SECONDARY METABOLITES BY CHEMICAL SCREENING 14[1]

TRANSFORMATION OF ELAIOPHYLIN IN SUBUNITS OF NATURALLY OCCURRING ACID IONOPHORES: SYNTHESIS, ANTICOCCIDIAL ACTIVITY AND STUDIES CONCERNING THE IONOPHORIC PROPERTIES

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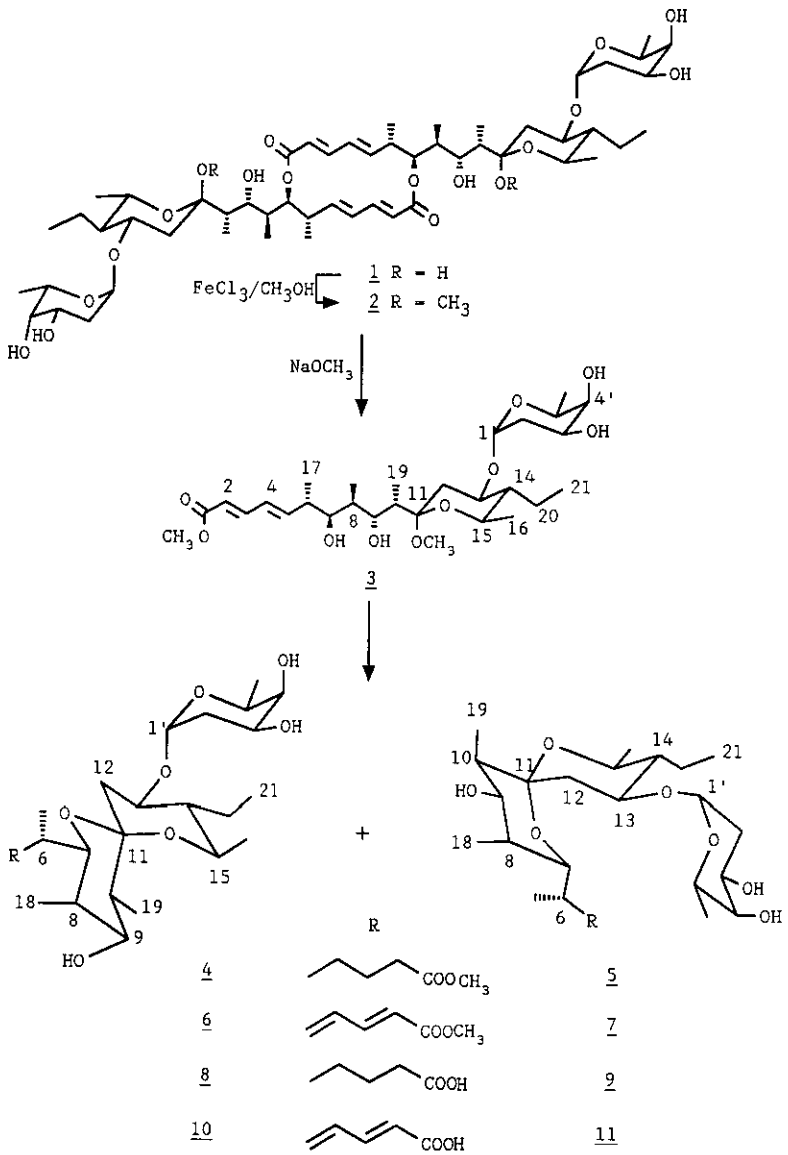
Abstract-The synthesis of the polyether subunits (**8** - **11**), starting from elaiophylin (**1**), as well as their biological properties, nmr spectra and conformational studies were described and discussed. Based upon the missing ability of potassium transport it was deduced that compounds (**8** - **11**) were new lead structures concerning their anticoccidial activity.

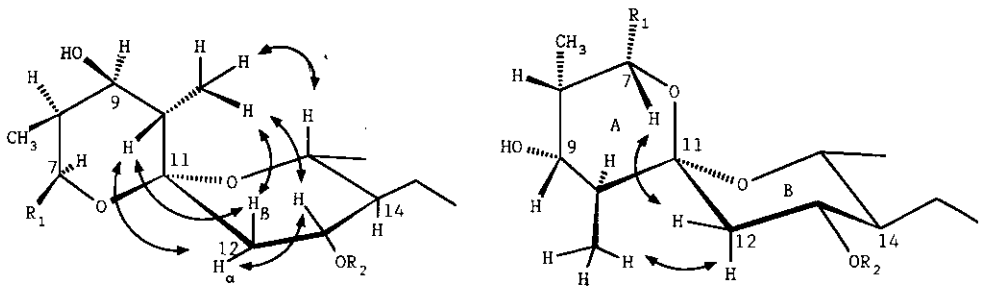
Polyethers like salinomycin, nigericin and lasalocid[2] are complex molecules possessing spiroacetal and cyclic ether units forming a cyclic structure, which is stabilized by the asymmetric centres in the backbone and a hydrogen bond between the carboxylic acid and a hydroxyl moiety.[2] Many polyethers are antibacterially, antivirally and anticoccidially active compounds.[3] Their mode of action was explained by cation complexation and transportation of cations through biological membranes.[3] A disadvantage of polyethers is their high toxicity in animals and cytotoxicity in different cell systems, as well as their low selectivity concerning their biological activities. Thus, there is still a need for new members of this interesting class of compounds, which have improved pharmacological properties.


FeCl3/CH3OH
1 R = H
2 R = CH3
NaOCH3
3
+
R
4 COOCH3 5
6 COOCH3 7
8 COOH 9
10 COOH 11

Griseochelin for example, an ionophore antibiotic with one tetrahydropyran unit, a carboxylic acid moiety and a polyhydroxy side chain exhibited potent activity against bacteria and viruses.[4] This demonstrated that smaller structures containing polyether subunits may also exhibit some valuable pharmacological activities. This prompted us to synthesize polyether subunits starting from elaiophylin (**1**) in order to study their biological effects and ionophoric properties. Elaiophylin (**1**), an antibacterially active macrodiolide from our pool of natural products derived by chemical screening,[5] possesses the structural elements required for the transformation process.

The reaction sequence started with acetalization to provide retro aldol cleavage in **2**.[6,7] Cleavage of **2** with sodium methoxide in methanol yielded the seco ester (**3**). The saturated spiroacetals (**4**) and (**5**) were generated by hydrogenation of **3** with Pd/C (Merck).[7] The spiroacetalization was carried out from **3** with $FeCl_3$ in methanol to **6** and **7**.[7]

The constitution and stereochemistry of the reaction products (**4** - **11**) were defined by means of nmr spectroscopy. The completely assigned ^1H and ^{13}C spectra provided corroboration for the assumed primary structure of individual isomeric product pairs while differences in their ^1H and ^{13}C nmr data (Tables 1 and 2) were readily attributable to the opposite stereochemistry at the spiro carbon atom, C-11. Definition of the actual (relative) configuration at that chiral centre was conveniently based on ^1H-{H} NOE difference spectroscopy. Selective saturation of the resonance due to 7-H_{ax} in **5** resulted in a significant enhancement of the 12-H_{eq} proton signal indicating that the pertinent protons were located on the same face of the plane defined by the C_7-O-C_{11}-C_{12} bonds (see Figure 1).



4 **5**

Figure 1: NOE effects in the spiroketal compounds (**4**) and (**5**).

Table 1: ^{1}H Nmr data of **4** and **5** in DMSO-d_6 solution at 30°C (400 MHz).

	4		**5**	
	δ(ppm)	$J_{H/H}$ (Hz)[a]	δ(ppm)	$J_{H/H}$ (Hz)[a]
2-H_2	2.28 2.29	J_{gem}=-15.5; $J_{2/3}$=7.3 and 7.5	2.27 2.30	J_{gem}=-16.0; $J_{2/3}$=7.2
3-H_2	1.48	m	1.48	m
4-H_2	1.15 1.35	m m	1.14 1.39	m m
5-H_2	1.10 1.70	m m	0.94 1.81	m m
6-H	1.45	$J_{6/7}$=10.0; $J_{6/17}$=6.5	1.47	$J_{6/7}$=9.7; $J_{6/17}$=6.6
7-H	3.42	$J_{7/8}$=2.0	3.05	$J_{7/8}$=2.0
8-H	1.78	$J_{8/9}$=4.7; $J_{8/18}$=6.5	1.83	$J_{8/9}$=4.8; $J_{8/18}$=6.7
9-H	3.47	$J_{9/10}$=10.8; $J_{9/OH}$=5.0	3.45	$J_{9/10}$=11.2; $J_{9/OH}$=5.0
10-H	1.36	$J_{10/19}$=6.6	1.45	$J_{10/19}$=6.7
12-H_α 12-H_β	1.60 2.10	J_{gem}=-14.5; $J_{12\alpha/13}$=3.5 $J_{12\beta/13}$=6.5	0.98 2.41	J_{gem}=-13.0; $J_{12\alpha/13}$=10.6 $J_{12\beta/13}$=4.0
13-H	3.64	$J_{13/14}$=8.5	3.78	$J_{13/14}$=10.6
14-H	1.68	$J_{14/15}$=9.8; $J_{14/20}$=4.5	1.13	$J_{14/15}$=10.4; $J_{14/20}$=4.0 and 3.0
15-H	3.50	$J_{15/16}$=6.3	3.93	$J_{15/16}$=6.2
16-H_3	1.15		1.07	
17-H_3	0.74		0.737	
18-H_3	0.72		0.741	
19-H_3	0.85		0.84	
20-H_2	1.35 1.44	J_{gem}=-14.0; $J_{20/21}$=7.5	1.38 1.68	J_{gem}=-14.0; $J_{20/21}$=7.3
21-H_3	0.83		0.78	
1'-H	4.90	$J_{1'/2'eq}$=1.0; $J_{1'/2'ax}$=3.8	4.96	$J_{1'/2'eq}$=1; $J_{1'/2'ax}$=3.5
2'-H_{eq} 2'-H_{ax}	1.46 1.77	J_{gem}=-12.5; $J_{2'eq/3'}$=5.0 $J_{2'ax/3'}$=11.5	1.48 1.81	J_{gem}=-12.4; $J_{2'eq/3'}$=4.9 $J_{2'ax/3'}$=12.0
3'-H	3.74	$J_{3'/4'}$=3.0; $J_{3'/OH}$=6.3	3.73	$J_{3'/4'}$=3.0; $J_{3'/OH}$=6.0
4'-H	3.37	$J_{4'/5'}$=1.0; $J_{4'/OH}$=4.5	3.38	$J_{4'/5'}$=1.0; $J_{4'/OH}$=4.7
5'-H	3.74	$J_{5'/6'}$=6.5	3.73	$J_{5'/6'}$=6.5
6'-H	1.07		1.06	
OCH_3	3.57		3.56	

[a] Mutual interproton couplings are given only once, at their first occurrence in the Table

Table 2: ^{13}C Nmr data of **4**, **5**, **6**, **7**, **10** and **11** in DMSO-d_6 (100 MHz, chemical shifts in ppm)

	4	**5**	**6**	**7**	**10**	**11**
1	175.45	173.15	166.65	166.44	167.62	167.45
2	33.27	33.16	118.27	118.75	119.27	120.29
3	25.01	24.90	150.03	148.25	149.04	147.23
4	26.12	26.03	126.24	127.70	126.40	127.87
5	32.53	32.64	145.57	145.00	144.93	144.30
6	34.20	34.06	37.45	38.80	37.39	38.84
7	73.62	76.36	73.55	75.26	73.56	75.26
8	35.94	36.05	36.05	35.80	36.04	35.86
9	71.26	71.39	71.17	71.29	71.16	71.22
10	41.07	40.44	41.00	40.44	41.00	40.44
11	100.03	100.86	100.01	101.05	100.00	101.02
12	35.36	27.96	35.24	28.17	35.40	28.25
13	70.25	66.77	70.17	66.90	70.22	67.62
14	43.92	47.68	44.19	48.53	44.01	48.14
15	70.40	66.66	70.26	66.72	70.29	66.92
16	21.09	19.19	21.05	19.12	21.04	19.15
17	14.07	14.14	14.19	15.45	14.27	15.53
18	4.61	4.85	4.64	4.83	4.65	4.85
19	11.85	12.28	11.70	12.21	11.71	12.20
20	21.63	18.48	21.67	18.75	21.59	18.73
21	9.85	7.83	9.82	8.50	9.77	8.67
1'	93.15	92.72	93.72	93.25	93.77	93.12
2'	32.52	32.47	32.83	32.73	32.86	32.64
3'	66.41	66.40	66.31	66.90	66.31	66.62
4'	70.23	70.17	70.17	70.32	70.20	70.34
5'	64.90	64.89	64.95	64.90	64.90	64.92
6'	16.99	16.91	16.96	17.00	16.95	17.05
CH_3	51.1	51.01	51.07	51.10		

Molecular models showed that such an arrangement of substituents at C-11 should result in the spatial proximity of 19-H and 12-H_{ax} protons, an expectation confirmed by mutual enhancements observed upon selective preirradation of the pertinent proton resonances. A series of NOE experiments performed with **4** revealed dipolar contacts between ring A and ring B protons (see Figure 1) in accordance with the altered configuration at C-11. Inspection of the ^{1}H-^{1}H coupling constants (Table 1) discloses that ring B assumes different conformations in the two isomeric spiroketals (**4**) and (**5**): while a regular chair form is seen to be characteristic for **5**, the coupling data measured for **4** are more compatible with a twisted chair arrangement. The latter distortion can be thought to be the result of the steric compression occurring between the 19-methyl group and the axially orientated protons 13-H_{ax}, 15-H_{ax} in the regular chair conformation. Spectral comparison indicates that the stereochemical conclusions made for **4** and **5** can be extented to the respective unsaturated analogs (**6**) and (**7**).

Conformational preferences in 1,7-dioxaspiro[5.5]undecanes are mainly influenced by steric interactions or anomeric effects. Normally, the thermodynamically more favoured equatorial position of the substituents prevailes the bisaxial arrangements of the spiro C-O bonds.[8] As expected, the nmr results confirmed that the conformations where 1,3-diaxial interactions occurred were minimized. This implied for **4** and **5** that one spiro C-O bond had to be orientated equatorially. Only for two naturally occurring compounds, aplysiatoxin and pectenotoxin, this conformation was described to date.[8] The four spiroacetals (**4** - **7**) could be saponified easily with 2N sodium hydroxide/ THF to the corresponding acids (**8** - **11**) within 2 hours in an almost quantitative yield. The products were separated by acidification and extraction with ethyl acetate and no further purification step was required. The saponification to **8** - **11** had no influence on the conformations described for the corresponding esters **4** - **7** as deduced from the comparison of the nmr spectra.

Compounds (**8** - **11**) contain a spiroacetal and a carboxylic acid moiety which show similarities to smaller polyethers like A 23 187 (calcimycin).[3] Although, the X-ray crystal structure of A 23 187 indicated that the

spiroacetal unit in this compound resided in the favoured bis-axial C-O conformation.

An investigation of the biological activities of **8** - **11** showed no effects against bacteria at a concentration of 100 μg/ml[5] and **9** and **11** exhibited only a weak effect against herpes viruses, prepared in VERO cells,[9] at a concentration of 40 μg/ml. The low toxicity was remarkable against VERO cells expressed in the maximal tolerated dose (MTD >400 μg/ml) compared to the naturally occurring polyethers salinomycin and lasalocid (MTD = 1.65 μg/ml). On the other hand, for **8** - **11** in contrast to the starting molecule elaiophylin (**1**), a distinct anticoccidial effect against E. tenella in vivo in chicken[10] was observed (Table 3).

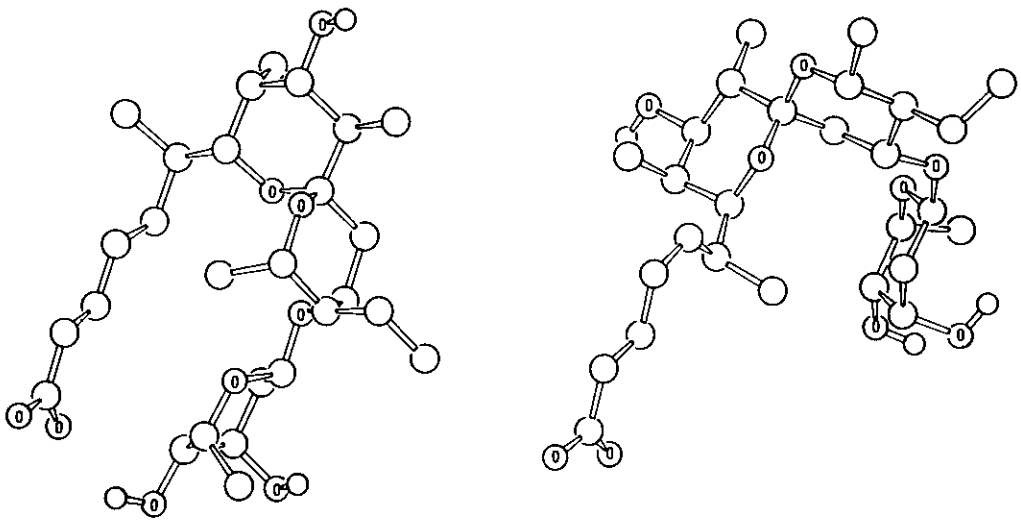
Table 3: Anticoccidial effect of **8** - **11** at 60 ppm in comparison with salinomycin at 60 ppm and lasalocid at 90 ppm in 8 chicken per group infected with Eimeria tenella

anticoccidial parameters compared to uninfected controls	sali-nomycin	lasa-locid	**8**	**9**	**10**	**11**	infected control
morbidity (%)	0	0	12.5	12.5	12.5	12.5	50
average weight gain (%)	98	90	40	70	50	80	33
lesion scores	0.5	1.4	3.1	3.5	2.8	2.6	4.0
reduction of oocysts (%)	82	72	32	30	33	43	-

In order to obtain information about the three dimensional structure and to study their polyether character the compounds (**10**) and (**11**) have been subjected to a conformation search using the valence force field method for calculating conformational energies.[12] In vacuum, compound (**10**) shows a preference for the formation of a cyclic structure stabilized by a OH-4'/O-1 hydrogen bond.

In contrast to **10**, the spiroketal (**11**) prefers a noncyclic conformation. The conformational energy of the noncyclic structure has been calculated to be 2 kcal/mol lower than that of the corresponding cyclic conformation of **11**. The latter is stabilized by an OH-3'/O-1 hydrogen bond.

To get deeper insight into the conformational behavior of compounds (**10**) and (**11**) molecular dynamics simulation with 40 water molecules and periodic boundary conditions have ben performed. Each dynamics run corresponds to a



10 **11**

Figure 2: Calculated conformations of **10** and **11**.

time period of 50 picoseconds at a temperature of 500 Kelvin. During the simulation compound (**10**) undergoes several small conformational changes. In most of these conformations the OH-4'/O-1 distance is larger than is typical for hydrogen bonds. However, they all exhibit a hairpin-like shape as is shown for the lowest energy conformations of **10** in Figure 2. In compound (**11**) the noncyclic conformation is essentially retained througout the simulation. If potassium ions are added to the simulated systems the conformational properties of both **10** and **11** are not affected considerably. The potassium complexation primarily takes place at the carboxylic acid moiety without significant participation of other groups.

In vacuum, ring B in compound (**10**) assumes a twist chair conformation which is due to the steric hindrance of the C-10 methyl group. In the dynamics simulation with water however, the average structure of **10** shows only a slightly distorted chair conformation of ring B. The fact that a twist chair conformation of ring B in the compounds (**4**), (**6**), (**8**) and (**10**) in DMSO, as suggested by nmr studies might thus be attributed to solvent effects.

Furthermore, the complex stability constant K_{mL} of **11** was estimated. The log (K_{mL}) value of **11** with sodium cation was 16.4 and for potassium cation 13.1, determined by acid-base titration of the free ligand and the ligand-metal mixtures in water/methanol solution and subsequent iterative optimization using the fortran program BEST.[11]

Table 4: Complex stability constants log (K_{mL}) of **11**, nigericin and salinomycin with sodium and potassium

compound	log (K_{mL}) Na^+	log (K_{mL}) K^+
11	16.4	13.1
salinomycin	1.8	2.2
nigericin	4.1	5.1

These values were remarkably high, compared to naturally occurring polyethers especially when taking into account that only one ionic bond is involved in cation binding (Table 4).

To assess the efficiency of **8** - **11** as polyether ionophores, the H^+/K^+-antiport activities of compounds (**10**) and (**11**) in the membranes of liposomes were measured. Liposomes (average diameter 110 nm) were prepared from soy bean phospholipids (Sigma No. P5638) as described.[13] The liposomes were suspended (0.6 g phospholopid/l) in a medium (pH 6.5, 22° C) containing 4-morpholino-ethanesulphonate, glycylglycine and KCl, 0.1 M each. The liposomes contained the same medium supplemented with 0.1 mM 8-hydroxy-1,3,6-pyrenetrisulphonate. After addition of the compounds (**10**) or (**11**), the external pH was shifted to 7.5. The velocity of internal pH increased (dpH/dt), which was caused by the external pH shift, was recorded fluorometrically. The proton flux (J) was calculated according to equation (a) using the internal buffering capacity (B) of liposomes.

$$(a) \qquad J/N = (dpH/dt) \cdot B/N$$

The turnover numbers in Table 5 represent the ratios (J/N) of the initial proton flux ($pH_{int.}$ 6.5; $pH_{ext.}$ 7.5) and the amounts (N) of compounds (**10**) or (**11**) within the membrane.

The turnover number of nigericin was high (1170s^{-1}) with N ranging between

0.5 and 50 nmol/g phospholipid (Table 5). When compound (**10**) or (**11**) was applied in the same amounts, the proton flux was equal to that measured in the absence of an ionophore. Stimulating effects on the proton flux were only observed with much greater ammounts. The turnover numbers of compound (**10**) and (**11**) given in Table 5 refer to N=5000 nmol/g phospholipid. They are more than three orders of magnitude smaller than that of nigericin.

Table 5: Compounds (**10**) and (**11**) as H^+/K^+-antiporters in liposomal membranes, compared to nigericin.

Compound	N (nmol/g phospholipid	turnover number (s^{-1})
nigericin	0.5 - 50	1170
10	5000	0.3
11	5000	0.01

This result is consistent with the biological activities of the three compounds. When compound (**10**) or (**11**) was present together with the protonophore 4,5,6,7-terachloro-2-trifluoromethylbenzimidazol (5 μmol/g phospholipid), the turnover numbers were not increased. This ruled out the possibility that compound (**10**) or (**11**) would act as electrogenic uniporters of K^+.

An universal property of polyether ionophores is the central orientation of the liganding site, whereas the surface of the complex is built up by nonpolar hydrocarbons to minimize interactions of the bound cation with lipid bilayers during the transport through a membrane. Based on the calculated structures the lack of potassium transport can be explained for **10** and **11**, and because of the structural similarities also for **8** and **9**, by the oxygen atoms as well as the cation orientated to the surface of the molecule. In **11** the noncyclic conformation causes an additional polarity directed towards the outside of the molecule, so that the hydrophility disturbs the transport through lipophilic membranes. This should be the fact for cations other than potassium, too. In summary, as the activity of **8** - **11** against coccidia can not be explained by an ionophoric activity it must be based upon a different mechanism. Therefore, the spiro-acetal acids represent new lead compounds in this indication. So one can speculate now, if the anticoccidial activity of

natural occurring polyethers may not only be caused by the ionophoric effect but also, in addition be associated to specific interactions with an enzyme, for example.

Furthermore, these results demonstrate how a complex molecule like **1** can be used as a building block for a polyether subunit and can therefore be chemoactivated with regard to a new biological activity.

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