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Simocyclinones, Novel Cytostatic Angucyclinone Antibiotics Produced by *Streptomyces antibioticus* Tü 6040

II. Structure Elucidation and Biosynthesis

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The simocyclinones D4 (**1**) and D8 (**2**), members of a novel class of antibiotics, were isolated from the mycelial extract of *Streptomyces antibioticus* Tü 6040 and consist of angucyclinone, deoxysugar, octatetraene dicarboxylate and aminocoumarin structural elements. The structure elucidation was done by one and two dimensional NMR experiments, and other spectroscopic methods in combination with incorporation experiments using ^{13}C labelled precursors.

The well established HPLC-DAD-screening for secondary metabolites isolated from actinomycetes resulted in the discovery of two new antibiotics named simocyclinones D4 (**1**) and D8 (**2**). In the previous paper[1] the taxonomy of *Streptomyces antibioticus* (strain Tü 6040) as well as the fermentation, isolation, characterisation and biological activities of the antibiotics were described. In this part we present the structure elucidation mainly done by NMR analysis and report the first results on biosynthetic investigations of these cytostatically active compounds.

Structure Elucidation

Simocyclinones D4 (**1**) and D8 (**2**) were obtained as yellow powders with an UV absorption maximum near 340 nm, which exhibited characteristic bathochrome shifts in alkaline solution typical for phenols. The IR spectra show absorptions at $\nu = 1695\,cm^{-1}$ (**1**) and $1698\,cm^{-1}$ (**2**), respectively, caused by α, β unsaturated ketones. The similarity of their spectra suggested their structural analogy. Therefore, the structure elucidation of **1** is described first, and afterwards the differences between **1** and **2** will be analysed.

FAB-MS and HRESI-MS established the molecular formula of **1** as $C_{46}H_{43}NO_{18}$. The ^1H and ^{13}C NMR spectra (Table 1 and 2) in connection with an APT spectrum exhibit the presence of three methyl groups, three methylene groups, six aliphatic, nine olefinic and five aromatic methine groups and 20 quaternary carbon atoms. COSY and HMBC experiments showed that the molecule can be divided into four parts, one of which displayed the typical ^1H NMR signal pattern of deoxysugars.

The carbon skeleton of the aglycone of simocyclinone D4 (**1**) consists of 19 carbon atoms. The APT spectrum indicates the presence of two carbonyl groups, one phenolic hydroxy group, six aromatic carbon atoms and five carbon atoms connected to oxygen atoms, two methylene and one methyl group. Detailed analysis of the HMBC experiments led to the angucyclinone part of structure **1** (Fig. 2). The compound confirms a new non-quinoid angucyclinone core

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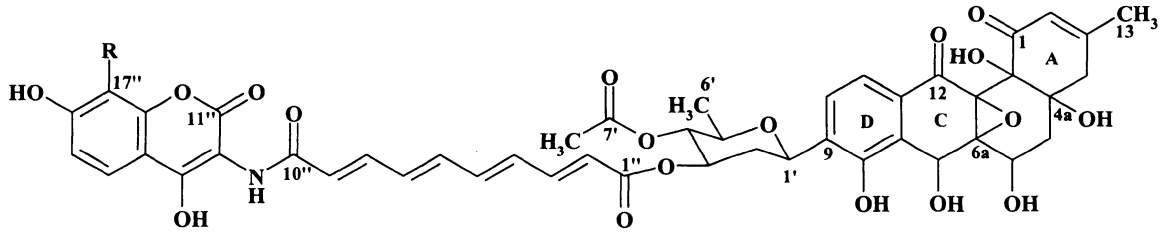
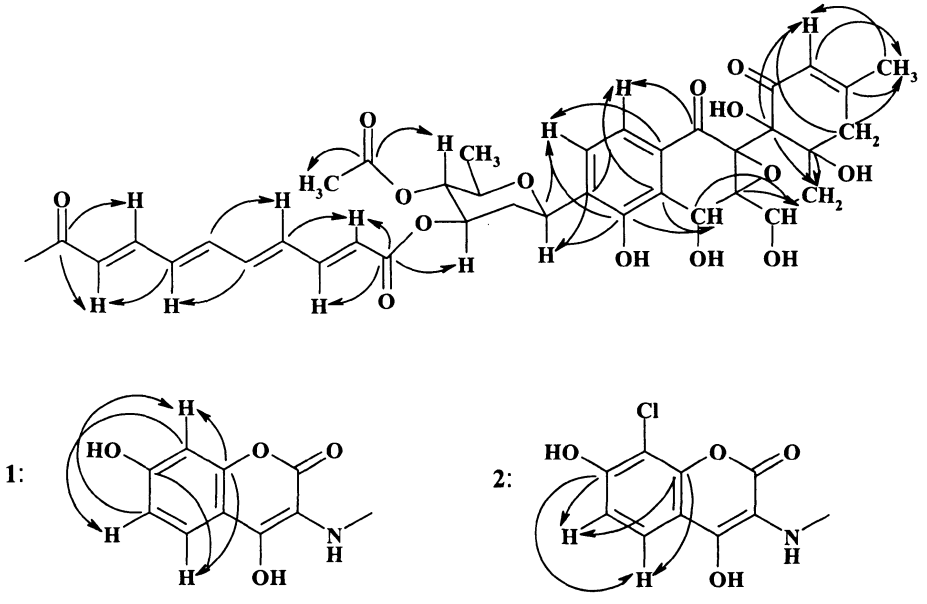
Fig. 1. Structure of simocyclinones D4 (**1**) and D8 (**2**).



1 : R = H (simocyclinone D4)

2 : R = Cl (simocyclinone D8)

Fig. 2. Selected HMBC-correlations of **1** and **2**.



of the SS-228Y type[2], characterised by an α, β unsaturated ketone in ring A, an angular connection to rings A and B *via* *O*-substituted quaternary carbon atoms, an epoxide at the linkage of rings B and C, the non-quinoid ring C as well as the aromatic ring D with a phenolic hydroxy group at C-8. Although mass and elementary analysis proved the number of carbons of **1** to be 46, only 45 could be detected by ^{13}C NMR spectroscopy. The quaternary carbon C-12a bearing an epoxy function cannot be seen in NMR experiments. Unfavourable parameter for NMR measurement are responsible for this phenomenon[3]. Similar effects may also be the reason why the carbon atoms C-12 and C-13″ can sometimes not be detected in ^{13}C NMR experiments. This caused also difficulties in the analysis of the NMR spectra of labelled substances.

The *C*-glycosidically attached deoxysugar was identified as β-D-olivose, acylated at 3′-OH and 4′-OH. The relative configuration was proved by coupling constants and COSY correlations, the connection with C-9 of the aglycone and with an acetyl group at 4′-OH by HMBC experiments (Fig. 2). Cross signals from C-1″ (δ_C 165.3) to 3′-H_{ax} (δ_H 5.17) and to 2″-H (δ_H 6.00) in the HMBC spectrum demonstrate the linkage of the sugar moiety to the octatetraene dicarboxylate. The all-(*E*)-configuration of the tetraene was

Table 1. ^{13}C NMR data of simocyclinone D4 (**1**) and D8 (**2**) in DMSO-d_6.

Position	Assignment	1 δ_C (ppm)	2 δ_C (ppm)
1	CO	196.0	196.0
2	CH	121.8	121.8
3	C	157.8	157.7
4	CH_2	42.7	42.7
4a	C	72.1	72.0
5	CH_2	38.9	38.7
6	CH	62.7	62.6
6a	C	65.4	65.4
7	CH	63.5	63.5
7a	C	124.7	124.8
8	C	152.6	152.6
9	C	134.2	134.2
10	CH	125.8	125.8
11	CH	118.1	118.1
11a	C	127.6	127.6
12	CO	190.2[a]	190.0[b]
12a	C	n.d.[c]	65.7[d]
12b	C	75.1	75.1
13	CH_3	23.1	23.1
1′	CH	70.6	70.6
2′	CH_2	36.5	36.5
3′	CH	71.6	71.6
4′	CH	74.1	74.1
5′	CH	73.2	73.1
6′	CH_3	17.7	17.7
7′	CO	169.7	169.6
8′	CH_3	20.5	20.5
1″	CO	165.3	165.2
2″	CH	121.5	121.5
3″	CH	144.4	144.4
4″	CH	133.2	133.1
5″	CH	140.2	140.2
6″	CH	139.2	139.0
7″	CH	134.4	134.3
8″	CH	141.3	141.1
9″	CH	124.6	124.6
10″	CO	166.1	166.1
11″	CO	159.7	158.7
12″	C	104.2	105.3
13″	C	155.5	158.7
13a″	C	117.1	115.3
14″	CH	117.0	119.6
15″	CH	120.0	115.8
16″	C	153.8	151.0
17″	CH / C	107.7	114.4
17a″	C	144.1	145.6

[a] Only visible through HMBC correlations.
[b] Only visible through feeding of [2-^{13}C]malonic acid.
[c] Not determined.
[d] Only visible through feeding of [1,3-$^{13}C_2$]malonic acid.

Table 2. ^1H NMR signals of simocyclinone D4 (**1**) and D8 (**2**) in DMSO-d_6.

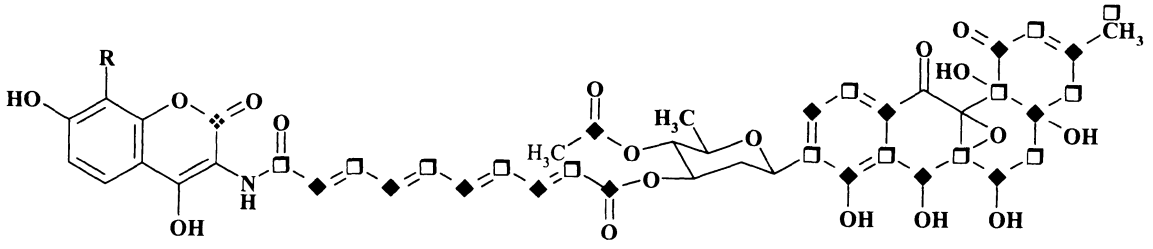
Proton(s)	1 δ_H (ppm, J in Hz)	2 δ_H (ppm, J in Hz)
2-H	5.90 (s)	5.90 (s)
4-H_2	2.24 (d, 19.0)	2.23 (d, 19.0)
	2.70 (d, 19.0)	2.70 (d, 19.0)
5-H	1.91 (m)	1.91
		(ddd, 13.5, 7.5, 1.0)
	1.99 (m)	1.98 (m)
6-H	4.42	4.42
	(dd, 7.5, 7.5)	(dd, 7.5, 7.5)
7-H	5.75 (s)	5.75 (s)
10-H	7.46 (d, 8.0)	7.45 (d, 8.0)
11-H	7.34 (d, 8.0)	7.33 (d, 8.0)
13-Me	1.81 (s)	1.81 (s)
1′-H	4.97 ax	4.96 ax
	(dd, 1, 11.5)	(dd, <1, 11.5)
2′-CH_2	1.54 ax	1.55 ax
	(ddd, 11.5, 11.5, 11.5)	(ddd, 11.5, 11.5, 11.5)
	2.42 eq	2.42 eq
	(ddd, 1.0, 4.5, 11.5)	(ddd, 1.0, 4.5, 11.5)
3′-H	5.17 ax	5.18 ax
	(ddd, 5.0, 9.0, 11.5)	(ddd, 4.5, 9.0, 11.5)
4′-H	4.75 ax	4.74 ax
	(dd, 9.0, 9.0)	(dd, 9.0, 9.0)
5′-H	3.78 ax	3.78 ax
	(dq, 6.0, 9.0)	(dq, 6.0, 9.0)
6′-Me	1.17 (d, 6.0)	1.16 (d, 6.0)
8′-Me	2.00 (s)	2.01 (s)
2″-H	6.00 (d, 15.0)	6.00 (d, 15.0)
3″-H	7.26	7.26
	(dd, 15.0, 11.0)	(dd, 15.0, 11.0)
4″-H	6.59-6.71 (m)	6.61-6.73 (m)
5″-H	6.79-6.88 (m)	6.79-6.88 (m)
6″-H	6.79-6.88 (m)	6.79-6.88 (m)
7″-H	6.59-6.71 (m)	6.61-6.73 (m)
8″-H	7.30	7.23 (m)
	(dd, 15.0, 11.0)	(dd, 15.0, 11.0)
9″-H	6.59-6.71 (m)	6.59 (d, 15.0)
14″-H	7.22 (d, 9.0)	7.23 (m)
15″-H	7.04	7.23 (m)
	(dd, 3.0, 9.0)	
17″-H	7.19 (d, 3.0)	-
13″-OH	12.90	12.60
NH	9.91	10.33
OH*	4.80, 5.27, 5.58, 9.57, 9.74	4.82, 5.27, 5.58, 9.58, 9.85

* Detectable but not assignable.

deduced from ^3J coupling constants of the double bonds (J=15 Hz) (Table 2).

Finally, a $C_9H_6NO_4$ residue with six quaternary carbon atoms forms the fourth part of **1**. With regard to HMBC-correlations eight substructures of this residue were possible, but only an aminocoumarin substructure allows chelation of 13″-OH (δ_H 12.60) with the carbonyl group C-10″ (δ_C 166.1) and fits the high field position of C-12″ (δ_C 104.2). The position of the second hydroxy group could either be at C-15″ or C-16″, but only position C-16″ is compatible with biosynthetic considerations assuming

Fig. 3. Enrichments in ^{13}C NMR spectra determined for **2** after feeding different precursors.



◆ [1,3-$^{13}C_2$]malonic acid, ❐ [2-^{13}C]malonic acid, ❖ L-[1-^{13}C]tyrosine

L-tyrosine is a precursor for this part of the molecule[4]. Also, the chemical shifts of the assigned carbons are in agreement with chemical shifts of the aminocoumarin part of novobiocin[5].

The ^1H and ^{13}C NMR data of simocyclinone D8 (**2**) are identical with those of **1**, except for the signals of the aminocoumarin part. The aromatic methine group C-17″ (δ_C 107.7) of **1** is replaced by a quaternary aromatic carbon atom at δ_C 114.4 (Table 1). The difference of the chemical shifts ($\Delta\delta_C$ 6.7) indicates a chlorine substitution at this carbon atom, confirmed by FAB-MS and elemental analysis, establishing the molecular formula of **2** as $C_{46}H_{42}NO_{18}Cl$[1].

Biosynthesis

Biosynthetic experiments carried out with the angucyclinones[2,6] and fumagillin[7] led to the proposal that acetate units form the aglycone of the simocyclinones *via* a decaketide and the octatetraene dicarboxylate *via* a pentaketide. To confirm this hypothesis, [1,3-$^{13}C_2$]- and [2-^{13}C]malonic acid were fed to growing cultures of the strain *Streptomyces antibioticus*, because acetate itself inhibits the production of the simocyclinones. The enrichments as shown in Table 3 and Figure 3 are in agreement with the proposed polyketide pathways. Worth mentioning is the observation that C-8′ only showed a slight enrichment by feeding [2-^{13}C]malonic acid. In addition, L-[1-^{13}C]tyrosine was fed and gave an enrichment of C-11″ only, in connection with an increased yield of **1** and **2**, respectively, proving L-tyrosine to be the precursor of the aminocoumarin moiety.

Table 3. Specific incorporations of **2** after feeding with [2-^{13}C]malonic acid (I), [1,3-$^{13}C_2$]malonic acid (II) and L-[1-^{13}C]tyrosine (III).

Position	I[a]	II[b]	III[c]
1	0	**1.36**	-0.09
2	**1.28**	0.57	0.06
3	0.08	**1.45**	-0.18
4	**1.64**	**1.07**	0.11
4a	0.28	**1.38**	-0.04
5	n.d.[d]	n.d.[d]	
6	0.43	**1.71**	0.14
6a	**1.20**	0.48	0.11
7	0.39	**1.73**	0.15
7a	**1.60**	-0.40	0.13
8	0.41	**1.71**	0.20
9	**1.39**	0.39	0.06
10	0.19	**1.68**	0.02
11	**1.83**	0.84	0.30
11a	0.30	**1.47**	0.12
12	n.d.[e]	n.d.[f]	
12a	n.d.[f]	n.d.[e]	
12b	**1.47**	0.39	-0.02
13	**1.23**	0.31	0
7′	0.34	**1.45**	0.12
8′	0.69	0.14	-0.07
1″	0.21	**1.31**	-0.05
2″	**2.28**	0.77	0.59
3″	0.03	**1.04**	-0.03
4″	**1.51**	0.21	0.09
5″	0.17	**1.67**	0.11
6″	**1.88**	0.18	0.18
7″	0.22	**1.04**	0.09
8″	**1.91**	-0.71	0.16
9″	0.44	**1.09**	0.25
10″	**1.51**	-0.05	-0.11
11″	0.28	0.13	**99.33**
12″	-0.35	-0.24	0

[a] Relative enrichments were normalized to the peak intensity of the C-1 signal.
[b] Relative enrichments were normalized to the peak intensity of the C-17a″ signal.
[c] Relative enrichments were normalized to the peak intensity of the C-13 signal.
[d] Signal covered by solvent.
[e] Reference signal detected only through HMBC correlations.
[f] Not determined.

Discussion

The simocyclinones D4 (**1**) and (**2**) are novel natural products with angucyclinone, deoxysugar, octatetraene dicarboxylate and aminocoumarin structural elements assembled to give an unique structure derived from different biogenetic roots[8]. New combinations of known building blocks might exhibit interesting new features and offer further insight into the connection of biosynthetic pathways used by talented microorganisms. The simocyclinones are an example of a strain applying metabolic combinatorial biosynthesis[9,10]. The aglycone shows the same features in ring A and B as WS009 A and B[11,12] and a non-quinoic ring C with an epoxide group found in elmycin C[13] and rubiginone I[14]. The phenolic ring D represents the conserved structural element of the angucyclinones. The aglycone as found in **1** and **2** has not been described before. Concerning the *C*-glycosidically bound deoxysugar, there are only four examples found in the literature for an acylation exclusively at 4′-OH especially with a decadienoic acid[15~18] and no example for an acylation with an unsaturated acid at 3′-OH or with an acylation at both, 3′-OH and 4′-OH. In **1** and **2**, the polyene dicarboxylic acid connects the D-olivose with the aminocoumarin moiety, which can be found as a substructure in *e.g.* rubradirin[19], novobiocin[5,20] and TPU-0031-A[21].

Feeding experiments confirmed that the angucyclinone skeleton of the simocyclinones consists of 10 acetate units typically built up as described for the angucyclinones[3]. We assume that the D-olivose is derived from D-glucose as described for aquayamycin and the urdamycins[22]. Also, the feeding experiments showed that the octatetraene dicarboxylic acid derives from five acetate units, starting from the left side (C-10″). Therefore, we conclude that the oxidation of C-10″ takes places after the regioselective esterfication of olivose at 3′-OH, a symmetrical intermediate can be excluded due to the enrichment pattern. Why C-8′ shows no significant incorporation might be due to the fact that the acetylation is one of the last biosynthetic steps and the remaining concentration of labelled malonic acid in the culture broth is too low. The lower incorporation could also hint that the acetyl group is being derived from acetyl CoA and not malonyl CoA. Finally, feeding of L-[1-^{13}C]tyrosine proved L-tyrosine to be the precursor of the aminocoumarin moiety. Recently it has been questioned, whether the formation of the aminocoumarin part derives *via* an oxidative cyclization[23], which was previously assumed to be the case[24], or, as indicated by recent enzyme studies with the novobicin producing strain *S. spheroides*, *via* an intramolecular cyclization of an enzyme-bound 2,4-dihydroxy-tyrosine derivative[25]. This question should be solved by a biosynthetic experiment in an $^{18}O_2$-rich atmosphere[26].

Experimental

General

MP's were determined on a Reichert hot-stage microscope and are not corrected. The ^{1}H and ^{13}C NMR spectra were measured in DMSO-d_6 and acetone-d_6 on a Varian VXR 500 spectrometer with solvents as internal standard. The ^{1}H and ^{13}C NMR shifts are listed in Tables 2 and 3.

Labelled Compounds

^{13}C labelled compounds were 99% ^{13}C atom purity. 3.0 mmol/liter L-[1-^{13}C]tyrosine (Isotec Inc.), 10.0 mmol/liter [1,3-$^{13}C_2$]malonic acid (CIL Inc.) and 10.0 mmol/liter [2-^{13}C]malonic acid (Isotec Inc.).

Incorportation of Isotope-labelled Compounds to **2**

S. antibioticus Tü 6040 was cultivated in a 1-liter fermenter (Biostat M, Braun) and 900 ml medium consisting of: glycerol 25 g, L-lysine 3.65 g, NaCl 1 g, K_2HPO_4 1 g, $MgSO_4 \cdot 7H_2O$ 0.5 g (feeding of tyrosine: plus Amberlite® XAD-2 33 g, feeding of malonic acid: plus Vitamin B_{12} 5 mg) and 2 ml trace element concentrate (TEC) in 1 liter deionised water (pH 7.0 prior to sterilisation). TEC (per liter): $FeSO_4 \cdot 7H_2O$ 1 g, $CuSO_4 \cdot 5H_2O$ 0.1 g, $MnSO_4 \cdot H_2O$ 0.1 g and $ZnSO_4 \cdot 7H_2O$ 0.1 g. The fermenter was inoculated with 10 vol-% of cultures grown for 48-hours in 300 ml Erlenmeyer flasks without baffles on a rotary shaker at 120 rpm and at 27°C in the same medium. The shake flask cultures were inoculated with a 48-hour liquid pre-culture (10 vol-%, 27°C, 120 rpm, 300 ml Erlenmeyer flasks without baffles) of a complex medium consisting of: soybean meal 20 g and mannitol 20 g (pH 7.0 prior to sterilisation) in 1 liter deionised water. The fermentations were carried out at 27°C with an aeration of 1.6 vvm and at 700 rpm. L-[1-^{13}C]tyrosine was added to the fermenter using a pulse feeding method with 5 ml portions every half hour from 16 hours until 21 hours of incubation. [1,3-$^{13}C_2$]malonic acid and [2-^{13}C]malonic acid, respectively, were fed to the fermenters constantly in small portions by a pump (mercedos SP-GLV-7) between the 16th and 26th hour of the incubation.

Isolation of Simocyclinone D8 (**2**)

The cultures were harvested after 40 hours by centrifugation. The mycelium was extracted twice with acetone and the aqueous phase was extracted at pH 4 with ethyl acetate three times. The combined organic phases were concentrated and applied to a LiChroprep Diol column (2×55 cm) with CH_2Cl_2 - methanol (99 : 1) as eluent. After Sephadex LH-20 chromatography (100×2.5 cm, MeOH) the labelled simocyclinone D8 (**2**) was obtained in the following amounts: 6.9 mg ([1,3-$^{13}C_2$]malonic acid), 15.3 mg ([2-^{13}C]malonic acid) and 8.9 mg (L-[1-^{13}C]-tyrosine).

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