Combining sugar biosynthesis genes for the generation of L- and D-amicetose and formation of two novel antitumor tetracenomycins

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Supporting information

Microorganisms, culture conditions and vectors

Saccharopolyspora spinosa (spinosyn producer) was used as source of DNA. pLN2, pLNR, pLNRho and pLNBIV[2c] were used as sources of sugar DNA cassettes. *Streptomyces lividans* 16F4[5] was used as host for gene expression. Growth was carried out on trypticase soya broth (TSB; Oxoid) or R5A medium[8] for product isolation. For sporulation we used agar plates containing A medium[8] for 7 days at 30ºC. *Escherichia coli* DH10B (Invitrogene) was used as a host for subcloning and was grown at 37º C in TSB medium. pCRBlunt (Invitrogene), pUC18, and pIAGO[9] were used as vectors for subcloning experiments and DNA sequencing. When antibiotic selection of transformants was needed, 50 μg/ml of thiostrepton, 25 μg/ml of apramycin, 50 μg/ml of kanamycin, or 100 μg/ml of ampicillin were used.

DNA manipulation and sequencing

Plasmid DNA preparations, restriction endonuclease digestions, alkaline phosphatase treatments, ligations and other DNA manipulations were according to standard procedures for *Streptomyces*[10] and for *E.coli*[11]. Sequencing was performed by using the dideoxynucleotide chain-terminator method[12] and the Thermo Sequenase Labelled Primer Cycle Sequencing Kit with 7-deaza-dGTP (Amersham Biosciences). Both DNA strands were sequenced with primers supplied in the kits or with internal oligoprimers (18-mer) using an ALF-express automatic DNA sequencer (Pharmacia). Computer-assisted data base searching and sequence analyses were carried out using the University of Wisconsin Genetics Computer Group programs package[13] and the BLAST program[14].

PCR amplification

Primers for *spnQ* amplification, including extra *Xba*I sites at both extremes were: FL-Sq-up: 5´-**TCTAGA**AAGACTGATCGTCGCCTG-3´. FL-Sq-rp: 5´-**TCTAGA**CTAGGAACTCTTGGCCAC-3´. The resulting PCR amplicon was subcloned into plasmid pCR-blunt (Invitrogene), generating pFL838.
PCR reaction conditions were as follows: 100 ng of template DNA were mixed with 30 pmols of each primer and 1.25 units of Platinum-Pfx DNA Polymerase (Invitrogene) in a total reaction volume of 50 μl containing 1 mM $MgSO_4$, 0.3 mM of each dNTP, 1x Pfx-buffer, and in some cases PCRx Enhancer Solution. The polimerization reactions were performed in a thermocycler (PT-100, MJ Research). General conditions for PCR amplification were as follows: 2 min at 94º C; 30 cycles composed of 30 s at 94º C, 1 min at the primers annealing temperature (58º C), and 80 s at 68º C; 5 min at 68º C; and 15 min at 4º C. The PCR products were purified with GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences), subcloned into pCRBlunt and sequenced.

Production conditions and chromatographic techniques

Spores of *S. lividans* 16F4 containing the different constructs were grown in R5A medium according to conditions previously described.[2c] HPLC analyses were performed as previously described.[2c]

Mass spectra and NMR analysis

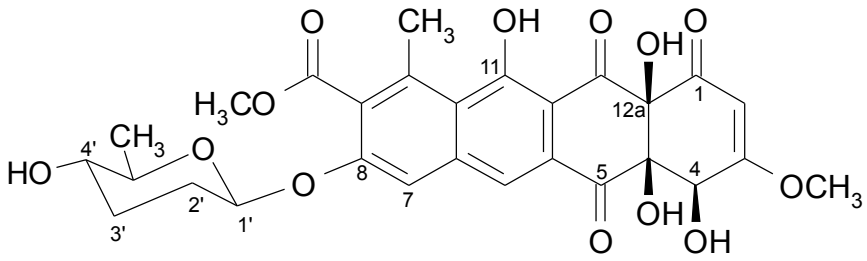
HPLC-MS analysis of the glycosylated compounds was carried out using a chromatographic equipment coupled to a ZQ4000 mass spectrometer (Waters-Micromass), using electrospray ionization in the positive mode, with a capillary voltage of 3 kV and a cone voltage of 20 V. Chromatographic conditions were as previously described,[2c] except for the column size (2.1 x 150 mm) and flow rate (0.25 ml/min).
A SymmetryPrep C18 column (7.8 x 300 mm, Waters) was used for compound purification, with acetonitrile and 0.1% trifluoroacetic acid as solvents. The new compounds were eluted in isocratic mode with 40%.
Standards with pure compounds were used for L-olivosyl, L-digitoxosyl-, D-olivosyl-TCM and -ELM comparisons.

Isolation and physico-chemical characterization of the new amicetosyltetracenomycins

Final purification was achieved by semi-preparative HPLC. Semi-preparative HPLC was run on a Waters Delta 600 instrument with a Waters 996 photodiode array detector (Waters symmetry C_{18} column, 19 x 150 mm, particle size 7 μm; solvent system: linear gradient H_2O:CH_3CN 80:20 to 40:60 within 30 min, flow rate 10 ml/min). HPLC/MS was performed on a Waters Alliance 2695 system with Waters 2996 photodiode array detector and a Micromass ZQ 2000 mass spectrometer equipped with an APCI ionization probe (Waters symmetry C_{18}, 4.6 x 50 mm, particle size 5 μm; solvent system: linear gradient H_2O:CH_3CN = 75:25 to 30:70 within 12 min; flow rate = 0.5 mL/min).

8-Demethyl-8-β-D-amicetosyl-tetracenomycin C (**1**)

Yield: 10 mg; R_{rel} = 4.15 min (analytical HPLC-MS)
MW = 572 g/mol ($C_{28}H_{28}O_{13}$)
Positive mode APCI-MS (from HPLC-MS): m/z = 573 (10%, $M+H)^+$; 459 (100%, aglycon), 441 (60%), 423 (30%), 409 (45%), 346 (100%), 313 (30%).
UV maxima (from HPLC-diode array): 250 (60%), 288 (100%), 390 (35%), 413 (35%)



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NMR data of 8-Demethyl-8-β-D-amicetosyl-tetracenomycin C (**1**). Given are d-values in ppm relative to TMS, in d_6-DMSO.

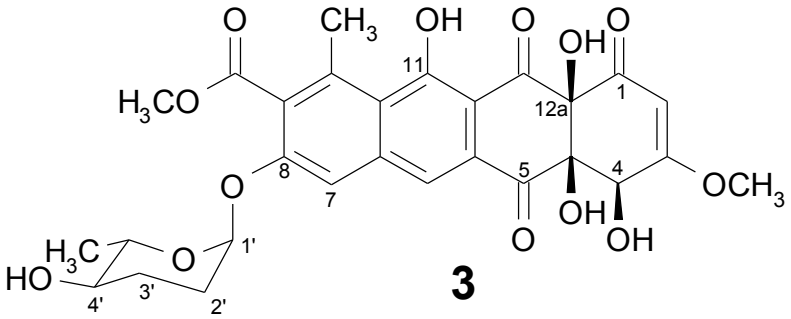
position	**^{1}H, 400 MHz d/ppm (multiplicity, *J*/Hz)**	**^{13}C (100 MHz)**
1		190.9
2	5.47 (d, *J* = 1.5 Hz)	100.6
3		173.2
3-OCH_3	3.70 (s)	56.7
4-H	4.77 (dd, *J* = 8; 1.5 Hz)	71.0
4-OH	5.91 (d, *J* = 8 Hz)	
4a		87.4 [a]
4a-OH	5.55 (s)	
5		191.4 [b]
5a		139.8
6	7.94 (s)	119.8
6a		129.2 [c]
7	7.80 (s)	111.0
8		153.7
9		128.3
9-CO		167.3
9-OCH_3	3.89 (s)	55.5
10		136.9
10-CH_3	2.77 (s)	20.6
10a		120.2 [c]
11		166.0
11-OH	13.83 (br s)	
11a		109.3
12		193.7 [b]
12a-OH	6.09 (s)	
12a		85.1 [a]
1’	5.88 (dd, *J* = 12; 2 Hz)*	94.5
2’ax	1.90 - 2.10 (m, compl.)#	35.1 [d]
2’eq	1.90 - 2.10 (m, compl.)#	

3'ax	1.82 (m, compl.)[#]	26.8 [d]
3'eq	1.63 (dddd, *J* = 17; 5; 5; 5)	
4'	3.15 (dddd, *J* = 10; 10; 6.5; 5 Hz)	52.7
4'-OH	4.71 (d, *J* = 6.5 Hz)	
5'	3.39 (dq, *J* = 6.5; 5 Hz)[&]	64.9
6'	1.06 (d, *J* = 6.5 Hz)	18.0

br = broad; * coupling constants are not exact due to overlap with 4-OH; [#]compl. = complex due to overlap with other signals; [&] partly obscured by water signal; [a,b,c,d] assignments mutually interchangeable

8-Demethyl-8-α**-L-amicetosyl-tetracenomycin C** (**3**)

Yield: 7 mg; R_{rel} = 4.33 min (analytical HPLC-MS)
MW = 572 g/mol ($C_{28}H_{28}O_{13}$)
Positive mode APCI-MS (from HPLC-MS): m/z = 573 (5%, M+H)$^+$; 459 (100%, aglycon), 441 (70%), 423 (30%), 409 (30%), 346 (90%), 313 (25%).
UV maxima (from HPLC-diode array): 240 sh (60%), 287 (100%), 390 (35%), 412 (35%)



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NMR data of 8-Demethyl-8-α-L-amicetosyl-tetracenomycin C **3**. Given are δ-values in ppm relative to TMS, in d_6-DMSO.

compound **3**	**^{1}H (400 MHz)**	**^{13}C (100 MHz)**
1		190.9
2	5.48 (d, *J* = 1.5 Hz)	100.4
3		172.9
3-OCH_3	3.70 (s)	56.6
4-H	4.77 (dd, *J* = 8; 1.5 Hz)	71.0
4-OH	5.89 (d, *J* = 8 Hz)	
4a		87.3[a]
4a-OH	n.o.[*]	
5		192.9[b]
5a		139.7
6	7.94 (s)	119.8
6a		129.1[c]
7	7.82 (s)	111.0
8		153.7
9		127.5
9-CO		167.0
9-OCH_3	3.91 (s)	55.5
10		136.9

10-CH_3	2.79 (s)	20.6
10a		120.2[c]
11		165.7
11-OH	13.81 (br s)	
11a		109.2
12		196.1[b]
12a-OH	6.13 (s)	
12a		85.1[a]
1’	5.91 (s)	94.0
2’ax	1.85 - 2.00 (m, compl.)[#]	28.7[d]
2’eq	1.85 - 2.00 (m, compl.)[#]	
3’ax	1.82 (m, compl.)[#]	26.8[d]
3’eq	1.62 (dddd, *J* = 17; 5; 5; 5 Hz)	
4’	3.14 (dddd, *J* = 10; 10; 5; 5 Hz)	52.5
4’-OH	5.01 (d, *J* = 5 Hz)	
5’	3.40 (dq, *J* = 6.5, 5 Hz, obsc.)[&]	69.9
6’	1.05 (d, *J* = 6.5 Hz)	18.1

br = broad; ^ n.o. = not observed; [#] compl. = complex, due to overlap with other signals; [&] partly obscured by water signal; [a,b,c,d] assignmanets mutually interchangeable

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