Supplementary Materials

Discovery of Stealthin Derivatives and Implication of the Amidotransferase FlsN3 in the Biosynthesis of Nitrogen-containing Fluostatins

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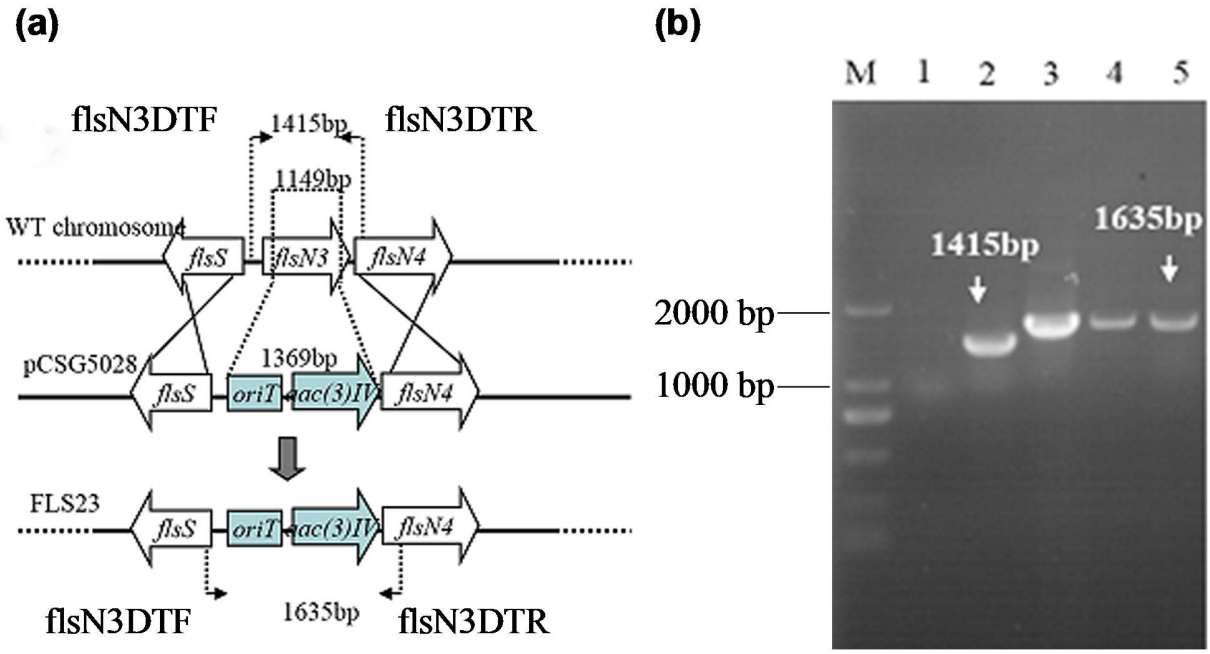
Figure S1. Construction *flsN3* inactivation mutant Δ*flsN3*



(a) Description of *flsN3* inactivation. FLS23 was constructed by replacing a 1149 bp internal *flsN3* fragment with a 1369 bp DNA fragment containing *oriT* and *acc3(IV)* in pCSG5028, resulting from a double cross-over recombination event. The location of the diagnostic PCR primers were indicated. Sizes of PCR products were also indicated: 1415 bp for the wild type strain *M. rosaria* SCSIO N160 and 1635 bp for the mutant FLS23.

(b) Gel electrophoresis of PCR products. DNA templates were from: ddH_2O (negative control, lane 1), pCSG5001 (negative control, lane 2), pCSG5017 (positive control, lane 3), Δ*flsN3* clone #1 (lane 4), Δ*flsN3* clone #2 (lane 5) and DNA marker D2000 (GenStar, lane M).

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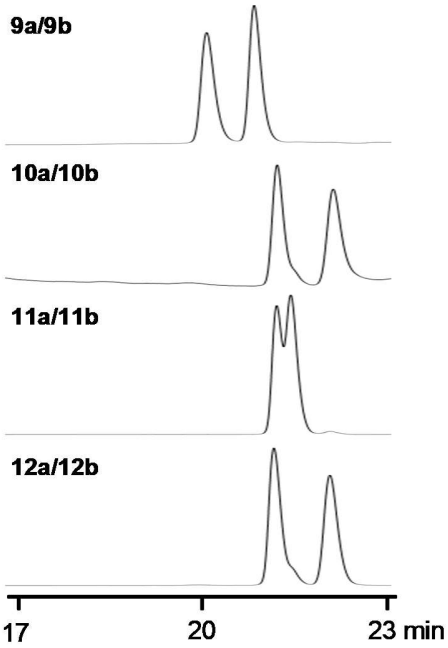
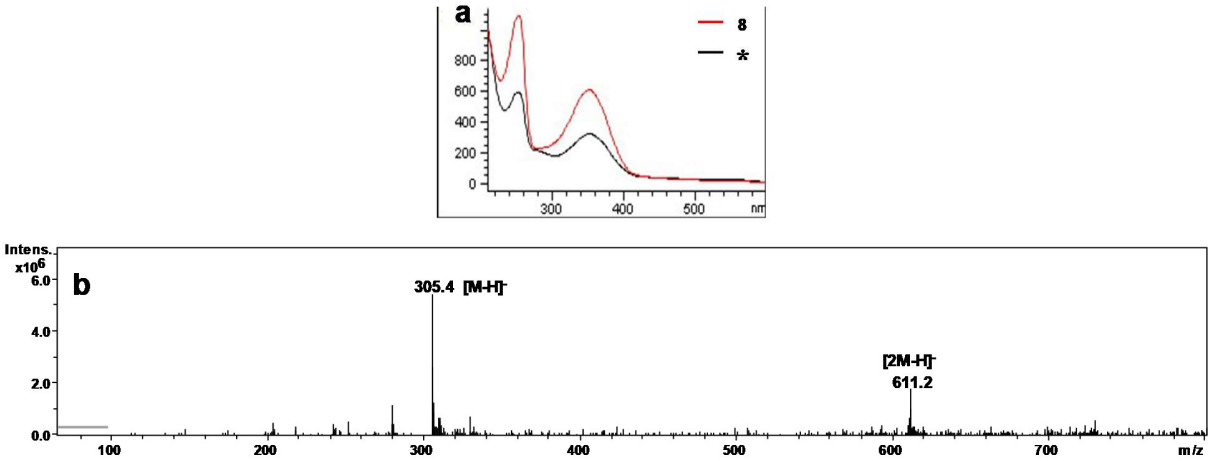
Figure S2. Chiral HPLC analysis of **9**−**12**



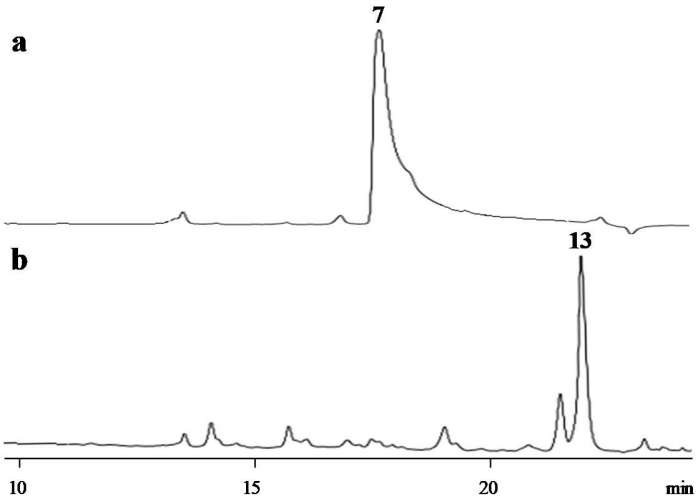
The chiral HPLC analysis was performed on an Agilent 1260 Infinity series instrument with a Chiral ND 5u (4.6 × 250 mm) chiral column (Phenomenex, Washington, CD, USA). The elution process runs the following program: 5% B to 80% B (linear gradient, 0–20 min), 80% B to 100% B (20–21 min), 100% B (isocratic elution, 21–24 min), 100% B to 5% B (24–25 min), 5% B (isocratic elution, 25–30 min). The solvent system comprises solvent A (10% acetonitrile in water supplemented with 0.08% formic acid) and B (90% acetonitrile in water). The monitoring wavelength at 430 nm.

Figure S3. UV and LC-MS analysis of the analogue related to salinipyrone A (**8**)



a UV comparison of salinipyrone A (**8**) with the product with the symbol "*"; **b** Negative mode ESI-MS data for the product with the symbol "*".

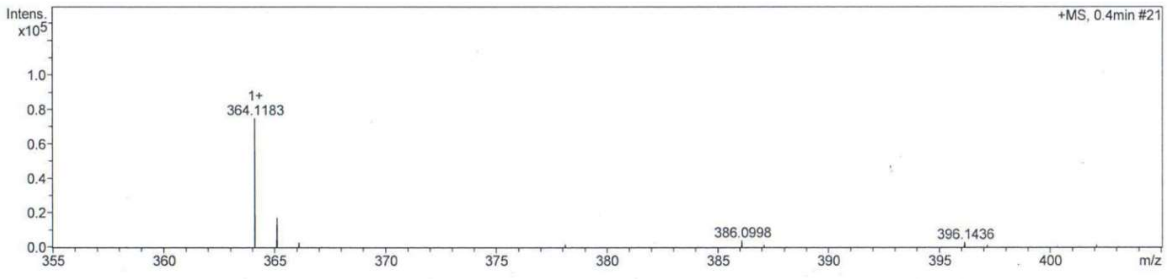
Figure S4. HPLC traces of the methylation of stealthin C (**7**)



a Stealthin C (**7**) standard; **b 7** was treated with methyl iodide.

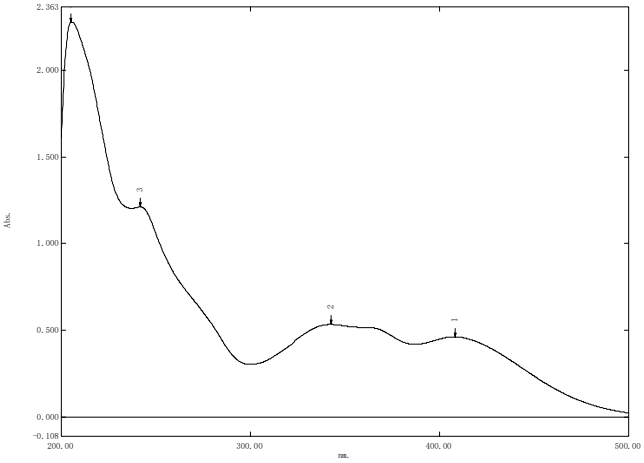
Figure S5. HRESIMS (**a**), UV (**b**), IR (**c**) of stealthin D (**9**)

(**a**). **HR-ESI-MS**



Meas. m/z	#	Ion Formula	Score	m/z	err [ppm]	err [mDa]	mSigma	rdb	e⁻ Conf	N-Rule
364.118291	1	C21H18NO5	100.00	364.117949	0.9	0.3	1.2	13.5	even	ok
386.099771	1	C21H17NNaO5	100.00	386.099893	0.3	0.1	16.0	13.5	even	ok
396.143566	1	C22H22NO6	100.00	396.144164	-1.5	-0.6	22.2	12.5	even	ok

(**b**). **UV**



(**c**). **IR**

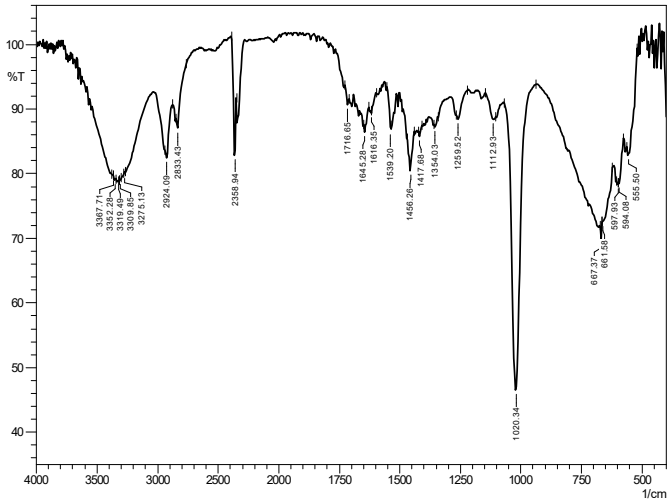
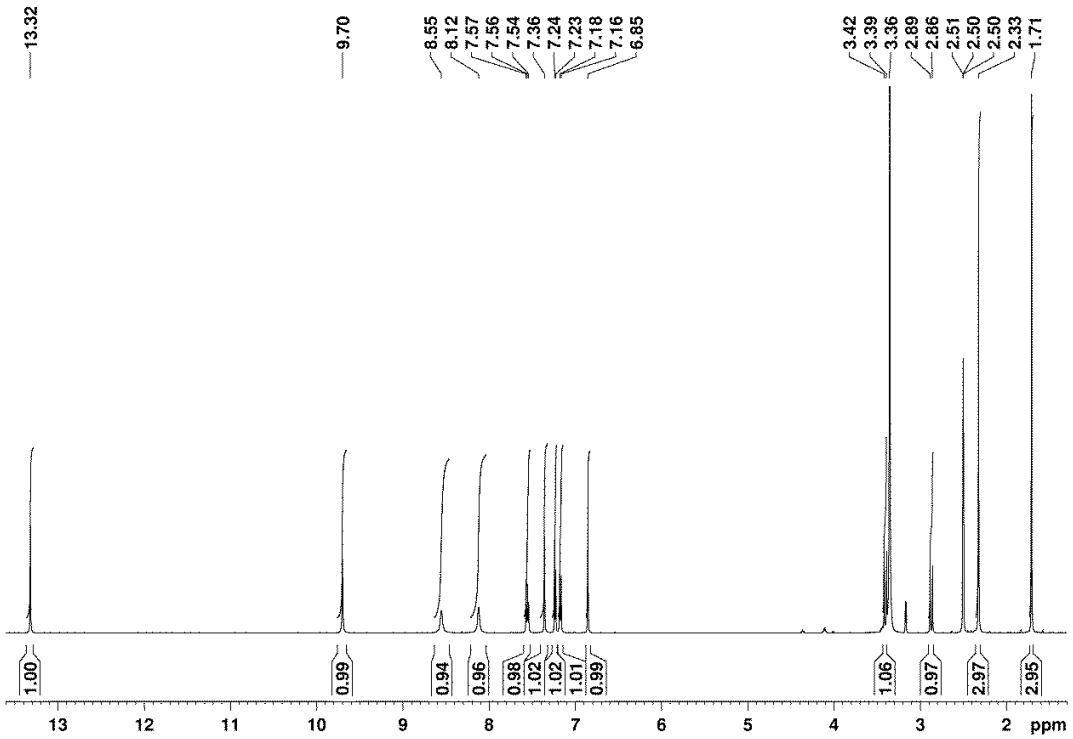


Figure S6. The ^{1}H NMR spectrum of stealthin D (**9**) in DMSO-d_6



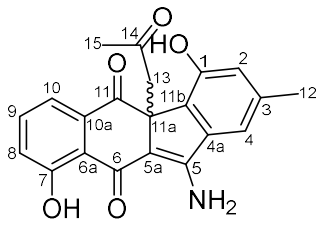


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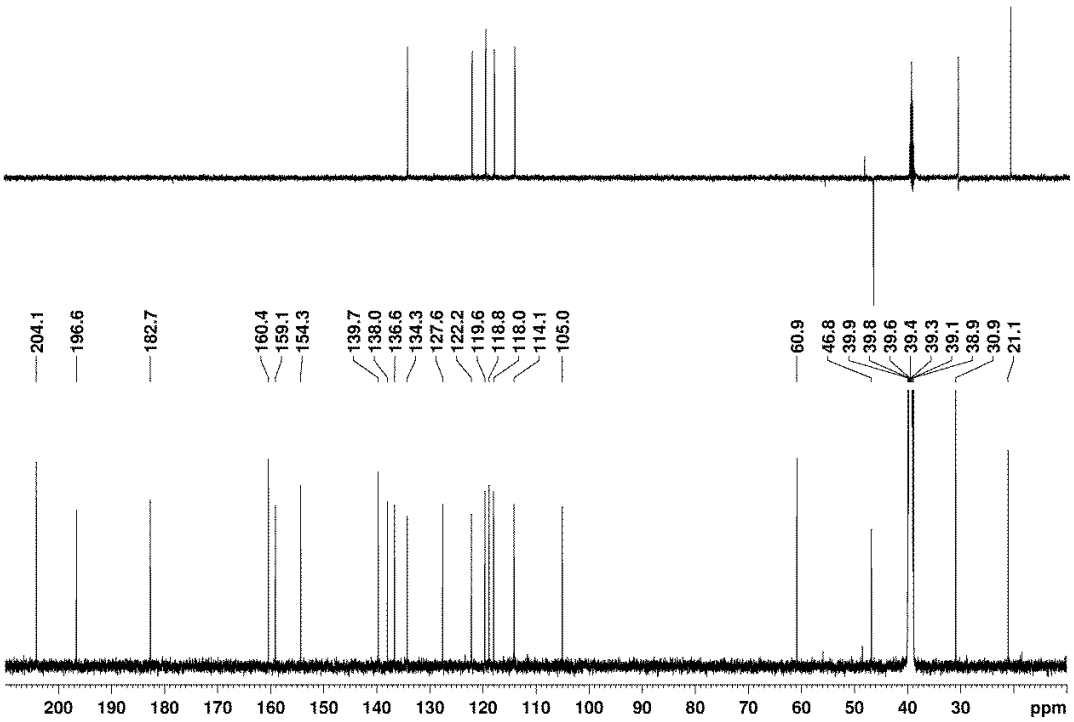
Figure S7. The ^{13}C and DEPT 135 NMR spectra of stealthin D (**9**) in DMSO-d_6



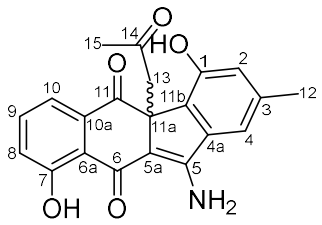


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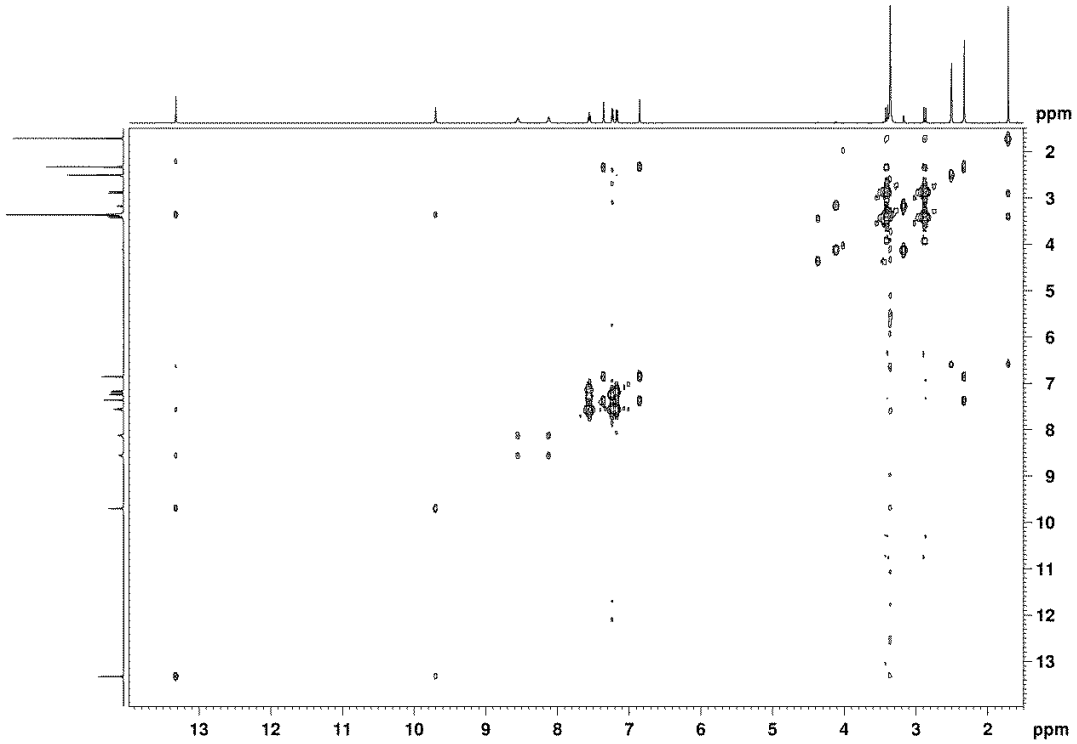
Figure S8. The ^{1}H-^{1}H COSY spectrum of stealthin D (**9**) in DMSO-d_6



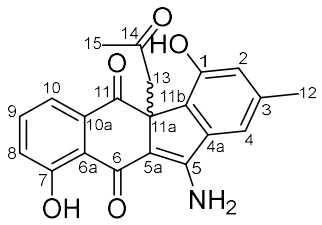


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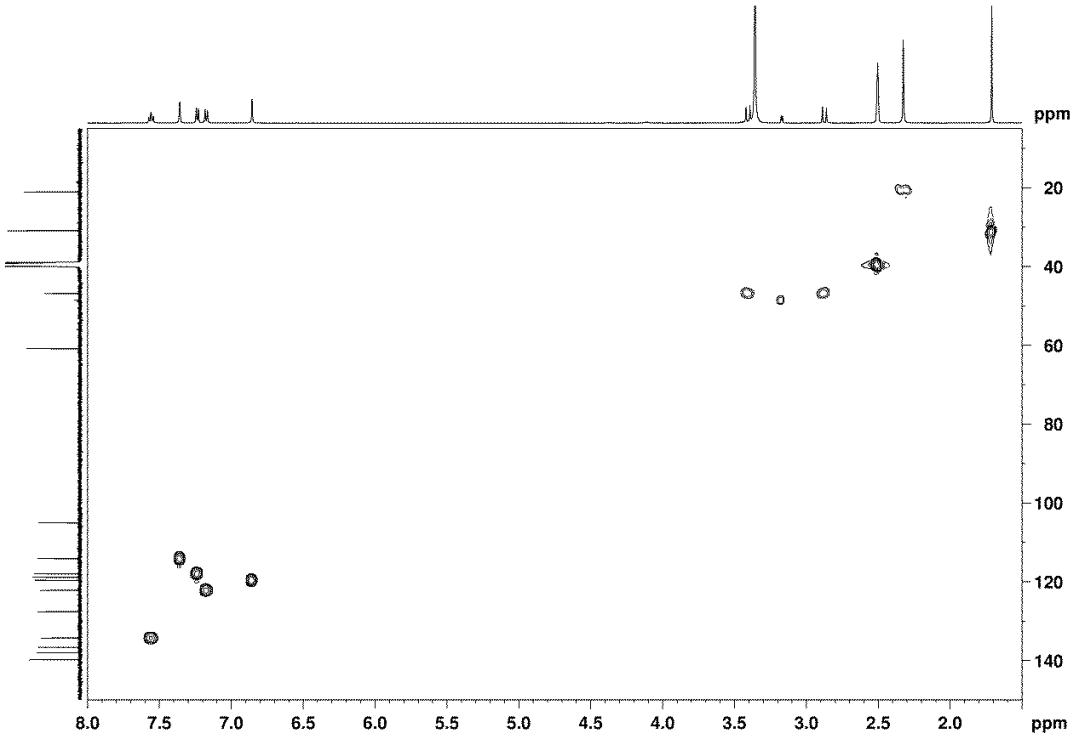
Figure S9. The HSQC spectrum of stealthin D (**9**) in DMSO-d_6



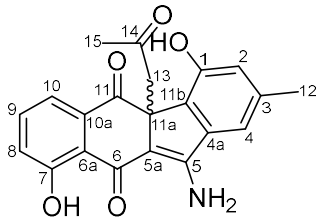


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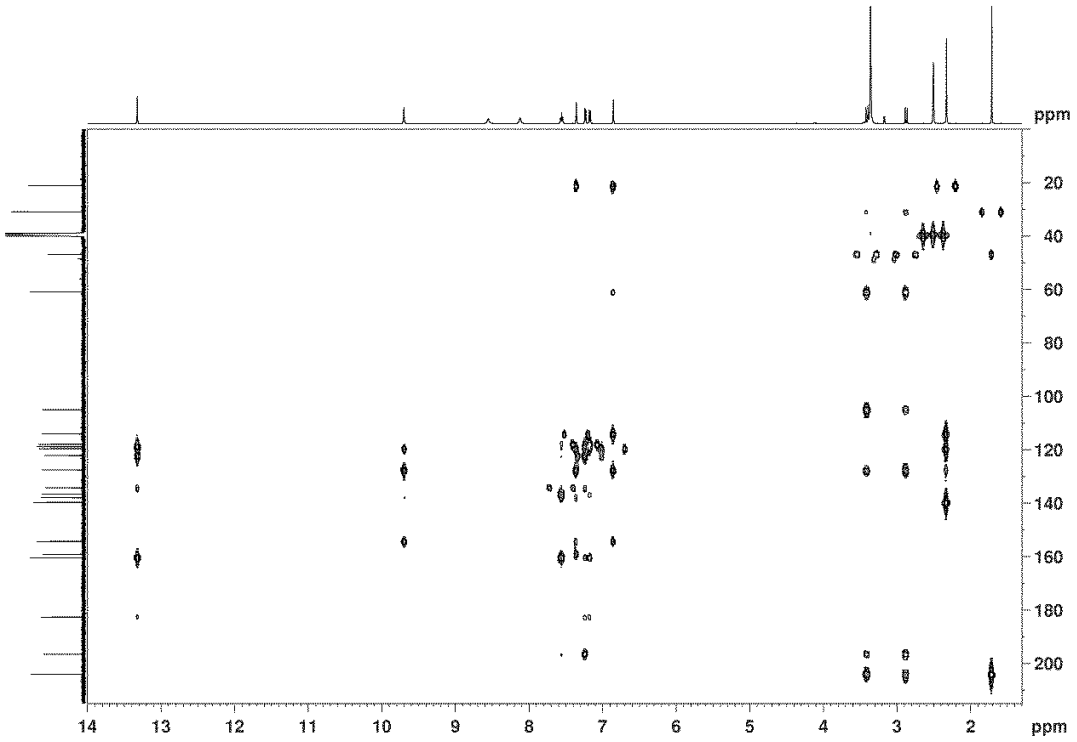
Figure S10. The HMBC spectrum of stealthin D (**9**) in DMSO-d_6



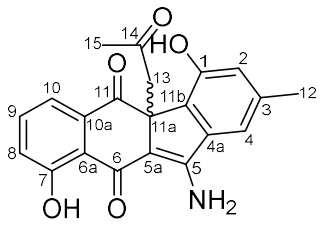


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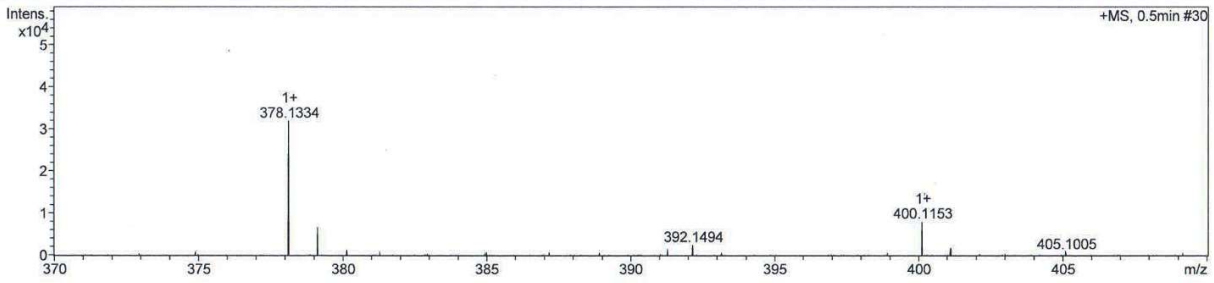
Figure S11. The HRESIMS spectrum of stealthin E (**10**)



Meas. m/z	#	Ion Formula	Score	m/z	err [ppm]	err [mDa]	mSigma	rdb	e⁻ Conf	N-Rule
378.133362	1	C22H20NO5	100.00	378.133599	-0.6	-0.2	18.5	13.5	even	ok
	2	C23H16N5O	24.31	378.134937	-4.2	-1.6	35.0	18.5	even	ok
400.115293	1	C22H19NNaO5	100.00	400.115543	-0.6	-0.3	5.7	13.5	even	ok
	2	C23H15N5NaO	36.37	400.116881	-4.0	-1.6	19.5	18.5	even	ok
777.240142	1	C40H34N8NaO8	85.01	777.239181	1.2	1.0	65.6	27.5	even	ok
	2	C44H38N2NaO10	48.87	777.241866	-2.2	-1.7	67.5	26.5	even	ok

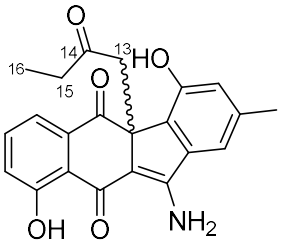


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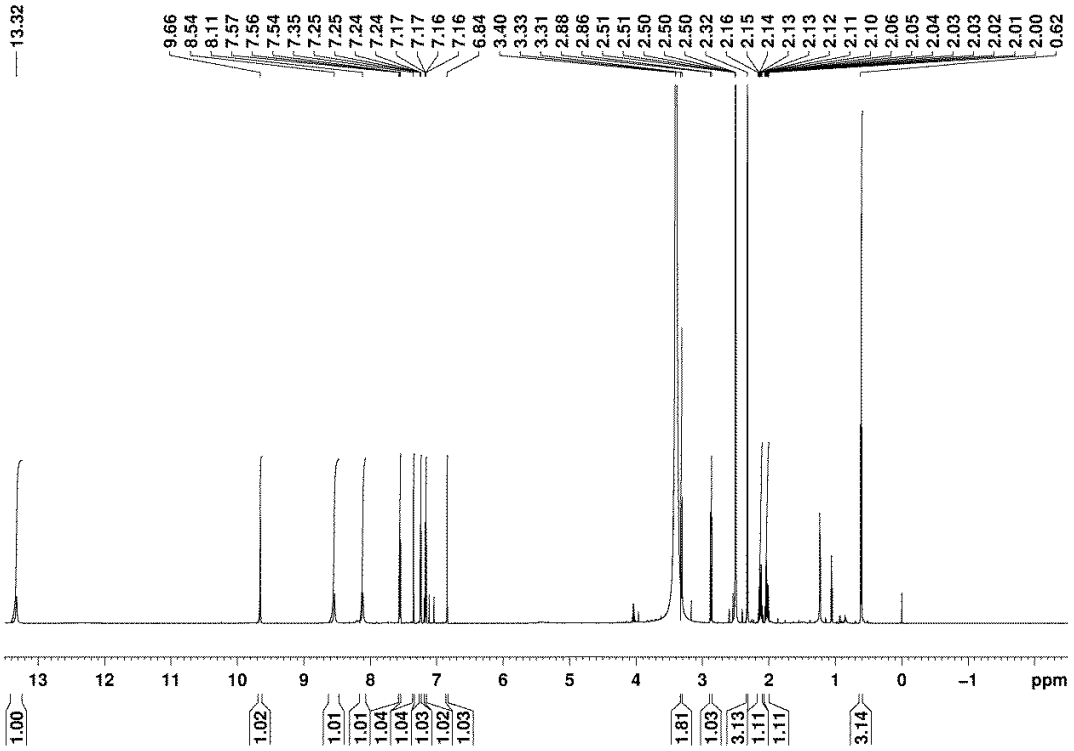
Figure S12. The ^{1}H NMR spectrum of stealthin E (**10**) in DMSO-d_6



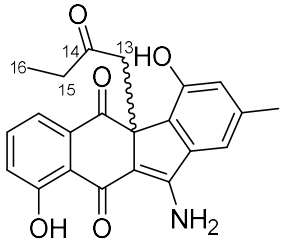


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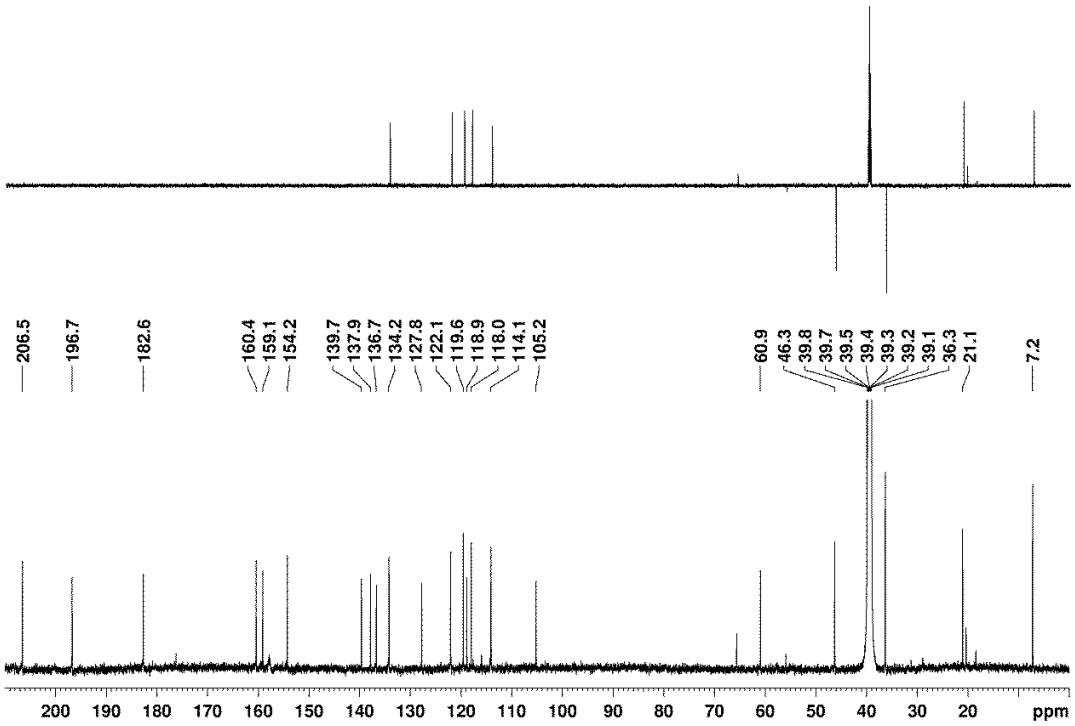
Figure S13. The ^{13}C NMR and DEPT 135 spectra of stealthin E (**10**) in DMSO-d_6



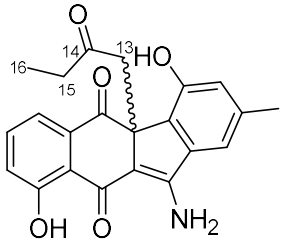


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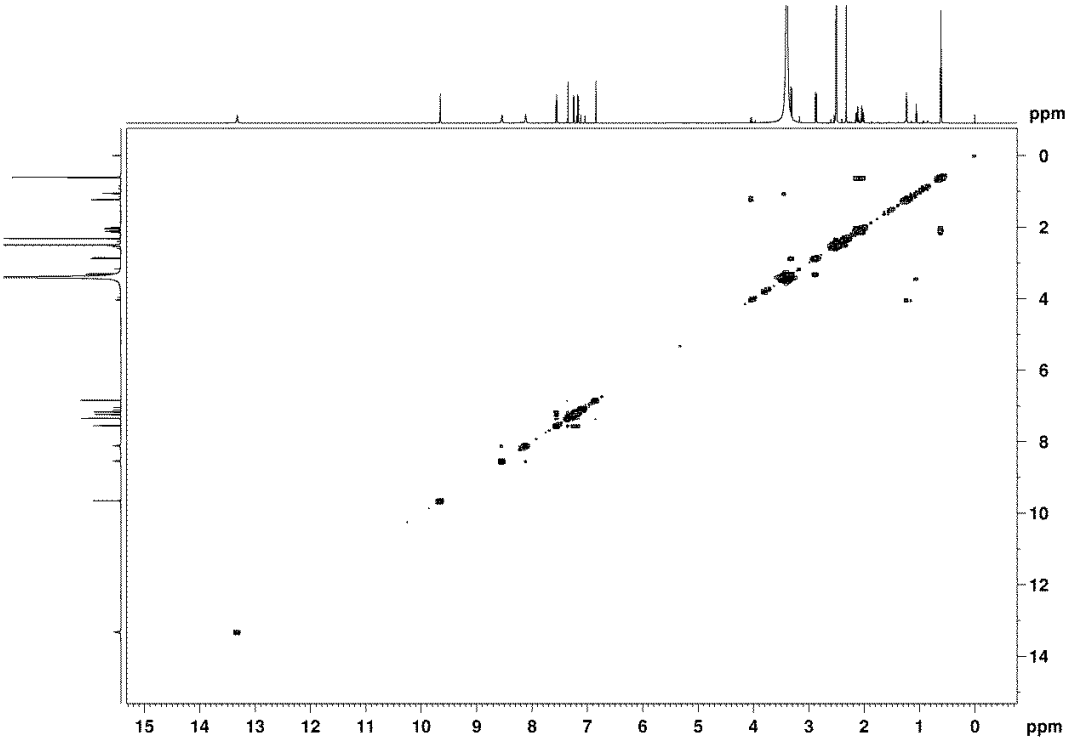
Figure S14. The ^{1}H-^{1}H COSY spectrum of stealthin E (**10**) in DMSO-d_6



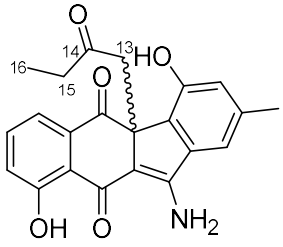


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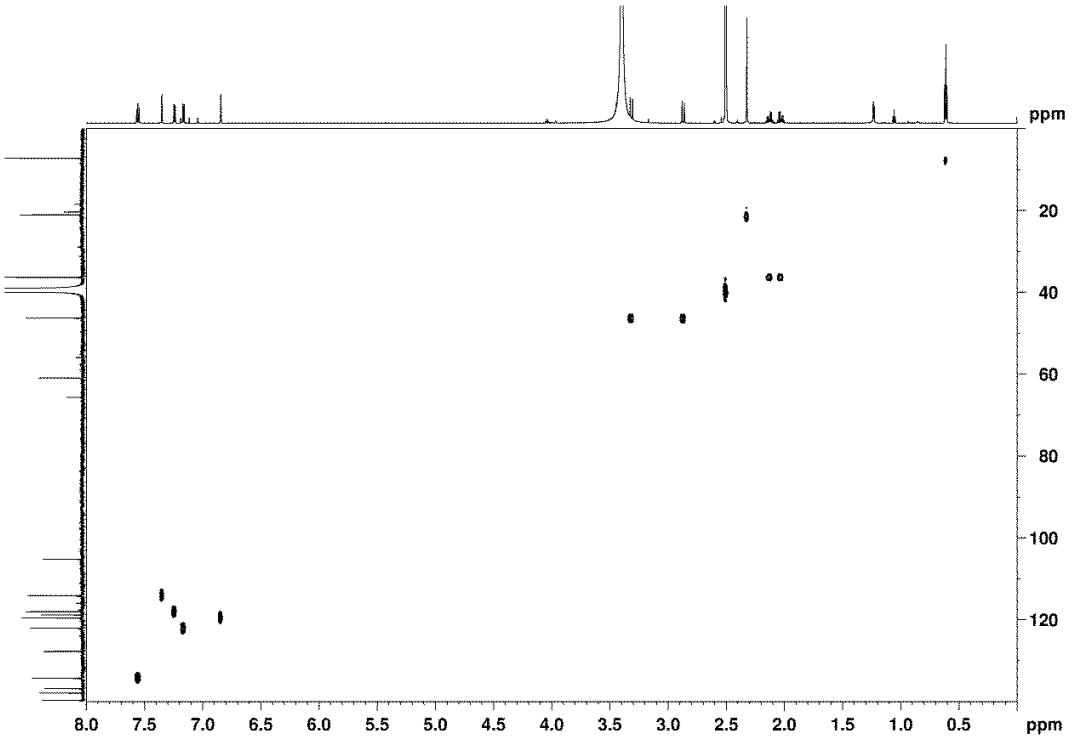
Figure S15. The HSQC spectrum of stealthin E (**10**) in DMSO-d_6



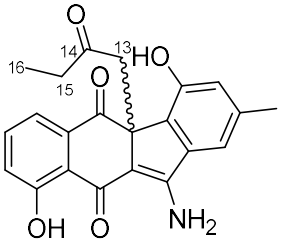


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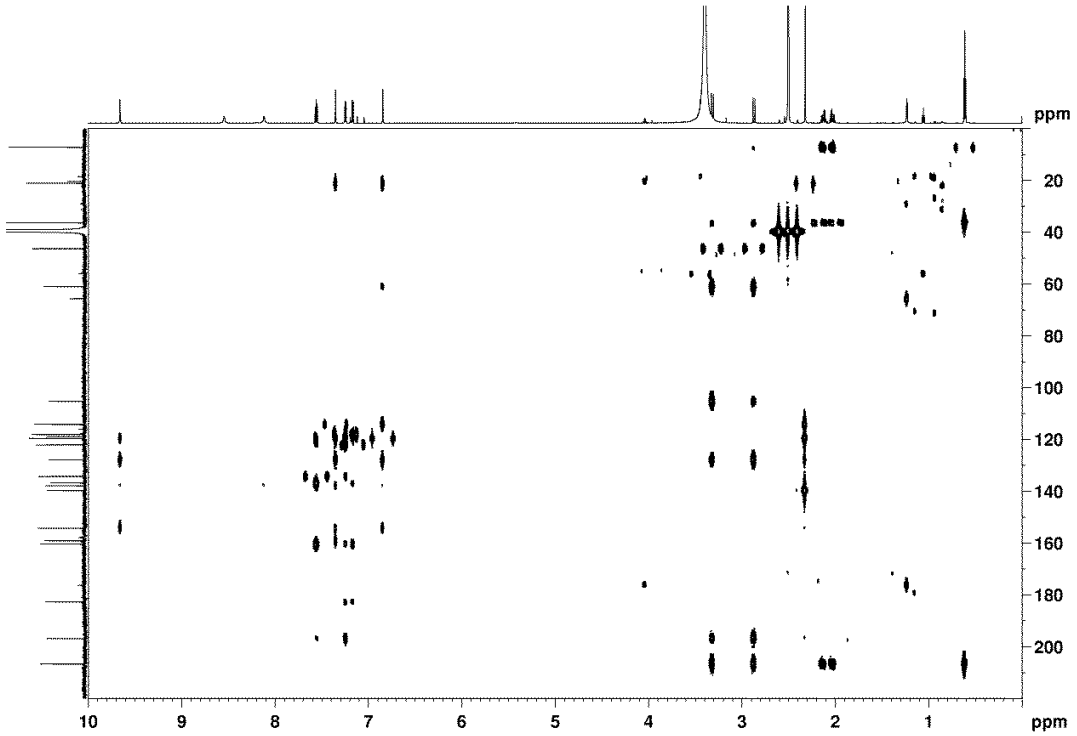
Figure S16. The HMBC spectrum of stealthin E (**10**) in DMSO-d_6



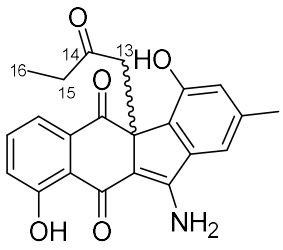


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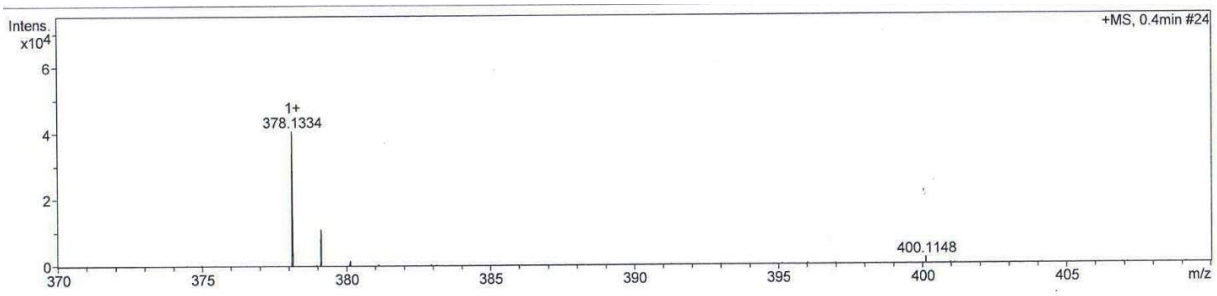
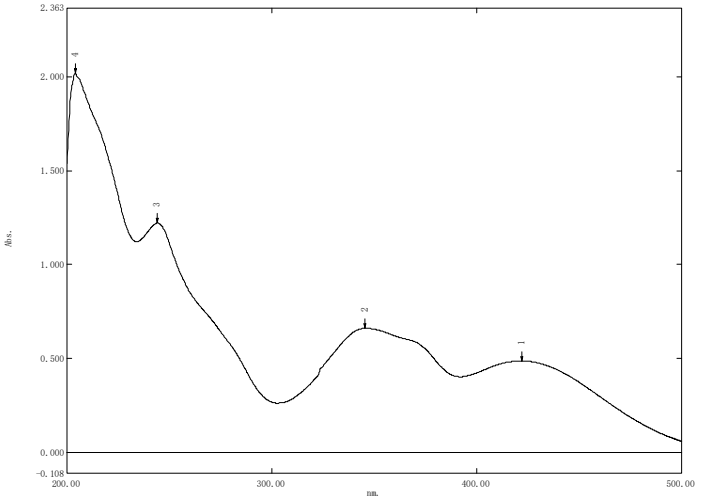
Figure S17. HRESIMS (**a**), UV (**b**), IR (**c**) of stealthin F (**11**)

(**a**). **HR-ESI-MS**



Meas. m/z	#	Ion Formula	Score	m/z	err [ppm]	err [mDa]	mSigma	rdb	e⁻ Conf	N-Rule
378.133429	1	C22H20NO5	100.00	378.133599	-0.5	-0.2	13.1	13.5	even	ok
400.114831	1	C22H19NNaO5	100.00	400.115543	1.8	0.7	38.7	13.5	even	ok
755.258947	1	C44H39N2O10	100.00	755.259922	1.3	1.0	18.7	26.5	even	ok

(**b**). **UV**



(**c**). **IR**

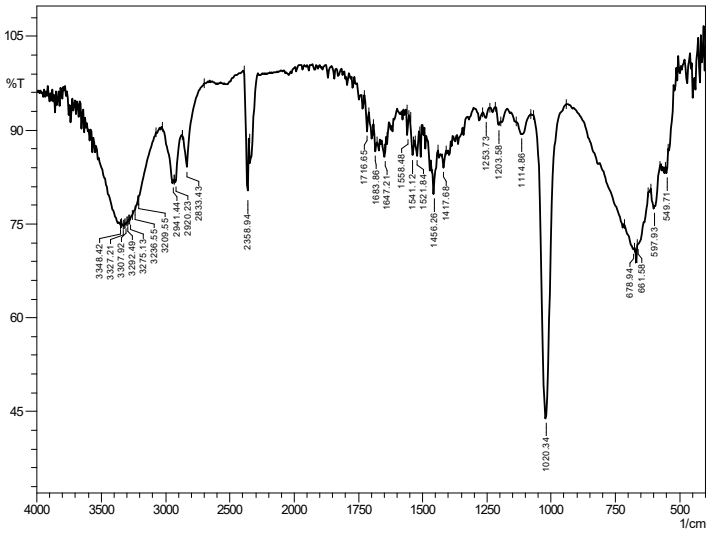
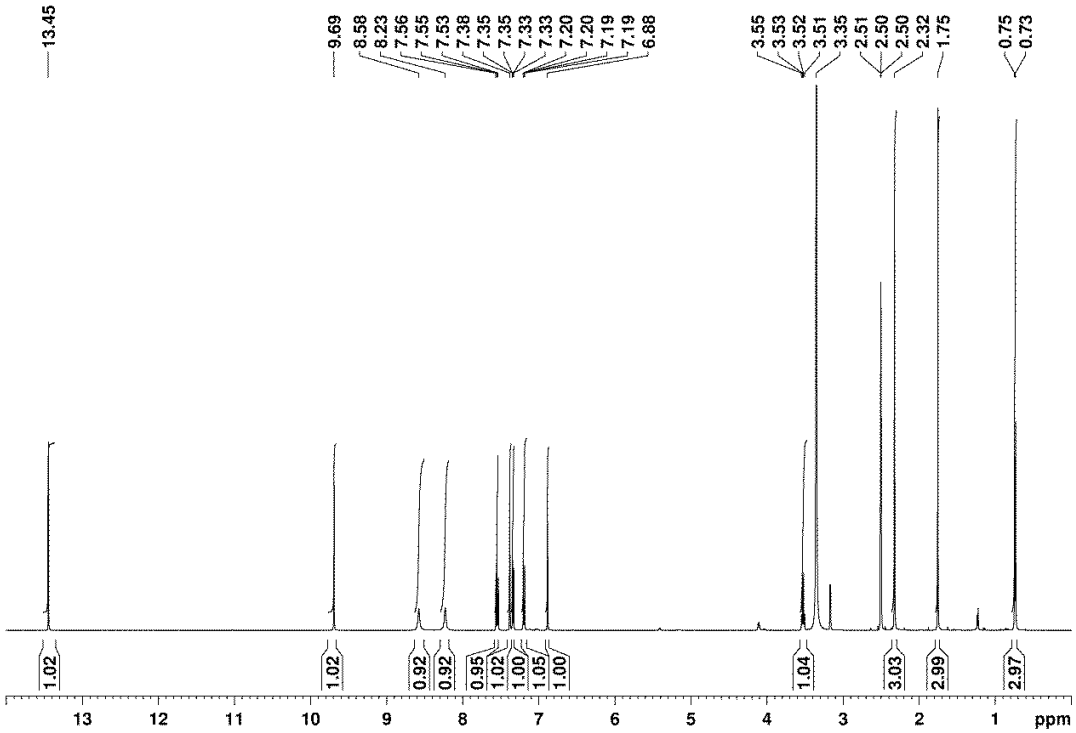
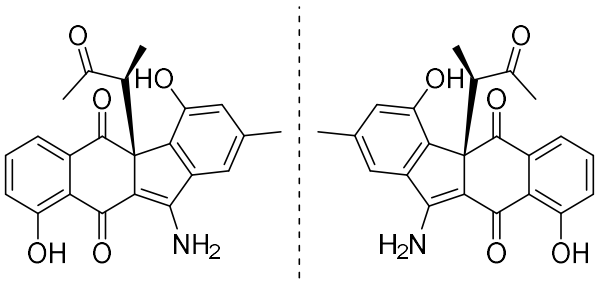


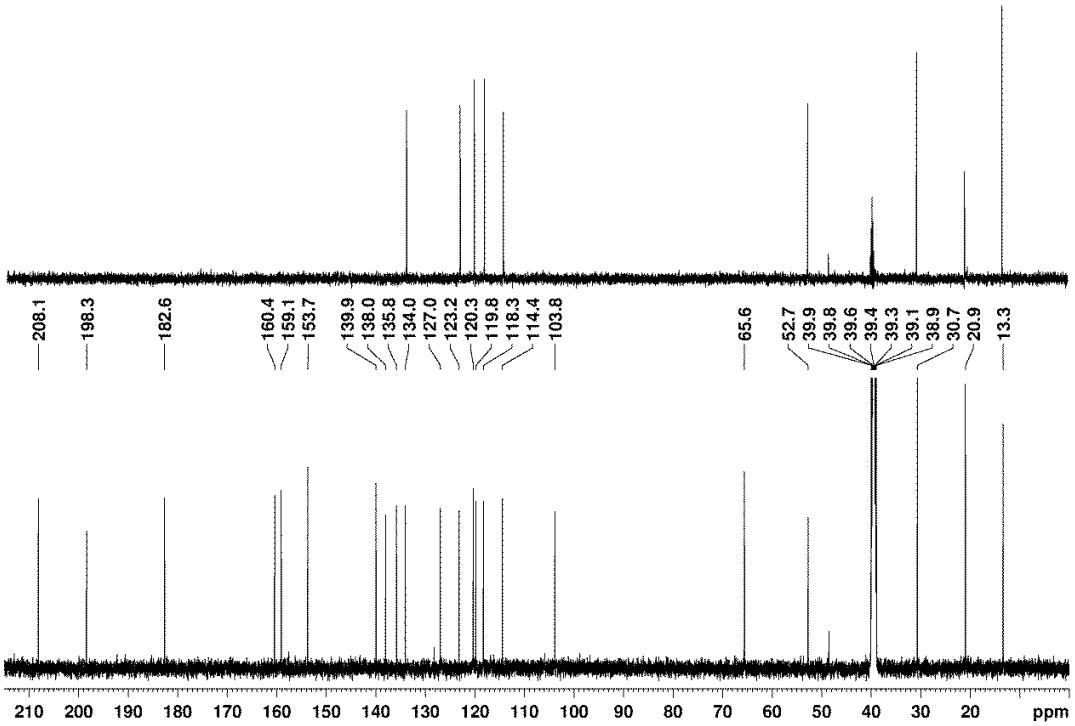
Figure S18. The ^{1}H NMR spectrum of stealthin F (**11**) in DMSO-d_6

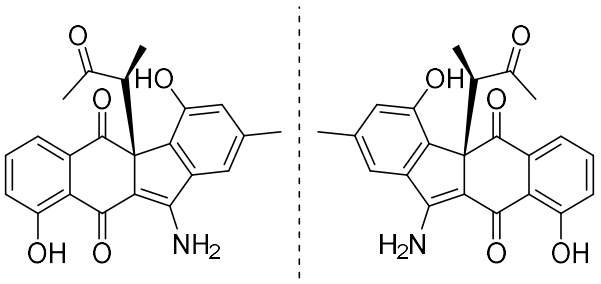




11a/11b

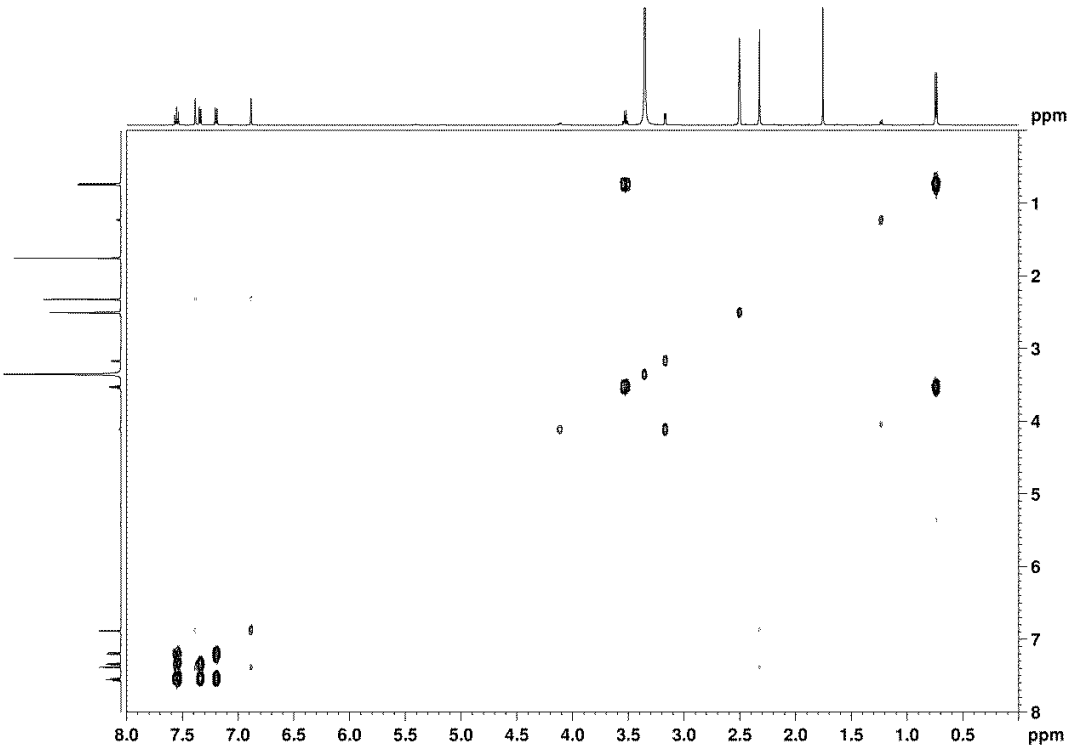
Figure S19. The ^{13}C NMR and DEPT 135 spectra of stealthin F (**11**) in DMSO-d_6

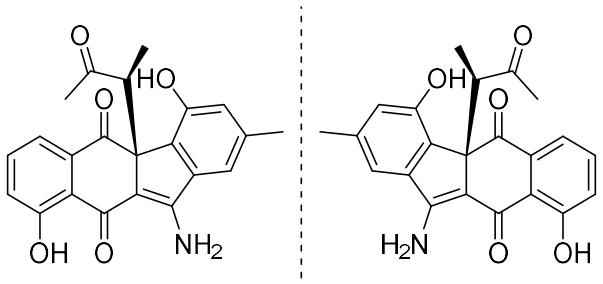




11a/11b

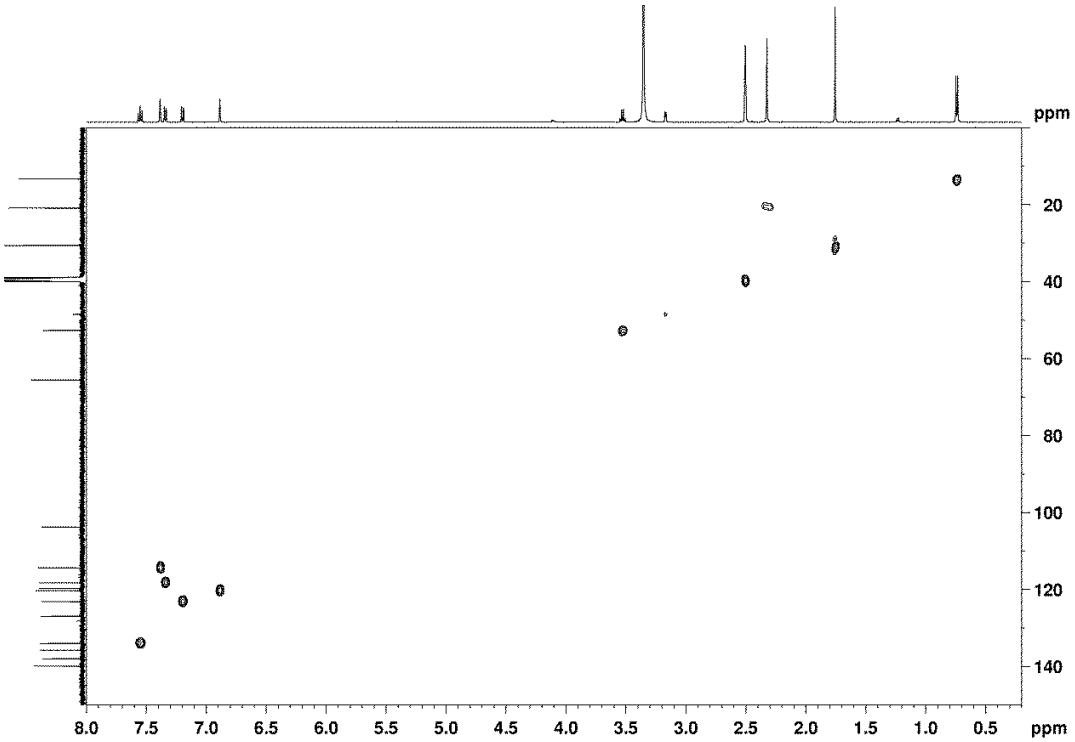
Figure S20. The ^{1}H-^{1}H COSY spectrum of stealthin F (**11**) in DMSO-d_6

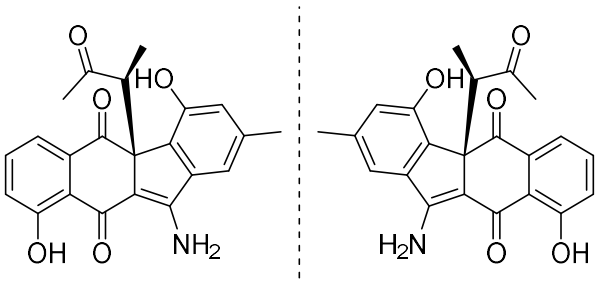




11a/11b

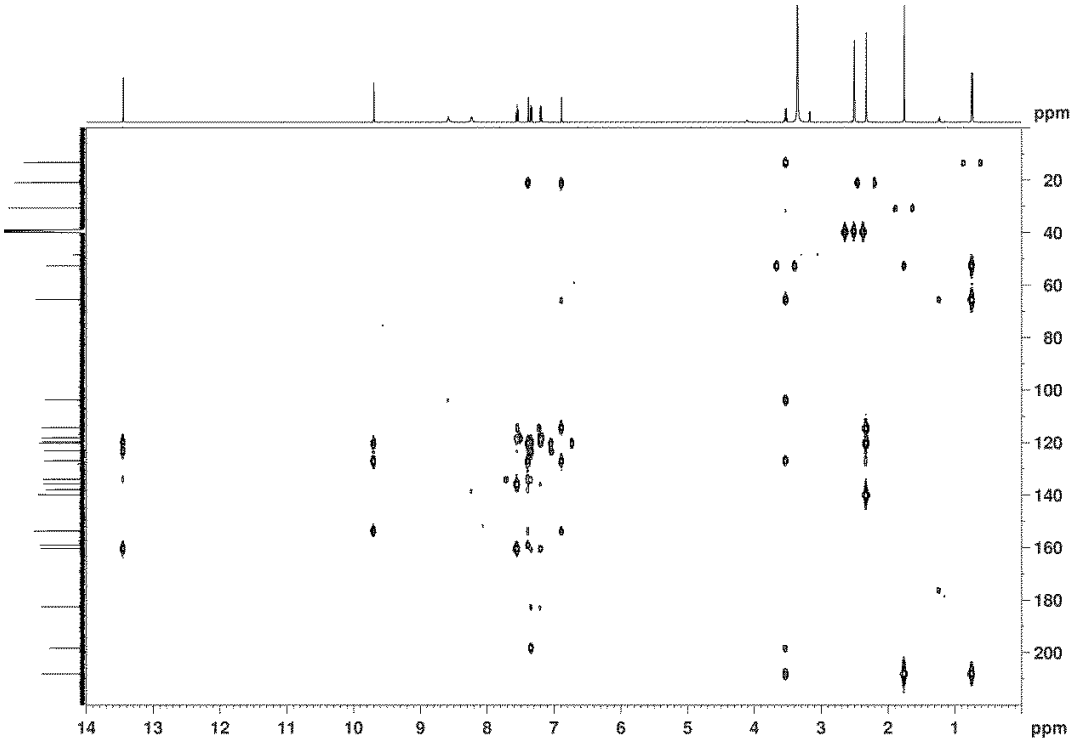
Figure S21. The HSQC spectrum of stealthin F (**11**) in DMSO-d_6

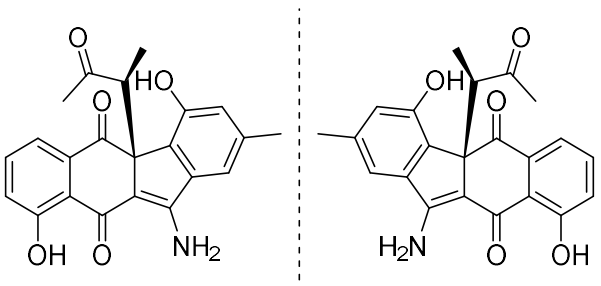




11a/11b

Figure S22. The HMBC spectrum of stealthin F (**11**) in DMSO-d_6

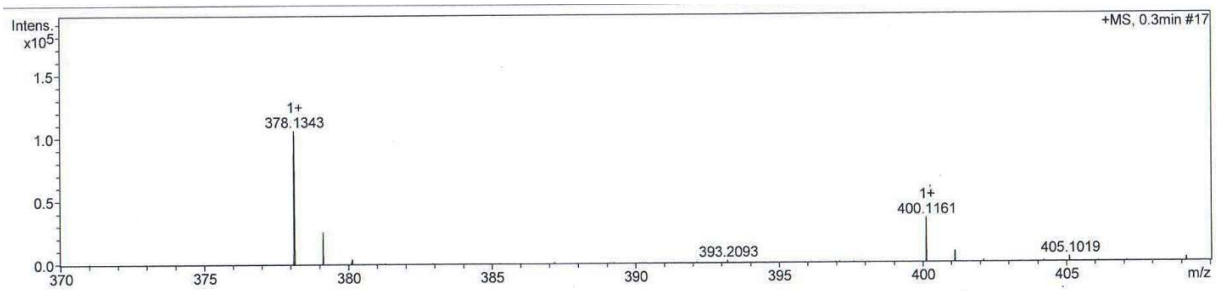




11a/11b

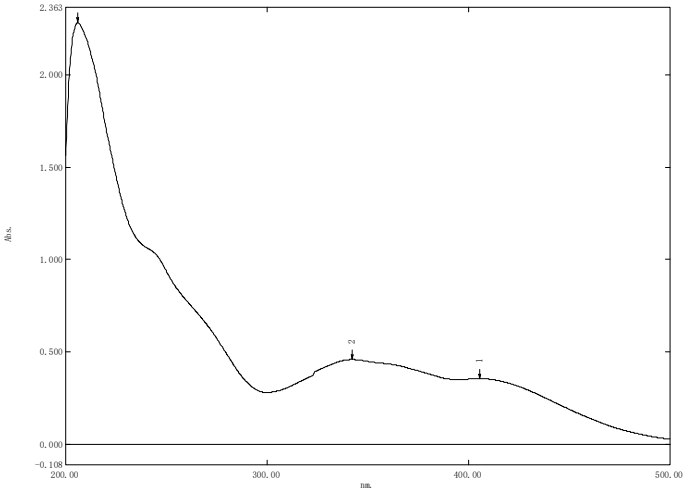
Figure S23. HRESIMS (**a**), UV (**b**), IR (**c**) of stealthin G (**12**)

(**a**). **HR-ESI-MS**



Meas. m/z	#	Ion Formula	Score	m/z	err [ppm]	err [mDa]	mSigma	rdb	e⁻ Conf	N-Rule
378.134279	1	C22H20NO5	100.00	378.133599	-1.8	-0.7	0.4	13.5	even	ok
	2	C23H16N5O	59.55	378.134937	-1.7	-0.7	13.9	18.5	even	ok

(**b**). **UV**



(**c**). **IR**

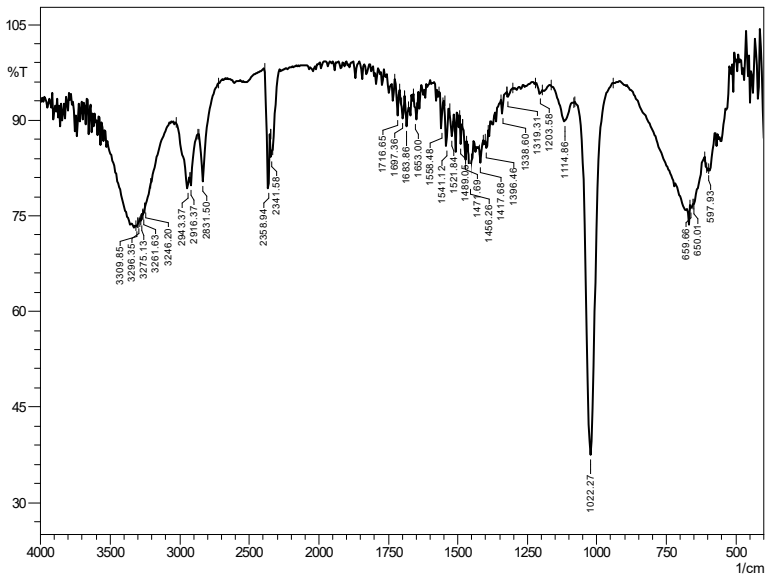
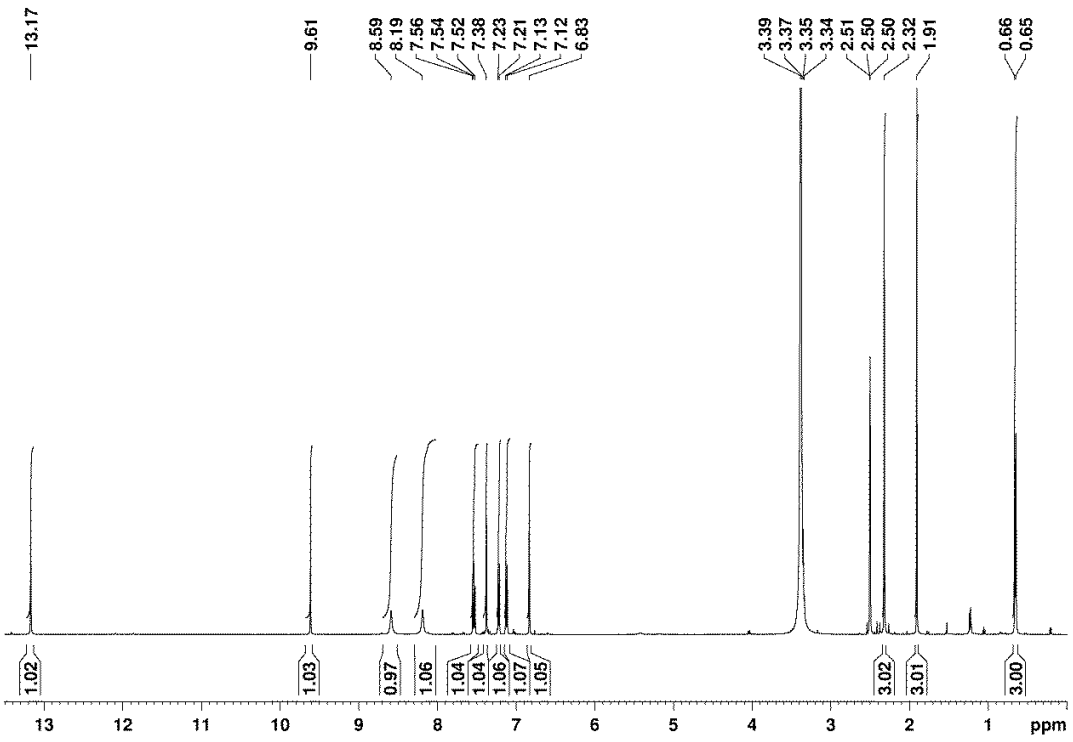
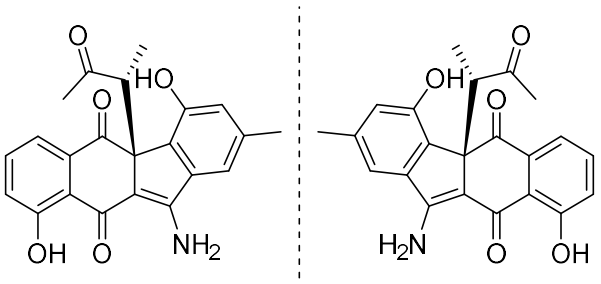


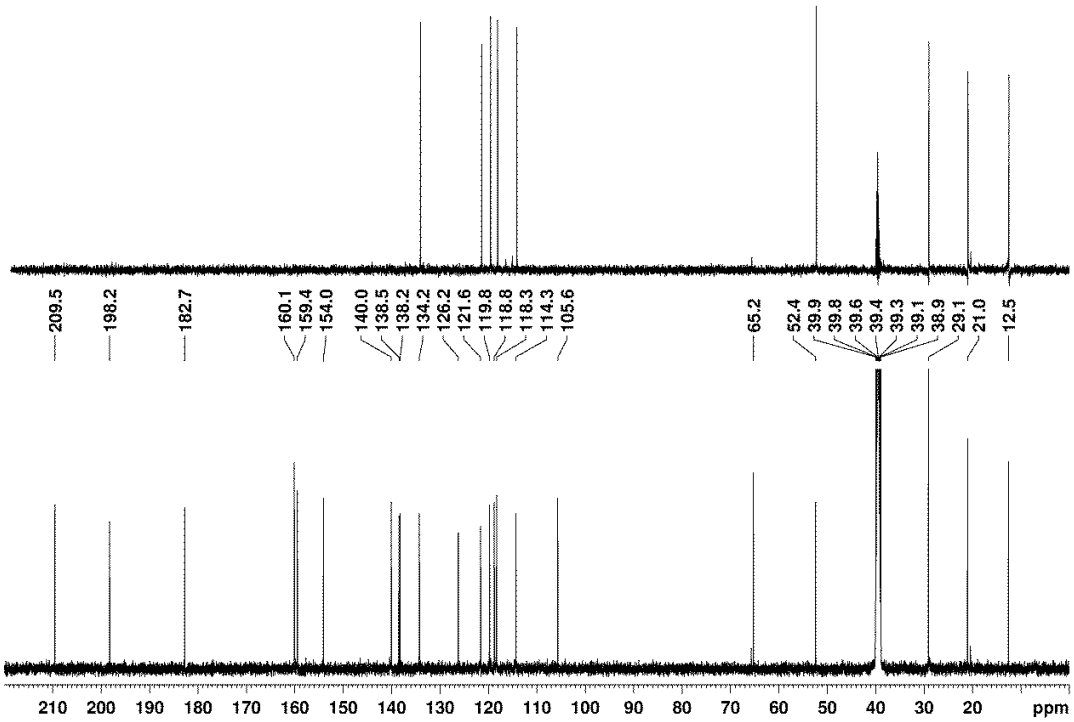
Figure S24. The ^{1}H NMR spectrum of stealthin G (**12**) in DMSO-d_6

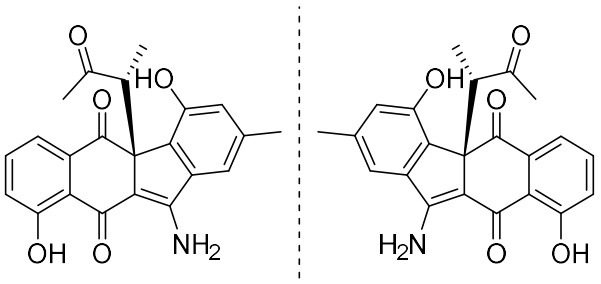




12a/12b

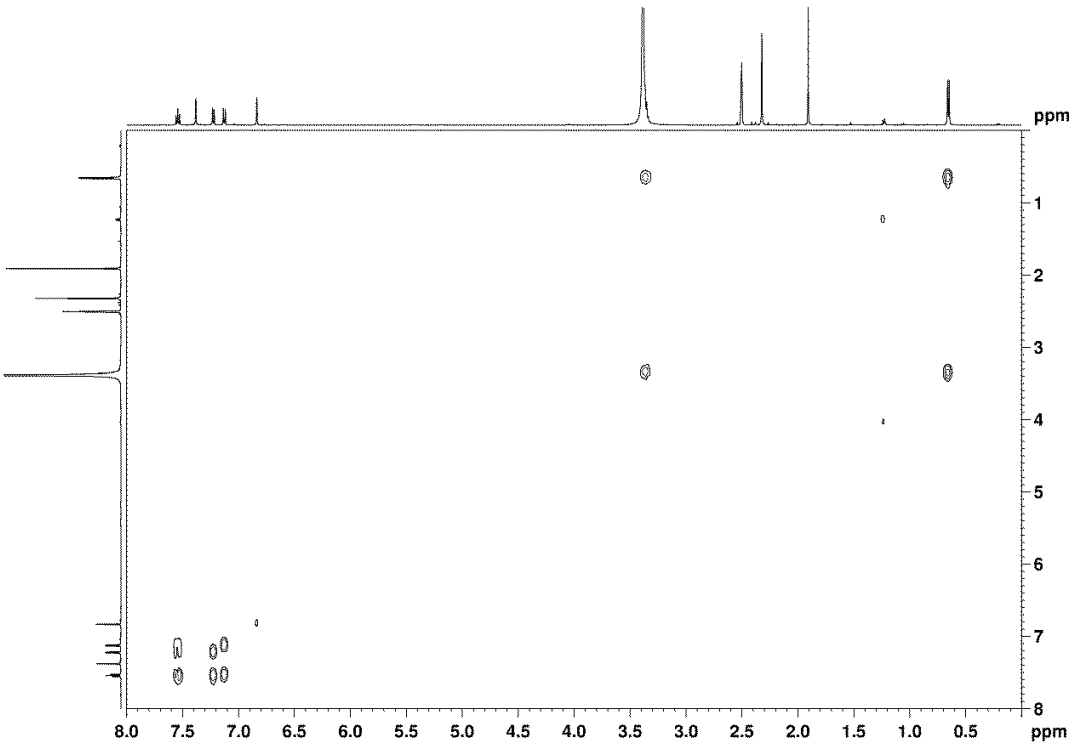
Figure S25. The ^{13}C NMR and DEPT 135 spectra of stealthin G (**12**) in DMSO-d_6

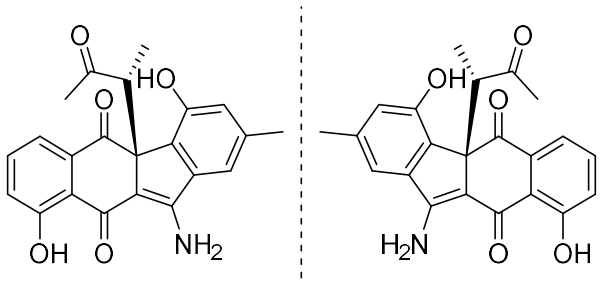




12a/12b

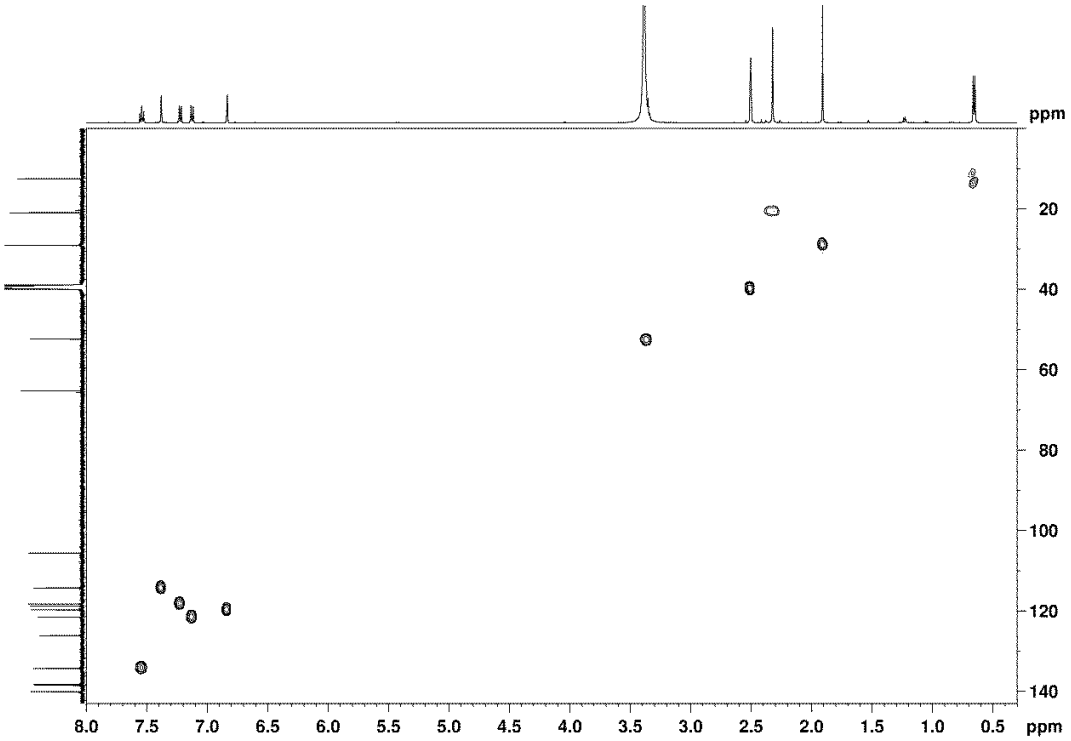
Figure S26. The ^{1}H-^{1}H COSY spectrum of stealthin G (**12**) in DMSO-d_6

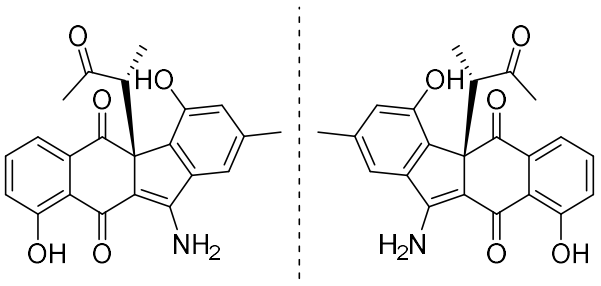




12a/12b

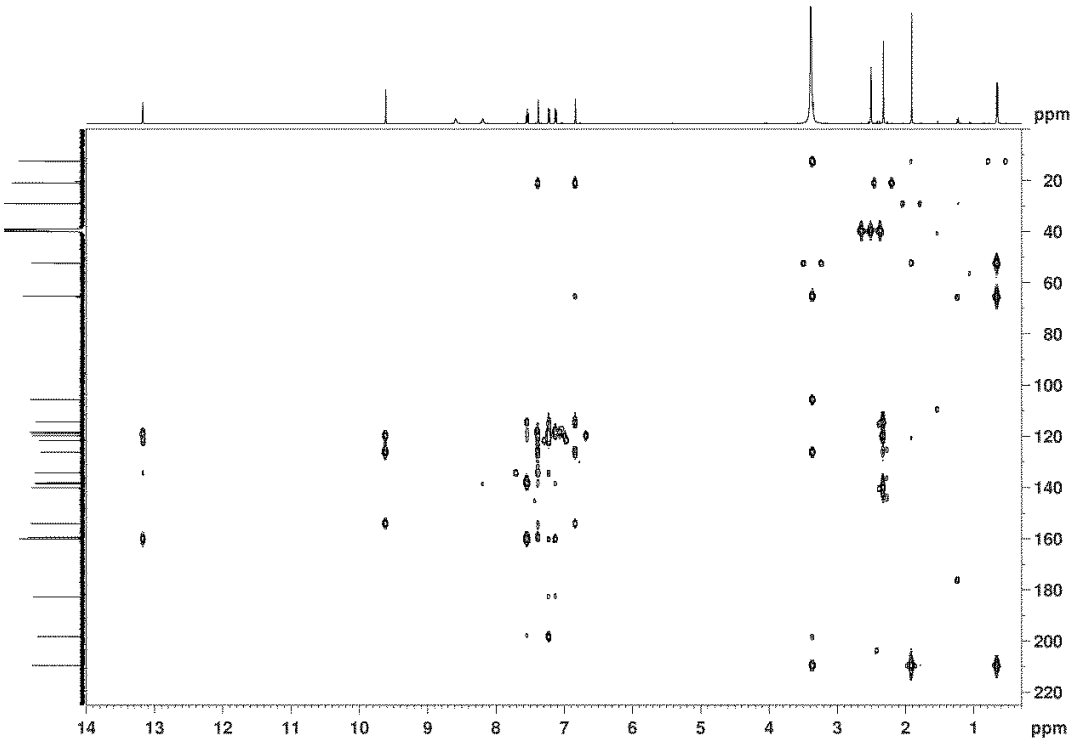
Figure S27. The HSQC spectrum of stealthin G (**12**) in DMSO-d_6

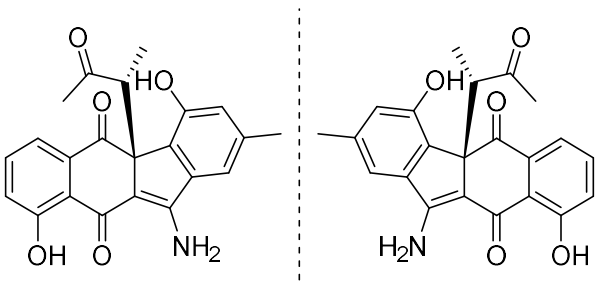




12a/12b

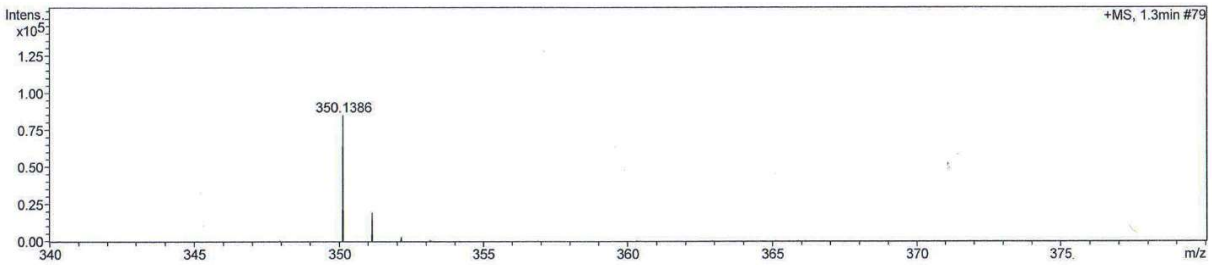
Figure S28. The HMBC spectrum of stealthin G (**12**) in DMSO-d_6



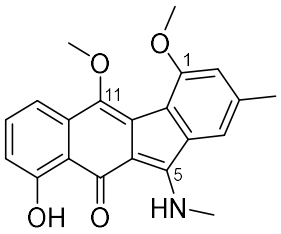


12a/12b

Figure S29. The HRESIMS spectrum of trimethylstealthin C (**13**)

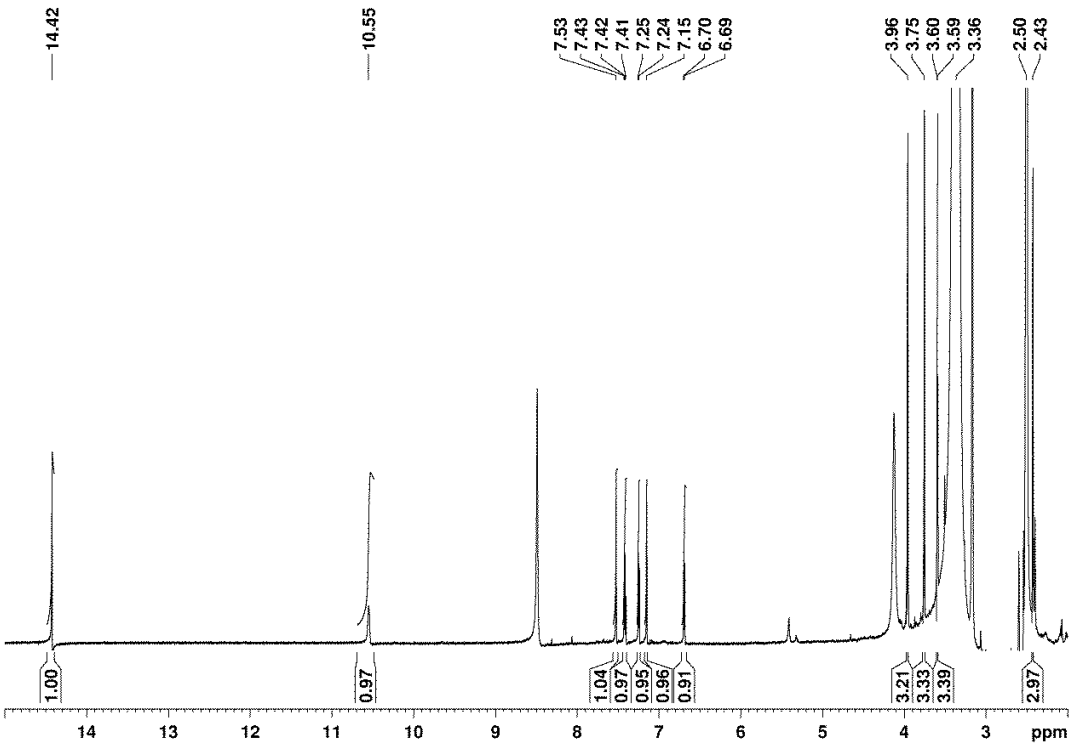


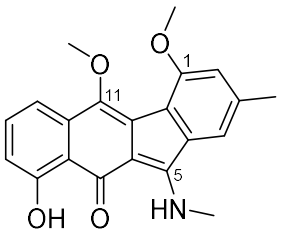
Meas. m/z	#	Ion Formula	Score	m/z	err [ppm]	err [mDa]	mSigma	rdb	e⁻ Conf	N-Rule
350.1386	1	C21H20NO4	100.00	350.1387	-0.1	-0.0	3.3	12.5	even	ok
699.2699	1	C42H39N2O8	100.00	699.2701	-0.2	-0.1	26.2	24.5	even	ok



trimethylstealthin C (**13**)

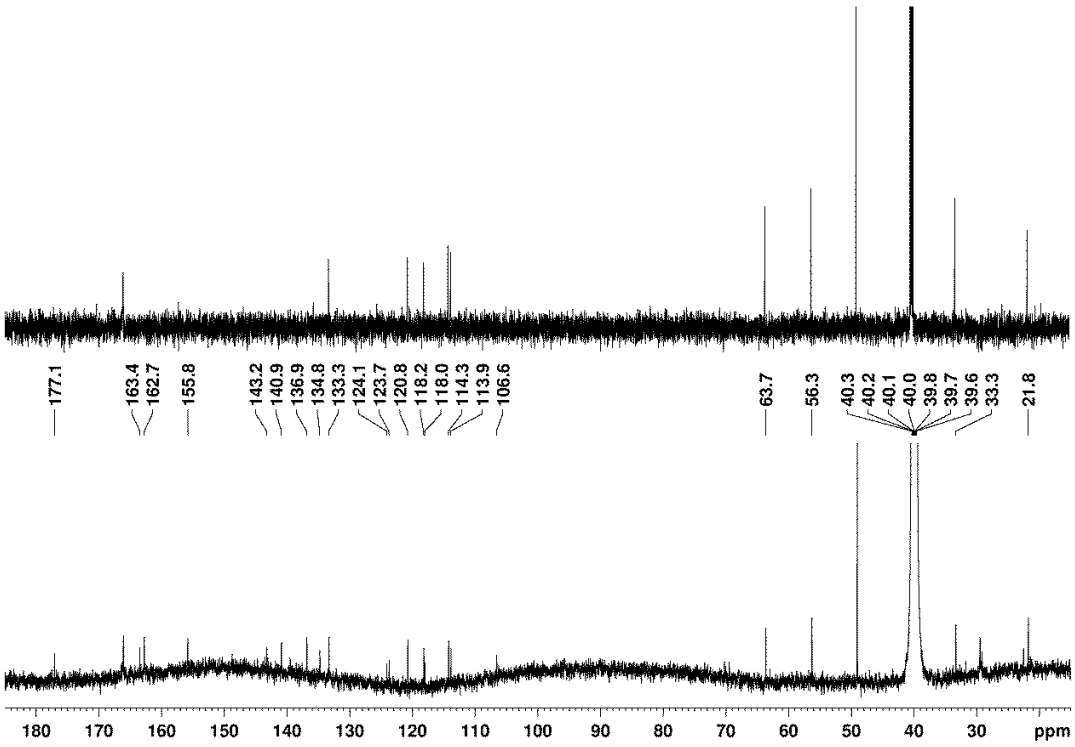
Figure S30. The ^{1}H NMR spectrum of trimethylstealthin C (**13**) in DMSO-d_6

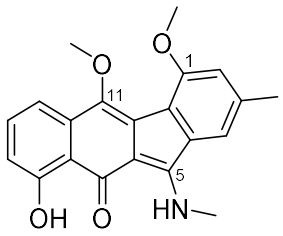




trimethylstealthin C (**13**)

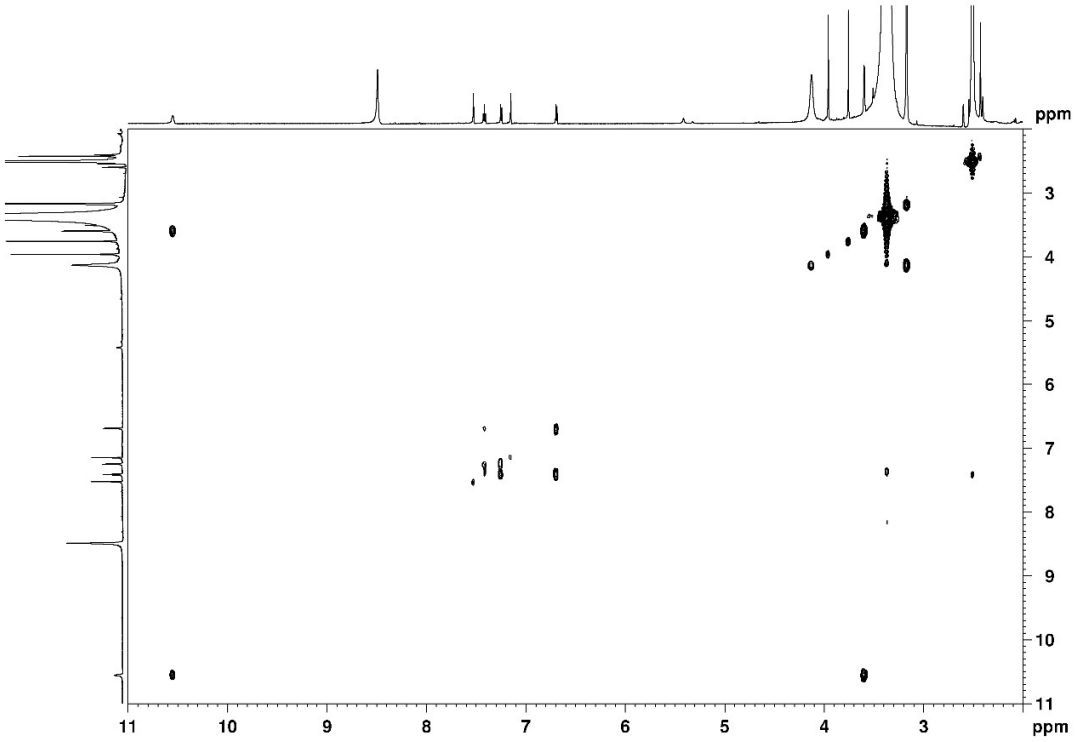
Figure S31. The ^{13}C NMR and DEPT 135 spectra of trimethylstealthin C (**13**) in DMSO-d_6

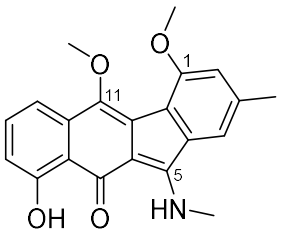




trimethylstealthin C (**13**)

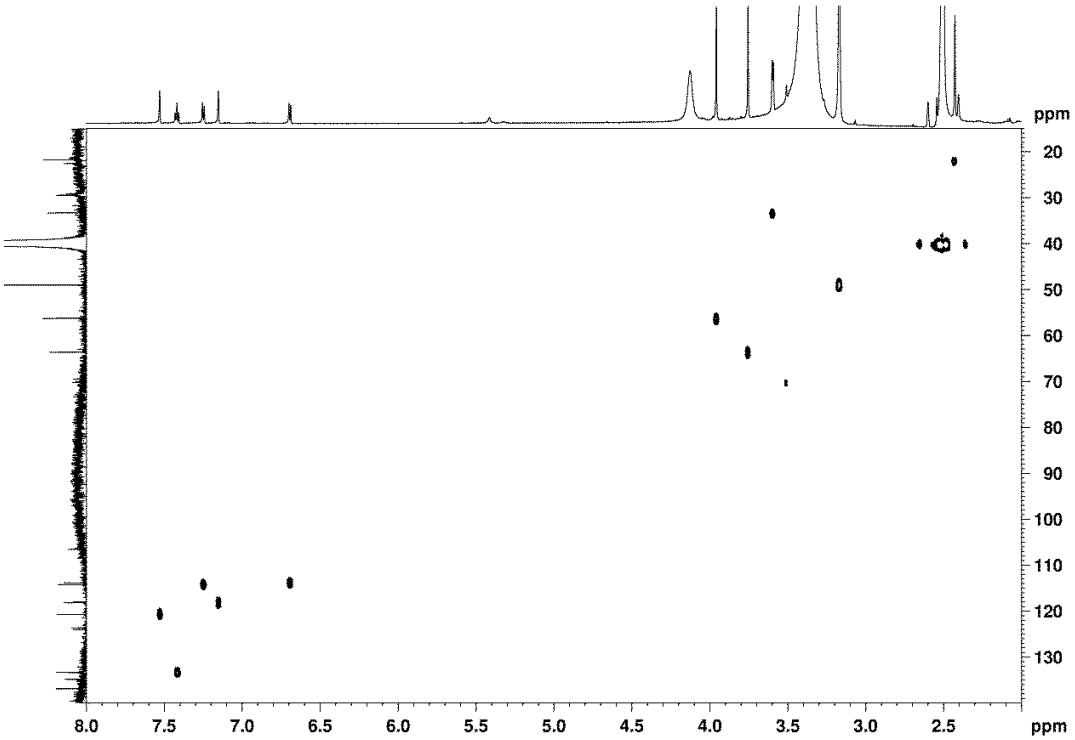
Figure S32. The ^{1}H-^{1}H COSY spectrum of trimethylstealthin C (**13**) in DMSO-d_6

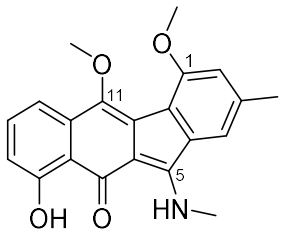




trimethylstealthin C (**13**)

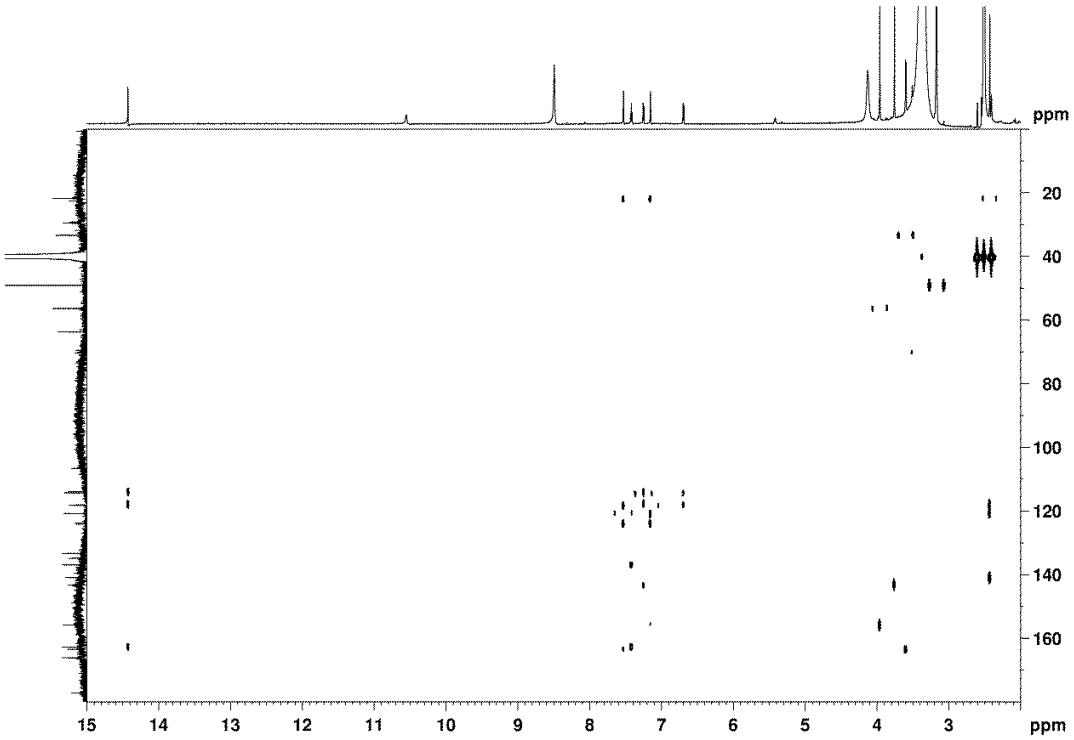
Figure S33. The HSQC spectrum of trimethylstealthin C (**13**) in DMSO-d_6

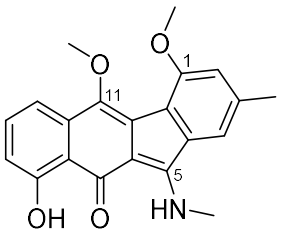




trimethylstealthin C (**13**)

Figure S34. The HMBC spectrum of trimethylstealthin C (**13**) in DMSO-d_6





trimethylstealthin C (**13**)

Table S1. Strains, plasmids and primers used in this study

Strains/Plasmids	**Characteristic(s)**	**Sources**
E.coli		
BW25113	Host strain for PCR targeting	[1]
ET12567	Donor strain for conjugation	[2]
DH5α	Host strain for cloning	Invitrogen
M. rosaria SCSIO N160	The producing strain of fluostatins	
FLS23	A mutant of *M. rosaria* SCSIO N160 where the *flsN3* gene was inactivated	This study
Plasmids		
pUZ8002	Km^r, includeing *tra* for conjugation	[3]
pIJ773	Apr^r, source of *aac(3)IV*	
pCSG5001	A cosmid of SuperCos1-based genomic library of strain SCSIO N160	
pCSG5028	pCSG5001 derivative where *flsN3* was replaced with *aac(3)IV* by insertional mutagenesis	This study
Primers	**Sequences**	
For *flsN3* disruption and confirmation of mutants' genotype		
flsN3DF	AAGGCCATCGCCGAGCGCGATCCGGCCCTGCGTGCCTTCattccggggatccgtcgacc	
flsN3DR	CAGCAACTGGTCGCCGCCGGCCGGCCCGACCAACTGGCCtgtaggctggagctgcttc	
flsN3DTF	CGGTGGAAGGAATGCCCGTT	
flsN3DTR	CGGGTGAACATGTCGACATC	

Table S2. Crystal data and structure refinement for stealthin D (**9**)

Identification code	1887925
Empirical formula	$C_{21}H_{17}NO_5$
Formula weight	363.36
Temperature/K	100.00 (10)
Crystal system	triclinic
Space group	P-1
a/Å	7.77070(10)
b/Å	8.6632(2)
c/Å	13.5470(2)
α/°	92.2060(10)
β/°	103.699(10)
γ/°	109.139(2)
Volume/Å^3	830.33(3)
Z	2
ρ_{calc}g cm^{-3}	1.453
μ/mm^{-1}	0.865
F(000)	380.0
Crystal size/mm^3	0.2 × 0.1 × 0.1
Radiation	Cu Kα (λ = 1.54184)
2Θ range for data collection/°	10.898 to 148.476
Index ranges	$-9 \leq h \leq 9$, $-10 \leq k \leq 10$, $-16 \leq l \leq 15$
Reflections collected	17405
Independent reflections	3294 [R_{int} = 0.0272, R_{sigma} = 0.0182]
Data/restraints/parameters	3294/0/248
Goodness-of-fit on F^2	1.057
Final R indexes [I>=2σ (I)]	R_1 = 0.0359, wR_2 = 0.0971
Final R indexes [all data]	R_1 = 0.0383, wR_2 = 0.0990
Largest diff. peak/hole / e Å^{-3}	0.20/-0.26

Table S3. Crystal data and structure refinement for stealthin F (**11**)

Identification code	1887926
Empirical formula	$C_{22}H_{19}NO_5$
Formula weight	377.38
Temperature/K	150.00 (10)
Crystal system	triclinic
Space group	P-1
a/Å	9.7527(2)
b/Å	14.4380(2)
c/Å	26.1676(4)
α/°	77.566(10)
β/°	83.3470(10)
γ/°	86.362(10)
Volume/Å^3	3571.17(11)
Z	8
ρ_{calc}g/cm^3	1.404
μ/mm^{-1}	0.825
F(000)	1584.0
Crystal size/mm^3	0.45 × 0.3 × 0.03
Radiation	Cu Kα (λ = 1.54184)
2Θ range for data collection/°	6.956 to 149.474
Index ranges	$-12 \leq h \leq 11$, $-18 \leq k \leq 18$, $-32 \leq l \leq 32$
Reflections collected	66899
Independent reflections	14380 [R_{int} = 0.0294, R_{sigma} = 0.0198]
Data/restraints/parameters	14380/0/1049
Goodness-of-fit on F^2	1.034
Final R indexes [I>=2σ (I)]	R_1 = 0.0398, wR_2 = 0.1048
Final R indexes [all data]	R_1 = 0.0440, wR_2 = 0.1078
Largest diff. peak/hole / e Å^{-3}	0.27/-0.28

References

1. Datsenko, K. A.; Wanner, B. L., One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. U. S. A.* **2000,** 97, 6640-6645.
2. Macneil, D. J.; Gewain, K. M.; Ruby, C. L.; Dezeny, G.; Gibbons, P. H.; Macneil, T., Analysis of *Streptomyces avermitilis* genes required for avermectin biosynthesis utilizing a novel integration vector. *Gene* **1992,** 111, 61-68.
3. Paget, M. S. B.; Chamberlin, L.; Atrih, A.; Foster, S. J.; Buttner, M. J., Evidence that the extracytoplasmic function sigma factor ς^E is required for normal cell wall structure in *Streptomyces coelicolor* A3(2). *J. Bacteriol.* **1999,** 181, 204-211.