Kanchanamycins, New Polyol Macrolide Antibiotics Produced by *Streptomyces olivaceus* Tü 4018

II. Structure Elucidation†

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Kanchanamycins are a new group of polyol macrolide antibiotics isolated from *Streptomyces olivaceus* Tü 4018. They all share a common bicyclic carbon skeleton formed by a 36-membered lactone ring and a 6-membered hemiacetal ring. A feature unusual for that class of macrolides is the terminal urea moiety observed in kanchanamycin A. The structures of the kanchanamycins were determined by electrospray MS and modern 2D NMR techniques. Due to substantial overlap of the signals intensive use of inverse detected heteronuclear correlation experiments (HSQC, HMBC, 2D-HSQC-TOCSY) was made.

The kanchanamycins (Fig. 1) represent a family of new polyol macrolide antibiotics produced by *Streptomyces olivaceus* Tü 4018. They were found by a non-target screening strategy utilizing reversed-phase HPLC and diode array detection. Taxonomy of the producing strain, fermentation and isolation as well as the biological activities are described in the preceding paper[1]. The structure elucidation of the kanchanamycins focused primarily on the main congener kanchanamycin A, from which the structures of the other components were derived.

Structure Elucidation of Kanchanamycin A

In the electrospray[2] mass spectrum of kanchanamycin A (Fig. 2), the molecular ion peak $[M+H]^+$ at $m/z=1056$ is accompanied by at least seven fragment peaks with mass differences of 18 amu between each other. No further fragment ions were observed. Apparently, kanchanamycin A contains an extended, stable carbon skeleton that does not fragment in the transport region of the electrospray ion source. Furthermore, the elimination of water hints to the presence of hydroxy groups. The UV spectrum of kanchanamycin A shows absorption bands at 239 nm ($\varepsilon=31200$) and at 262 nm ($\varepsilon=18600$) indicating the presence of conjugated double bonds. These results suggest that kanchanamycin A belongs to the class of polyol-polyene macrolides. The optical rotation of kanchanamycin A was determined as $[\alpha]_D^{20}=35°$.

2D-NMR proved to be the method of choice for the structure elucidation of several antibiotics[3~5]. To obtain information also about labile protons, all spectra were acquired in the non-protic solvent dimethylsulfoxide-d_6. In the HSQC[6,7] spectrum of kanchanamycin A six methyl groups and 24 methylene or methine groups were identified. Five of the six methyl groups appear as sharp doublets in the 1D-proton spectrum while one is shifted downfield compared to the others and forms a singlet. Accordingly five $CH-CH_3$ fragments have to be present in the molecule. Between $\delta(^1H)=3$ ppm and 5 ppm twelve cross peaks were found in the HSQC that were assigned by means of their ^{13}C chemical shifts (62 ppm to 78 ppm) to carbon atoms bound to oxygen. ^{13}C chemical shifts alone do not allow us to distinguish between carbon atoms bound to a hydroxy group and carbon atoms that are part of an ester or acetal moiety.

† Art. No. 7 on biosynthetic capacities of actinomycetes. Art. No. 6: See ref. 1.

Fig. 1. Structural formulae of kanchanamycin A, C and D.

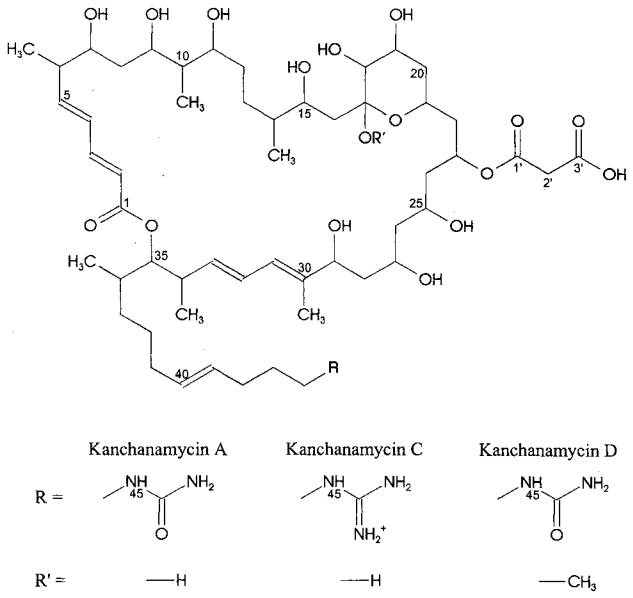
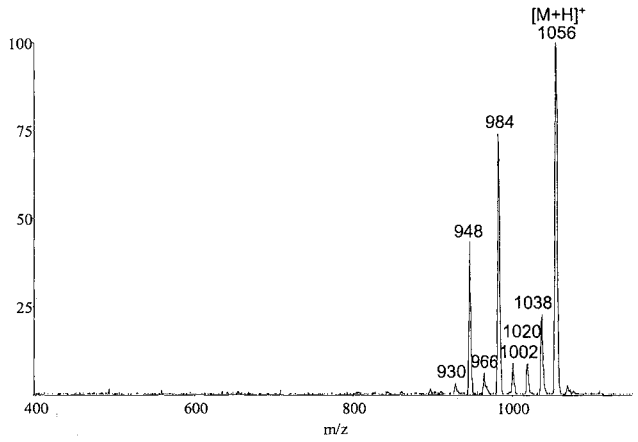


Fig. 2. Electrospray mass spectrum of kanchanamycin A.

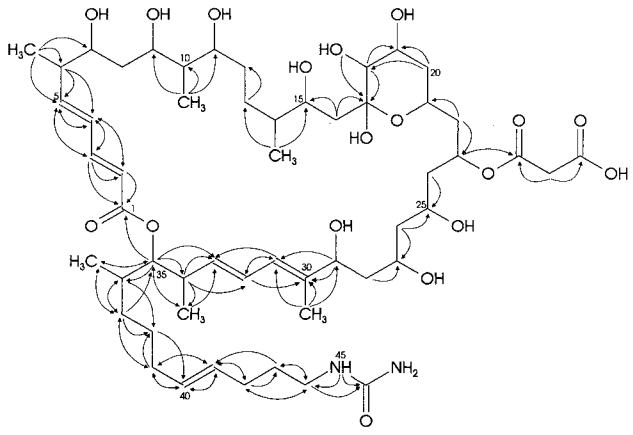


Therefore an HSQC of kanchanamycin A with the labile protons exchanged to deuterium was recorded. In that experiment hydroxymethylene carbon atoms (C-7, C-9, C-11, C-15, C-18, C-19, C-25, C-27, C-29) showed a characteristic highfield shift while the ^{13}C chemical shift values of all other carbon atoms remained unaffected[8]. These data indicate ester or ether groups at C-21, C-23 and C-35. A number of broad signals between 4 ppm and 5 ppm in the 1D-proton spectrum with no corresponding cross peaks in the HSQC were assigned to hydroxy protons. Finally nine cross peaks were identified in the region of the HSQC characteristic for C-C-double bonds (F2: 5.3~7.2 ppm; F1: 117~146 ppm). Overlapping signals (C-40 and C-41) could be unraveled in the HMBC spectrum. Two additional signals in the 1D spectrum with integrals corresponding to one and two protons, respectively, were assigned to one NH and one NH_2 group. In the HMBC[9,10] spectrum six quaternary carbon atoms were detected, four in the carbonyl region of the spectrum (158.2 ppm, 165.9 ppm, 166.3 ppm, 167.7 ppm), one at 139.9 ppm and one at 98.2 ppm. Two strong cross peaks from the methylene protons H-2′ to the carbonyl groups at 167.7 ppm and 166.3 ppm established the presence of a malonyl residue in kanchanamycin A. The position of the esterification is defined clearly by a cross peak between H-23 and the carbonyl group at 166.3 ppm (C-1′). The carbonyl group at 165.8 ppm shows cross peaks to H-2 and H-3 and was therefore assigned to C-1. The formation of a 36-membered lactone ring in kanchanamycin A is proved unambiguously by a strong cross peak between C-1 and H-35 in the HMBC spectrum. Cross peaks from H-44 and the amide proton H-45 allowed to identify the signal at 158.2 ppm as the carbonyl function (C-46) of the urea moiety. Because neither a *N*-methyl group nor any additional amide protons besides H-45 and H-47 were detected, the possibility of a (substituted) guanidino group was ruled out. In order to obtain chemical shift values comparable to published data of macrolides, an additional ^{13}C spectrum of kanchanamycin A in methanol-d_4 was recorded. The value of $\delta(^{13}C)_{MeOH}=$ 162.2 ppm for the urea carbon atom C-46 is in good agreement with that of homologous urea derivatives, *e.g.* *N*-(12,18,20,22,24,26,28-heptahydroxy-9,11,13,19-tetramethyloctacosa-4,14,16-trienyl) urea ($\delta(^{13}C)_{MeOH}=$ 162.2 ppm)[11]. In contrast, comparable guanidino groups like in azalomycin F show carbon chemical shifts of $\delta(^{13}C)_{MeOH}=157.4$ to 158.7 ppm[12]. In the HSQC spectrum of kanchanamycin A, an odd number of olefinic carbon atoms was detected indicating a trisubstituted C-C double bond. The corresponding quaternary carbon atom (C-30) was found at $\delta(^{13}C)=139.9$ ppm with cross peaks in the HMBC to H-29, H-30M and H-32. A signal at $\delta(^{13}C)=98.2$ ppm corresponds to the hemiacetal carbon atom C-17. This assignment was confirmed by cross peaks to H-16, H-18 and to the hydroxy proton at C-18. Since C-21 is the only carbon atom part of an ether or ester linkage that has not been assigned yet (see above), it has to be connected to C-17 giving rise to a six-membered hemiactal ring in kanchanamycin A.

Connectivities between proton containing functional groups were established by the combined use of HMBC, DQF-COSY[13,14] and HSQC-TOCSY[15]. By analysis of the HMBC spectrum, it was possible to determine

connectivities in almost all areas of the molecule (Fig. 3). Nevertheless between some fragments (*e.g.* C-7 to C-9, C-8 to C-9), no HMBC cross peaks were detected probably due to unfavorable values of $^2J_{C,H}$ and $^3J_{C,H}$. As an alternative method the comparison of the DQF-COSY with the TOCSY[16,17] spectrum revealed the structure of the molecule in the regions from H-2 to H-10M, H-13 to H-14M, H-18 to H-23 and from H-28 to H-45 (Fig. 4). Although cross peaks also occur between protons in other parts of the molecule they can not be assigned unambiguously due to signal overlap. To circumvent this problem, a 2D-HSQC-TOCSY with short spin lock time was recorded that yields an HMBC-like cross peak pattern. The occurrence of an HSQC-TOCSY cross peak depends on $^1J_{H,C}$ and $^3J_{H,H}$ rather than on $^2J_{C,H}$ or $^3J_{C,H}$. HSQC-TOCSY therefore represents a method that is complementary to HMBC in terms of the assignment of connectivities within a spin

Fig. 3. HMBC contacts found in kanchanamycin A.



Arrows point from proton to the corresponding carbon atom. Double arrows indicate contacts in both directions.

Fig. 4. Contacts found in the DQF-COSY (solid lines) and HSQC-TOCSY (dotted lines) spectra of kanchanamycin A.

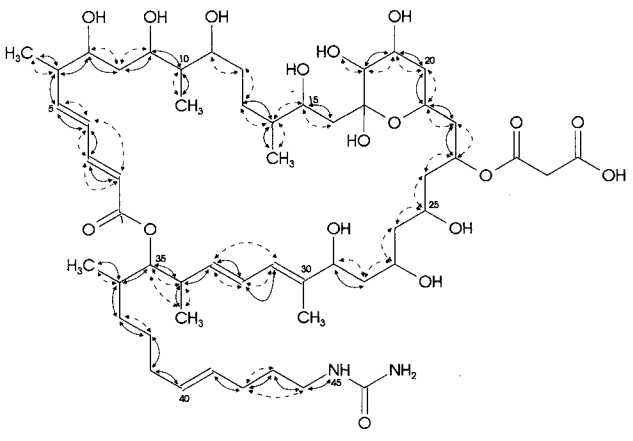


Table 1. ^1H and ^{13}C chemical shifts of kanchanamycin A, C and D in DMSO-d_6.

	$\delta(^1H)$			$\delta(^{13}C)$		
	A	C	D	A	C	D
1	—	—	—	165.8	165.7	165.9
2	5.82	5.82	5.80	118.8	118.8	118.8
3	7.14	7.15	7.14	144.5	144.6	144.6
4	6.20	6.20	6.19	127.9	127.8	127.9
5	6.17	6.18	6.17	146.0	146.1	146.1
6	2.35	2.35	2.35	41.4	41.2	41.5
6M	1.01	1.01	1.00	16.2	16.1	16.2
7	3.66	3.66	3.66	72.1	71.9	72.3
8	1.50	1.50	1.50	38.3	38.1	38.4
	1.33	1.33	1.32			
9	3.53	3.53	3.52	71.3	71.1	71.4
10	1.35	1.35	1.34	42.4	42.2	42.8
10M	0.70	0.70	0.71	9.6	9.4	9.6
11	3.68	3.68	3.57	69.5	69.4	69.4
12	1.39	1.40	1.41	32.1	31.8	31.9
	1.19	1.20	1.16			
13	1.30	1.30	1.30	28.1	28.1	27.7
	1.11	1.11	1.14			
14	1.44	1.45	1.46	38.3	38.2	38.5
14M	0.78	0.78	0.80	14.7	14.7	14.9
15	3.68	3.68	3.68	69.5	69.4	69.4
16	1.70	1.70	1.68	40.3	40.1	34.8
	1.54	1.54	1.61			
17	—	—	—	98.3	98.1	101.0
18	3.08	3.08	3.30	75.9	75.7	74.9
19	3.61	3.61	3.64	66.9	66.8	66.6
20	1.74	1.74	1.74	40.0	39.7	40.0
	1.12	1.12	1.12			
21	3.89	3.89	3.58	64.0	63.8	64.7
22	1.61	1.61	1.61	39.7	39.5	40.0
23	4.97	4.97	5.04	70.3	70.2	70.5
24	1.65	1.65	1.68	42.0	41.8	42.9
	1.58	1.59	1.54			
25	3.66	3.66	3.67	63.0	62.9	62.9
26	1.31	1.31	1.27	45.2	45.1	45.4
27	3.82	3.82	3.82	63.5	63.4	63.4
28	1.39	1.39	1.37	43.4	43.2	43.5
	1.26	1.26	1.22			
29	4.01	4.01	4.00	71.2	70.9	71.1
30	—	—	—	139.9	139.8	140.0
30M	1.58	1.58	1.56	12.6	12.6	12.7
31	5.89	5.89	5.88	122.0	121.9	121.9
32	6.19	6.19	6.18	126.4	126.4	126.4
33	5.41	5.41	5.39	133.7	133.7	133.7
34	2.52	2.52	2.51	38.2	38.0	38.2
34M	0.93	0.93	0.92	17.3	17.1	17.1
35	4.70	4.70	4.69	78.0	77.9	78.0
36	1.72	1.73	1.73	33.2	32.9	33.1
36M	0.81	0.81	0.82	13.3	13.3	13.4
37	1.23	1.23	1.22	32.2	32.1	32.4
	1.06	1.06	1.07			
38	1.31	1.31	1.32	25.8	25.7	26.0
39	1.88	1.90	1.89	31.7	31.5	31.8
40	5.36	5.40	5.36	129.5	130.3	129.4
41	5.36	5.36	5.36	129.5	128.5	129.4
42	1.91	1.96	1.90	29.1	28.4	29.1
43	1.37	1.50	1.37	29.5	27.8	29.6
44	2.91	3.05	2.91	38.4	39.7	38.5
45	5.91	7.51	5.83	—	—	—
46	—	—	—	158.2	156.3	158.5
47	5.29	—	5.28	—	—	—
1′	—	—	—	166.3	166.1	166.6
2′	3.27	3.28	3.26	41.6	41.4	41.9
3′	—	—	—	167.7	167.8	169.9

Values are referenced to the solvent peak ($\delta(^1H)_{DMSO}$ = 2.48 ppm, $\delta(^{13}C)_{DMSO}$ = 39.5 ppm).

system. The HSQC-TOCSY allowed to define connectivities in the regions between H-11 and H-16, H-18 and H-29 as well as some other parts of the molecule (Fig. 4). The chemical shifts of all protons and carbon atoms of kanchanamycin A are summarized in Table 1.

The configurations of the double bonds were established by the measurement of $^3J_{H,H}$ in the case of disubstituted double bonds. Coupling constants were determined both as active and as passive couplings[18] from P.E.COSY[19] cross peaks. Looking at the passive couplings allowed to unravel coupling constants even if chemical shifts of the two protons were virtually identical (*e.g.* H-4 and H-5). Large values of approximately 15 Hz in all cases ($J_{(H\text{-}2,H\text{-}3)}=15.5$ Hz, $J_{(H\text{-}4,H\text{-}5)}=15.9$ Hz, $J_{(H\text{-}32,H\text{-}33)}=15.3$ Hz, $J_{(H\text{-}40,H\text{-}41)}=16.0$ Hz) indicated an *E*-configuration for these double bonds. The stereochemistry of the trisubstitued double bond 30-31 was established by comparing the intensities of ROESY[20] cross peaks between H-30M and H-31 or H-32, respectively. Assuming an *E*-configured (*Z*-configured) double bond H-30M and H-31 are 3.81 Å (2.84 Å) apart while the distance between H-30M and H-32 amounts to 2.98 Å (4.63 Å). In the ROESY spectrum of kanchanamycin A a strong cross peak was observed between H-30M and H-32 while the cross peak between H-30M and H-31 was very weak. Accordingly the configuration of double bond 30-31 is also *E*.

Structure Elucidation of Kanchanamycin C

Electrospray MS revealed a relative molecular mass of 1055 for the $[M+H]^+$ ion of kanchanamycin C. This is one mass unit less than the mass of kanchanamycin A. Within a range of 0.02 (0.7) ppm the ^1H (^{13}C) chemical shifts of both antibiotics are identical for HC-1 to HC-39. Larger differences occur only in the terminal part of the molecule, most pronounced in the vicinity of the carbonyl atom C-46. These findings suggest that in kanchanamycin C the urea moiety of kanchanamycin A is replaced by a guanidino group (Fig. 1). The ^{13}C spectrum of kanchanamycin C in methanol-d_4 also shows a chemical shift value for C-46 ($\delta(^{13}C)_{MeOH}=158.6$ ppm) that is typical for a guanidino group (see above). The chemical shifts of all protons and carbon atoms of kanchanamycin C are listed in Table 1.

Structure Elucidation of Kanchanamycin D

The $[M+H]^+$ ion of kanchanamycin D has a relative molecular mass of 1070 which is 14 amu higher than that of kanchanamycin A. The largest differences in chemical shifts between kanchanamycin A and C with values above 0.1 ppm ($\Delta\delta(^1H)$) and 0.9 ppm ($\Delta\delta(^{13}C)$), respectively, are found in the region of the oxan ring (C-17 to C-21). In the HSQC spectrum of kanchanamycin D an additional cross peak appears at $\delta(^1H)=3.03$ ppm and $\delta(^{13}C)=46.4$ ppm. The integration of the corresponding peak in the proton spectrum indicates three protons for this signal. A strong HMBC cross peak from these protons to C-17 proves the methylation of hemiacetal yielding an acetal moiety in kanchanamycin D (Fig. 1). Chemical shifts of kanchanamycin D are summarized in Table 1.

Discussion

The kanchanamycins are a family of new macrolide antibiotics forming a 36-membered lactone ring. Due to the high flexibily of the molecule and the large number of stereocenters present, no stereochemical assignment on the basis of NOE data and vicinal coupling constants was possible. The most remarkable structural feature is the replacement of the terminal guanidino moiety commonly found in this class of macrolides by a urea group in the case of kanchanamycin A and D. Within the lactone ring, the structure of kanchanamycin A is identical to the one of RS-22 that was published recently[21]. This region is also highly homologous to other macrolides and differs *e.g.* from azalomycin[12] and shurimycin[22] just by one methyl group at C-2 or C-28, respectively. The region C-6 to C-27 is conserved compared to some other related macrolides like guanidylfungin[23], niphimycin[24] or malolactomycin[25]. This is in agreement with the observation that in particular the hemiacetal ring (C-17 to C-21) is essential for biological activity[26]. Interestingly, a methylation of the hemiacetal group as found in kanchanamycin D does not seem to affect the antibiotic activity. By contrast, the substitution of the guanidino group for an urea moiety as found in kanchanamycin C causes a considerably change in the biological activity[1].

Experimental

UV spectra were measured in methanol on a Varian DMS 100S spectrometer (Varian, Palo Alto, CA, U.S.A.). The optical rotation was determined in methanol (*c* 1.0) on a Perkin-Elmer 241MC (Perkin-Elmer GmbH, Überlingen, Germany). The electrospray mass spectra were recorded on a API III triple-quadrupol mass spectrometer equipped with a nebulizer-assisted electrospray (ion spray) ion source (Sciex, Thornhill, Canada). All spectra were acquired in the positive ion mode (step size 0.1 u, dwell time 2 msec). Daughter ion mass spectra were recorded using argon as collision gas. The accuracy of mass determination for all measurements was ± 0.2.

High-resolution NMR spectra were recorded on a Bruker AMX-II 600 spectrometer (Bruker Analytische Meßtechnik GmbH, Karlsruhe, Germany) using an inverse triple resonance probe with z-gradients. Measurements were carried out on a 10 mmol sample of kanch-

anamycin A in DMSO-d_6 at 305 K. All two-dimensional spectra except HMBC were acquired in phase-sensitive absorption mode with quadrature detection in both dimensions using the TPPI method[27]. Generally 512 experiments with 32 to 128 scans and a data size of 4 K complex points were collected for each two-dimensional experiment while the P.E.COSY was acquired with 8 K complex points in F2. In the case of DQF-COSY, HSQC and HMBC pulsed field gradients were used for coherence selection. For the determination of the dependence of $\delta(^{13}C)$ on the deuterium exchange of labile protons two samples of kanchanamycin A in DMSO-d_6-H_2O (9:1) and DMSO-d_6-D_2O (9:1) were prepared. An HSQC spectrum of the hydroxymethyl region with higher resolution was recorded for both samples (sweep with in F1: 20 ppm, 512 experiments). Data were processed and analyzed on iris indigo work stations (Silicon Graphics Inc., Mountain View, CA, U.S.A.) using FELIX software (Biosym Inc., San Diego, CA, U.S.A.).

Acknowledgments

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