6.3 Antiproliferation assay in LN229 cells

The human malignant glioma cell line LN229 was purchased from ATCC (ATCC® CRL-2611™) and cultured in DMEM medium (Corning, 10-013CV) supplemented with 10% fetal bovine serum (FBS) (Corning, 35-010CV). Cells were seeded at a density of 2,000 cells/well in clear bottom 96-well plates (CellBIND®, Corning, 3340). After 24 hours, cells were washed once with Dulbecco's phosphate buffered saline (DPBS) (Corning, 21-030CV). 200 µL F12K medium (Kaighn's Modification of Ham's F-12 Medium; Corning, 10-025CV) supplemented with 10% FBS with the indicated concentrations of test compounds in triplicates were added. Clonal populations were allowed to grow until the control wells (mock treatment) were near confluency (6 days). At end of the assay, 140 µL of supernatant medium were removed from each well and 60 µL of CellTiter-Glo® (Promega, G7570) were added. Cell lysis was induced by placing the plates for three minutes on an orbital shaker. Plates were incubated for 10 min at room temperature to stabilize the luminesce signal. Luminescence was recorded using an EnSpire® Multimode Plate Reader (Perkin Elmer). Background (luminescence from wells incubated in the presence of 10 µM melphalan (Sigma, M2011; CAS 148-82-3) was subtracted and data were normalized to the DMSO (JT Baker, 9224-01) control (proliferation in the absence of any competitor). JPH203, a LAT1 selective inhibitor also known as KYT-0353 (MedKoo, Inc., 406760; CAS 1037592-40-7) was used as positive control. Data were fitted to the Michaelis-Menten equation using GraphPad Prism (Version 8.2.0).

Table S6. Apparent IC_{50} for amino acid starvation in LN229 cells.

Compound	**IC_{50}[a]** **Mean ± SD [µM]**
Brasilicardin A (**1a**)	0.13 ± 0.02
Brasilicardin C (**3**)	15.7 ± 0.3
JPH203	15.0 ± 3

[a] n = 3

6.4 Gabapentin uptake competition assay

The ability of compounds to interact with LAT1 was measured using a radiolabeled competition uptake assay with [^{3}H]-gabapentin ([^{3}H]-GP) (Perkin Elmer, NET1182001MC). LN229 cells were cultured in DMEM medium (Corning, 10-013CV) supplemented with 10 % fetal bovine serum (Corning, 35-010CV). After trypsinization, cells were seeded in 96-well plates (Corning 3903) at a concentration of 10^{4} cells/well and allowed to grow for 72 hours. Cells were washed and then incubated with 50,000 counts per minute (cpm) of [^{3}H]-GP in Dulbecco's phosphate buffered saline (DPBS) (Corning, #21-030CV) with indicated concentrations of test compounds in triplicates for 15 min. Subsequently, cells were washed three times with 100 µl of ice-cold DPBS buffer. Cells were lysed with 150 µl of scintillation fluid (Perkin Elmer, Optiphase Hisafe 3, #1200.437) and radioactivity retained within the cells was measured using a 96-well scintillation counter. Background uptake ([^{3}H]-GP uptake in the presence of 10 mM non-radiolabeled GP was subtracted and data were normalized to the DMSO control ([3H]-GP uptake in the absence of any competitor). Data were fitted to the Michaelis-Menten equation using GraphPad Prism.

Table S7. Apparent affinities for [^{3}H]-gabapentin uptake competition in LN229 cells.

Compound	**IC_{50}[a] Mean ± SD [µM]**
Brasilicardin A (**1a**)	0.04 ± 0.01
Brasilicardin C (**3**)	0.08 ± 0.01
Brasilicardin E (**5**)	0.46 ± 0.04
Brasilicardin G (**7**)	0.25 ±0.04

[a] n = 3

6.5 Viability assays

The viability of tumor cell lines (A-549, HT-29, THP-1, HL-60, JURKAT) and 3T3 fibroblast was determined after 72 hours of drug incubation using Cell Counting Kit-8 according to the manufacturer´s instructions (Sigma Cat. No. 96992). Data were analyzed using GraphPad software in order to calculate the IC_{50} value for each drug.

Table S8. Results of viability assays at 72 h.

	IC_{50} (µM)					
	3T3	**A549**	**HT-29**	**THP-29**	**HL60**	**JURKAT**
Brasilicardin A (**1**)	>10	7.32	6.19	>10	>10	0.203
Brasilicardin C (**3**)	>10	>10	>10	>10	>10	5.66
Brasilicardin E (**5**)	>10	>10	>10	>10	>10	>10
Brasilicardin G (**7**)	>10	7.60	>10	6.06	>10	1.17

6.6 ADME Tests

All *in vitro* assaying for brasilicardin A (**1a**) and brasilicardin C (**3**) was conducted at Quintara Discovery (3825 Bay Center Place, Hayward, CA 94545, USA). Where applicable, all samples were analyzed by LC/MS/MS using an AB Sciex API 4000 instrument, coupled to a Shimadzu LC-20AD LC Pump system. Analytical samples were separated using a Waters Atlantis T3 dC18 reverse phase HPLC column (20 mm x 2.1 mm) at a flow rate of 0.5 mL/min. The mobile phase consisted of 0.1% formic acid in water (solvent A) and 0.1% formic acid in 100% acetonitrile (solvent B). Elution conditions are detailed in the tables below.

Table S9. Gradient conditions.

Time (min)	Flow (µL/min)	%A	%B
0	500	98	2
0.30	500	98	2
1.40	500	2	98
2.00	500	2	98
2.01	500	98	2
2.50	500	98	2

Table S10. MRM conditions.

Compound	Q1	Q3	Mode
Brasilicardin A (**1a**)	893.4	204.1	ESI+
Brasilicardin C (**3**)	570.4	424.2	ESI+

6.6.1. *in vitro* Stability in Plasma

The *in vitro* stability of the test compounds brasilicardin A (**1a**) and brasilicardin C (**3**) was studied in human and mouse plasma (BioIVT, Westbury, NY, USA). The reactions were initiated by the addition of the test compounds (1 mM in DMSO) to preheated plasma solution to yield a final concentration of 1 µM. Aliquots of samples (50 µL) were transferred to four 96-well microtiter plates and incubated at 37 °C with gentle agitation on an orbital shaker. The reaction was stopped at 0, 30, 60 and 240 min, by adding 300 µL quench mixture (50 vol-% acetonitrile, 50 vol-% methanol and 0.05 vol-% formic acid) and vortexed for 20 min. Samples were centrifuged at 4,000 rpm for 15 min. The clear supernatant was transferred to a fresh plate

for LC/MS/MS analysis. Propantheline (1 µM) was used in both plasma matrices to monitor assay performance.

Table S11. Plasma stability results of brasilicardins A and C (test concentration 1 µM).

Matrix	Compound	0 min	30 min	60 min	240 min
Human Plasma	Propantheline	100.0	35.0	6.0	0.0
	Brasilicardin A (**1a**)	100.0	99.2	90.8	97.1
	Brasilicardin C (**3**)	100.0	101.7	102.4	101.4
Mouse Plasma	Propantheline	100.0	71.3	49.0	5.3
	Brasilicardin A (**1a**)	100.0	94.6	98.0	99.5
	Brasilicardin C (**3**)	100.0	92.7	104.3	100.2

6.6.2 *in vitro* Stability in Simulated Intestinal and Gastric Fluids fluid (SIF & SGF)

Both, SIF and SGF were prepared following US Pharmacopeia protocols. Assessment of the stability for Brasilicardin A (**1a**) and Brasilicardin C (**3**) in simulated intestinal fluid (SIF) and in simulated gastric fluid (SGF) was performed is essence as described for the stability of **1a** and **3** in plasma (*vide infra*). For SGF assays, compounds were incubated in SGF at pH 1.2 with 3.2 g/L of pepsin. For SIF assays, compounds were incubated in SIF at pH 6.8 containing 1% of pancreatin. Omeprazole at 1 µM was used in the SGF assay and BUN (blood urea nitrogen) at 1 µM was used in the SIF assay to monitor assay performance.

Table S12. Results of the SIF and SGF analyses (test concentration 1 µM).

Matrix	Compound	0 min	30 min	60 min	240 min
SGF with pepsin	Omeprazole	100.0	0.0	0.0	0.0
	Brasilicardin A (**1a**)	100.0	100.2	102.5	102.9
	Brasilicardin C (**3**)	100.0	98.9	99.8	100.4
SIF with pancreatin	Bun	100.0	24.5	6.3	1.4
	Brasilicardin A (**1a**)	100.0	101.5	111.9	113.8
	Brasilicardin C (**3**)	100.0	98.0	98.9	101.4

6.6.3. Caco-2 Transwell Permeability

Caco-2 cells were maintained for 21 days at 37 ºC with 5% CO_2 and handled according to manufacturer's instructions (ReadyCell, Barcelona, Spain). Cells were washed with Hank's Balanced Salt Solution (HBSS) with 5 mM HEPES buffer ((4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) for 30 min before starting the experiment. Prior to the experiment integrity of the cell monolayer was verified by transendothelial electrical resistance (TEER) measurements. All the wells had high resistance above the acceptance cut-off (1 kΩ). Solutions of test compounds **1a** and **3** were prepared by diluting DMSO stock solutions (1 mM) into HBSS buffer resulting in a final DMSO concentration of 0.1 %. Transport experiments were initiated by adding test compounds to the apical (75 µL) or basal (250 µL) side with a final concentration of 5 µM. Transport plates were incubated at 37 ºC in a humidified incubator with 5 % CO_2. Samples (5 µL and 50 µL) were taken from the donor and acceptor compartments to a clean 96-well plate after one hour of incubation. Subsequently, quench solution (100 % acetonitrile plus 0.1% formic acid) containing internal standards (bucetin for positive ESI) was added to each well. Plates were sealed, vortexed, and centrifuged at 4 °C for 15 min at 4,000 RPM. The supernatant was transferred to fresh 96-well plates and analyzed by liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS). Digoxin (P-gp efflux substrate) at 5 µM and propranolol (high passive permeability) at 5 µM were used as the reference controls.

Table S13. Results of the Caco-2 transwell permeability test (test concentration 5 µM).

Compound	$P_{app, A\text{-}B}$ (x10^{-6} cm/s)		$P_{app, B\text{-}A}$ (x10^{-6} cm/s)		Ratio B-A/A-B	Recover Rate (%)
	Value	Mean	Value	Mean		
Digoxin	1.3	1.3	7.9	8.3	6.6	106
	1.3		8.7			
Propranolol	20.2	19.3	15.1	15.5	0.8	90
	18.4		15.9			
Brasilicardin A (**1a**)	0.01	0.01	0.08	0.05	4.6	87
	0.01		0.03			
Brasilicardin C (**3**)	0.07	0.06	0.07	0.07	1.2	92
	0.04		0,07			

6.6.4 Hepatocyte Stability

Cryopreserved hepatocytes were removed from the liquid nitrogen tank and thawed quickly in a 37 °C water bath. As soon as the cells pulled away from the vial wall, they were decanted into 48 mL of warm HT medium (Gibco® HT Supplement consisting of sodium hypoxanthine (10 mM) and thymidine (1.6 mM); ThermoFisher Scientific, USA). Cells were centrifuged for four minutes at 420 RPM (50 g). After removing the supernatant, the pellet was re-suspended in 4 mL of warm DMEM medium (Dulbecco's Modified Eagle Medium; Thermo Fisher Scientific, USA). Cell density was counted by a hemocytometer. The assay was carried out in 96-well microtiter plates. Test compounds **1a** and **3** were incubated at 1 µM with 0.5 million cells/mL hepatocytes in the DMEM medium for 0, 60, 120, and 180 minutes at 37 °C. The volume of the incubation mixture was 50 µL with a final concentration of 0.1 % DMSO. At each of the time points, incubation was stopped by adding 150 µL quenching solutions (100% acetonitrile plus 0.1% formic acid). Subsequently, the mixtures were vortexed vigorously for 20 min and centrifuged at 4,000 RPM at a temperature of 4 °C. The supernatants (80 µL) were transferred to a clean 96-well plate and analyzed by LC/MS/MS. Midazolam at 1 µM with a final concentration of 0.1 % of DMSO was included as a positive control to verify assay performance.

Table S14. Results of the hepatocyte stability test.

Species	**Compound**	**0 min**	**60 min**	**120 min**	**180 min**
		Value	**Value**	**Value**	**Value**
Human	Midazolam	100.0	30.7	10.5	3.1
	Brasilicardin A (**1a**)	100.0	97.9	87.0	86.0
	Brasilicardin C (**3**)	100.0	96.1	90.3	92.2
Mouse	Midazolam	100.0	18.8	5.3	0.9
	Brasilicardin A (**1a**)	100.0	102.5	73.8	69.8
	Brasilicardin C (**3**)	100.0	105.2	92.3	90.6

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