The Staurosporine Producing Strain *Streptomyces longisporoflavus* Produces Metabolites Related to K-252a

Proposal for Biosynthetic Intermediates of K-252a

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Alkaloids of the indolocarbazole type bind to ATP-binding sites of various enzymes. Staurosporine isolated from *Saccharothrix aerocolonigenes* subsp. *staurosporeus*[1] was found to be an inhibitor of protein kinase C (PKC) in the low nanomolar range[2]. Most other alkaloids of this type contain a six-membered sugar moiety like staurosporine[3~6]. A subgroup of these metabolites are K-252a (**6**)[7] and K-252b[8], which were isolated by Japanese researchers from two *Nocardiopsis* species strains as PKC inhibitors. In contrast to staurosporine these secondary metabolites have a five-membered sugar moiety and a carboxylate function attached to this ring. Although a weaker inhibitor of PKC than staurosporine, K-252b attracted considerable interest as it is able to potentiate neurotrophin-3 actions *in vitro*[9]. In previous publications we described several minor metabolites of *Streptomyces longisporoflavus* strain R-19 which were isolated as byproducts of a large scale staurosporine fermentation[6,10]. In the present communication the structure elucidation, physicochemical data, and biological properties of further minor metabolites are described. Fermentation[6] and isolation[10] of the new compounds were reported earlier.

Results and Discussion

Structure Elucidation

K-252a (**6**) and K-252c (**1**)

The two compounds gave virtually identical ^{1}H NMR and ^{13}C NMR spectra as the reported ones[8]. MS investigation confirmed the identity of the structures.

N-Methyl-staurosporine (**4**)

High resolution EI-MS discloses the molecular formula of $C_{29}H_{28}N_4O_3$ and suggests an additional methyl group compared to staurosporine. This substituent has to be located on the 4′ nitrogen, as the ^{1}H NMR signal of the *N*-methyl group shows an integral corresponding to six hydrogens. Structure **4** is confirmed by irradiation on the N–$(CH_3)_2$ group in $(CD_3)_2CO$ which gives rise to a NOE of 20% on the 4′ proton. The synthesis of *N*-methyl-staurosporine has already been described in a patent application[12].

N-Methyl-3′-amino-3′-deoxy-derivative of K-252a (**5**)

The ^{1}H NMR of **5** is almost identical to that of the synthetic derivative **3**[13] with the exception of an additional methyl group. This one must be located on the amino group as indicated by its chemical shift (1.9 ppm). As the coupling constants are also unchanged, the stereochemistry on center 3′†† should be the same as

Table 1. ^{1}H NMR and ^{13}C NMR chemical shifts (in ppm).

Carbon	^{1}H NMR				^{13}C NMR	
	4[a]		**5**		**5**	
1	7.54	d	7.90	d	109.0	d
2	7.47	t	7.48	t	125.4	d
3	7.32*	t	7.27	t	119.5	d
4	9.49	d	9.21	d	125.6	d
4a					122.5	s
4b					115.9	s
4c					119.6	s
5					171.7	s
6	7.51	brs	8.61	s		
7	5.07	s	4.99	brs	45.5	t
7a					132.9	s
7b					114.6	s
7c					124.0	s
8	8.04	d	8.03	d	121.3	d
9	7.28*	t	7.35	t	120.5	d
10	7.45	t	7.46	t	125.2	d
11	8.08	d	8.08	d	114.7	d
11a					139.6	s
12a					128.4	s
12b					124.1	s
13a					136.8	s
2′					100.4	s
3′	4.28	d			78.0	s
4′(a)	3.02	m	3.39	dd	40.1	t
4′b			2.03	dd		
5′(a)	≈2.8		7.18	dd	85.7	d
5′b	2.66	ddd				
6′	6.81	dd				
2′-CH_3	2.45	s	2.16	s	23.8	q
3′-OCH_3	3.05	s				
3′-C=O					172.8	s
N–CH_3	1.81	s	1.92	d	32.2	q
N–H			2.44	q		
1″			3.93	s	52.8	q
Some characteristic coupling-constants (Hz)						
3′-4′	2					
4′a-4′b			14			
4′-5′	4/8		7/5			
5′a-5′b	14					
5′a/b-6′	7/3					

Assignments with asterisks may be interchanged. Temperature ambient. Solvent: DMSO-d_6, except for [a] solvent: acetone-d_6.

†† Three different nomenclatures for the sugar part of staurosporine type alkaloids are used in the literature. We will use the numbering system for staurosporine assigning position 1′ to the oxygen.

in **3**, which leads to structure **5**. IR and ^{13}C NMR spectra are fully consistent with the proposed structure. As expected the ^{13}C NMR data of **3** and **5** are very similar with the exception of C-3′ which is shifted by 6 ppm to lower field in **5**. Comparison of the CD spectra of **5** and **3** shows that both compounds have the same absolute stereochemistry, which is identical to K-252a[13] and probably the same at the bridging atoms 2′†† and 5′ or 6′ as the recently determined absolute configuration of staurosporine[11].

Biological Properties

Compound **5** inhibits porcine PKC[14] at a very similar concentration as K-252a (Table 2). The selectivity pattern for the two other kinases tested is also very similar, suggesting that the 3′ amino group is not crucial for its biological activity. The PKC inhibition values of *N*-methyl-staurosporine and staurosporine itself are indistinguishable. The additional methyl group increases the selectivity towards c-AMP dependent protein kinases and phosphorylase kinase by a small factor of 3 to 6.

Biosynthetic Considerations

The main metabolite produced by the *S. longisporoflavus* strain R-19 is staurosporine. Most minor metabolites are closely related either to staurosporine or to the oxime TAN-1030A which was also isolated[6,10]. Based on the rather surprising result that the same strain produces also K-252a (**6**) and another metabolite with a five-membered sugar moiety (**5**) as minor metabolites, a pathway for the last steps in the biosynthesis of K-252a is proposed (Fig. 1).

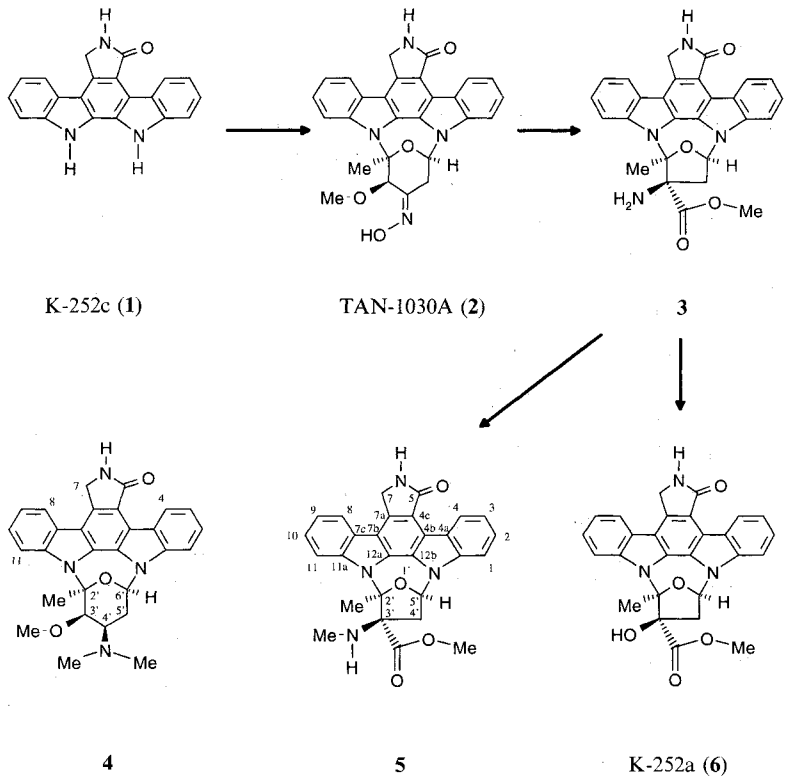
The biosynthesis of staurosporine was investigated by American researchers and two units of tryptophan were found to be incorporated into the aglycone moiety of this alkaloid[15]. The aglycone K-252c (**1**) is the first characterized intermediate of the biosynthesis. As K-252d, another metabolite from the *Nocardiopsis* sp. K-252[8], contains a L-rhamnose moiety, it seems to be likely that L-rhamnose is the biosynthetic precursor for the sugar moiety of most indolocarbazole type alkaloids. This hypothesis is supported by the fact that the absolute stereochemistry of L-rhamnose at C-4 and C-5 and of staurosporine at the centers 3′ and 2′ is identical[11].

Table 2. Enzyme inhibition of protein kinases by staurosporine and compounds **2**, **4**, **5** and **6**.

Name	IC_{50} (μM)		
	PKC	PKA	PK
Staurosporine	0.006	0.015	0.003
2 TAN-1030A	1.17	1.4	0.04
4 *N*-methyl-staurosporine	0.006	0.092	0.01
5	0.11	1.6	0.014
6 K-252a	0.22	0.4	0.004

PKC: protein kinase C.
PKA: c-AMP dependent protein kinase.
PK: phosphorylase kinase.

Fig. 1. The hypothetical biosynthetic pathway of compound **5** and of K-252a (**6**) starts from K-252c (**1**) and proceeds through TAN-1030A (**2**) and the proposed intermediate **3**, and structure of **4**.



TAN-1030A has the same absolute stereochemistry as K-252a at the bridging atoms 2′ and 5′[13]. TAN-1030A might be formed by oxidation of the corresponding ketone derivative, another minor metabolite of strain R-19[10], which could be easily formed by oxidation of a sugar hydroxy function.

In the course of synthetic studies of the oxime TAN-1030A (**2**) we found a new, synthetically useful, ring-contracting reaction which leads in one step to compound **3** under Beckmann conditions[13]. It is therefore conceivable that TAN-1030A (**2**) is a biosynthetic precursor which is converted by the microorganism to the proposed intermediate **3**. This compound itself was not detected in the fermentation broth, but instead we found its *N*-methyl derivative **5**. Compound **5** might be formed from **3** by the same enzyme which introduces the *N*-methyl group into staurosporine and which might to be unable to discriminate between substrates with five- and six-membered ring systems. The free amino group of intermediate **3** could be converted to the hydroxy group of K-252a (**6**). This metabolite was found to be co-produced in small amounts by our strain *S. longisporoflavus* R-19. It is noteworthy that the proposed biosynthetic reactions from **2** to K-252a proceed without loss and incorporation of a carbon atom despite their structural differences.

In conclusion our work suggests that most compounds of the staurosporine type are biosynthetically even more closely related than previously anticipated. Investigations of blocked mutants of the *Nocardiopsis* species which produces K-252a and of *S. longisporoflavus* should further clarify the biosynthetic pathway.

Physico-chemical Data

Data of **4**

Colorless crystals from CH_2Cl_2-2-PrOH, MP 133~137°C; HREI-MS Found: *m/z* 480.215 Cacld for $C_{29}H_{28}N_4O_3$: 480.216; EI-MS: *m/z* 480 (30), 466 (4), 409 (7), 381 (5), 350 (9), 337 (7), 311 (10), 282 (5), 144 (100), 102 (38), 101 (41), 85 (22), 58 (32), 45 (56), 44 (85); IR (CH_2Cl_2) cm^{-1} 3450, 2970, 2930, 1685, 1630, 1590, 1470, 1460, 1400, 1380, 1350, 1340, 1320, 1230, 1150, 1130, 1120, 1070.

Data of **5**

White powder crystalized from CH_2Cl_2, MP 152~155°C; HREI-MS Found: *m/z* 480.180 Cacld for $C_{28}H_{24}N_4O_4$: 480.181; EI-MS: *m/z* 480 (16), 437 (5), 406 (7), 353 (15), 311 (36), 170 (18), 128 (20), 111 (19), 87 (100), 68 (16), 55 (13), 44 (66); IR (CH_2Cl_2) cm^{-1} 3450, 2960, 2930, 1730, 1690, 1590, 1460, 1390, 1370, 1320, 1200, 1150, 1050; CD (λ^{EtOH} nm (θ): 330 (4300), 310 (1081), 299 (8850), 271 (−4900), 252 (15000), 240 (9500), 212 (−23500).

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