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Anti-cancer and Antibacterial Trioxacarcins with High Anti-malaria Activity from a Marine *Streptomycete* and their Absolute Stereochemistry†

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The ethyl acetate extract from the *Streptomyces* sp. isolate B8652 delivered the trioxacarcins A～C (**2a**～**2c**) and additionally three new derivatives designated as trioxacarcins D～F (**2d**～**2f**). All trioxacarcins showed high anti-bacterial and some of them high anti-tumor and anti-malaria activity. The structures of the new antibiotics were derived from mass, 1D and 2D NMR spectra and confirmed by comparison of the NMR data with those of known derivatives. The absolute configuration of the trioxacarcins is deduced from the X-ray analysis of gutingimycin (**2g**) and from the known stereochemistry of the L-trioxacarcinoses A and B.

Trioxacarcins are complex antibiotics isolated first in 1981 by Tomita *et al.*[1]; their structures were elucidated in the following year by Shirahata *et al.*[2]. Trioxacarcins A (**2a**), B (**2b**) and C (**2c**) were obtained from *Streptomyces ochraceus* and also *Streptomyces bottropensis*, trioxacarcin A_1 (O^{13}-deglyco-trioxacarcin A) and A_2 (O^4,O^{13}-deglyco-trioxacarcin A), however, only from the latter. Trioxacarcins are cytotoxic against various cancer cell lines and are also active against Gram-positive and Gram-negative bacteria, the trioxacarcins A_1 and A_2 exhibit also antifungal activity.

In the course of our screening program for novel bioactive compounds from marine Streptomycetes, the ethyl acetate extract of the marine *Streptomyces* sp. isolate B8652[3] drew our attention due to various yellow bands on TLC, which showed an intensive green fluorescence under UV light and gave a grey colouration with anisaldehyde/sulphuric acid. In addition, high biological activity against *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis*, and *Streptomyces viridochromogenes* Tü 57 deserved further interest. Purification of the non-polar fraction afforded parimycin[3] (**1**), while the middle polar fraction delivered the known trioxacarcins A～C (**2a**～**2c**) and the new derivatives D～F (**2d**～**2f**). The polar fraction delivered a novel structure which we named gutingimycin[4] (**2g**). We also discovered a so far unknown high anti-malaria activity of the trioxacarcins. In this paper we report on the isolation, the structure elucidation and the bioactivity of the trioxacarcins A～F (**2a**～**2f**) and the bioactivity of gutingimycin (**2g**).

Fermentation and Isolation

The taxonomic properties and the fermentation of *Streptomyces* sp. isolate B8652 have been reported previously[3]. The crude extract, obtained after usual work-

† Art. No. XXVIII on Marine Bacteria. Art. XXVII: R. N. Asolkar, D. Schröder, R. Heckmann, S. Lang, I. Wagner-Döbler & H. Laatsch: Helquinoline, a new tetrahydroquinoline antibiotic from *Janibacter limosus* Hel 1. J. Antibiotics 57, 17～23, 2004.

Dedicated to Prof. Dr. H. Anke on the occasion of her 60th birthday.

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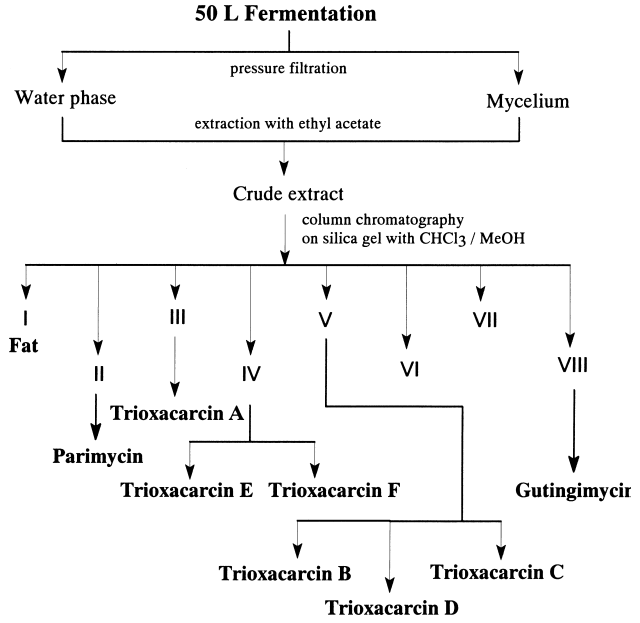
Fig. 1. Work-up scheme of the marine *Streptomyces* sp. isolate B8652.



up of 20 liters of culture broth, was subjected to flash chromatography on silica gel with a MeOH/$CHCl_3$ gradient. The fraction I contained only fats and was not further analysed. A second yellow, green fluorescent fraction II delivered parimycin[3] (**1**). The following fractions III~V were highly active in the agar diffusion test against *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis*, and *Streptomyces viridochromogenes* Tü 57. Further separation of these fractions yielded mainly trioxacarcin A (**2a**), and the minor components **2b**~**2f** as light yellow solids. The highly polar fraction VIII contained a compound designated as gutingimycin (**2g**).

Results and Discussion

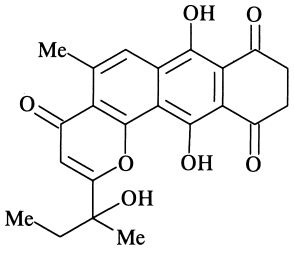
The colour, the fluorescence under 366 nm and the ^{1}H NMR spectrum of the yellow compound **2a** indicated similarities with the data of parimycin (**1**). A chelated hydroxy group gave an H/D exchangeable 1H singlet at δ 14.06 and the aromatic region showed a singlet at δ 7.49. The signals between δ 6.0~4.0 could be assigned to the presence of additional sugar moieties in the molecule. Three further methoxy signals at δ 3.81, 3.61 and 3.44 and three singlets of olefinic or aromatic methyl groups were visible at δ 2.60, 2.46 and 2.12. Furthermore, two methyl doublets at δ 1.21 and 1.08, a 3H singlet at δ 1.04, and several 1H doublets and multiplets between δ 3.00~1.00 were identified. The ^{13}C/APT NMR spectra delivered more than 40 signals. The signals at δ 210.3 and 202.8 could be assigned to ketone signals, that one at δ 170.3 results from an acid, ester or lactone. Ten signals between δ 163.1 and 107.4 were assigned to the naphthalene system of **2a**. The signals at δ 104.6, 101.5, 99.7, 97.9 and 94.8 are due to acetal carbons or electron rich sp^2 atoms. Thirteen carbon atoms with signals between δ 80.0~56.0 must be connected directly with oxygen. Below δ 48.0, three CH_2 and six CH_3 signals were identified. The (+)- and (−)-ESI mass spectra showed peaks at *m/z* 1775 ($[2M+Na]^+$) and 899 ($[M+Na]^+$), respectively at *m/z* 875 ($[M-H]^-$), which led to a molecular weight of 876.

The proton and ^{13}C NMR spectra of compound **2b** showed very close similarities with those of compound **2a**, except that the proton signals at δ 2.98 and 2.89 of a methylene group were shifted to δ 3.75 and the corresponding methylene carbon signal at δ 48.2 were shifted to lower field as well. The molecular weight, derived from the ESI mass spectrum, was 18 units higher than that of compound **2a**, which indicated compound **2b** to contain an additional molecule of water.

The ^{1}H and ^{13}C NMR spectra of compound **2c** were again very similar to those of compound **2a**. The only deviation was an additional 1H quartet at δ 3.93 and a 3H doublet in the region δ 1.40~1.20 instead of the methyl singlet at δ 2.46. In the ^{13}C NMR spectrum, the carbonyl signal at δ 210.3 was missing and an additional signal in the aliphatic region was detected. The missing ketone signal and the additional 2 mass units compared with compound **2a** indicated that the ketone group in the sugar moiety of compound **2a** had been reduced to an alcohol.

The search in AntiBase[5] with these data led to the trioxacarcins A~C[1,2] (**2a**~**2c**) and to the isomeric antibiotics of the LL-D49194 group[6], where the residue at C-13 in the **2** series and one of the methoxy groups at C-16 are exchanged. Therefore the structures were controlled by 2D experiments (Figs. 2 and 3) which confirmed, however, structures **2a**~**2c**.

Compound **2d** exhibited the same characteristic properties as shown by **2a**~**2c**. Comparison of the proton spectrum of this substance with that of **2a** demonstrated identity except for a missing acetate signal at δ 2.11. In the ^{13}C NMR spectrum, the signals of the acetate group at δ 170.3 and 27.8 were missing as well. The (+) and (−) ESI mass spectrum showed *quasi*molecular ion signals at *m/z* 1691 ($[2M+Na]^+$) and 857 ($[M+Na]^+$) or 1668 ($2M^-$) and 834 (M^-), respectively. ES HRMS measurements resulted in the molecular formula $C_{40}H_{50}O_{19}$, thus confirming the



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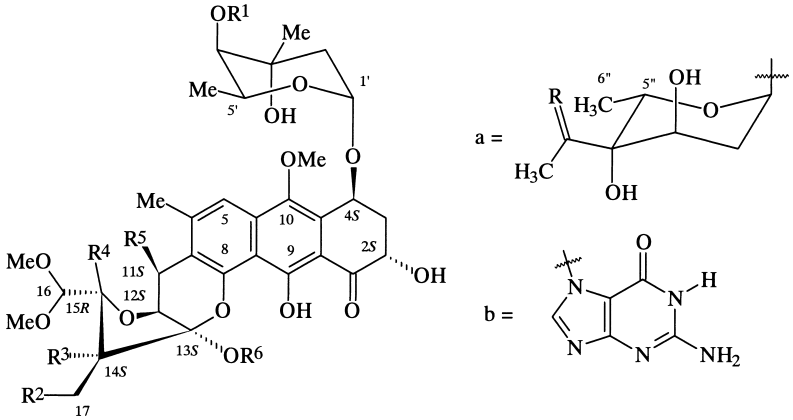
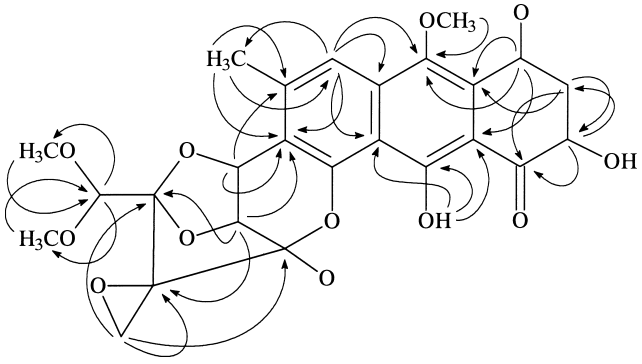
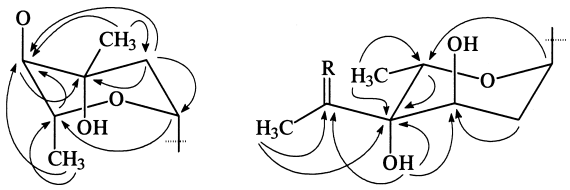


2a: $R^1 = COCH_3$; R^2-$R^3 = R^4$-$R^5 = O$; R^6 = a: R = O
2b: $R^1 = COCH_3$; $R^2 = R^3 = OH$; R^4-$R^5 = O$; R^6 = a: R = O
2c: $R^1 = COCH_3$; R^2-$R^3 = R^4$-$R^5 = O$; R^6 = a: R = OH, H
2d: $R^1 = H$; R^2-$R^3 = R^4$-$R^5 = O$; R^6 = a: R = O
2e: $R^1 = COCH_3$; $R^2 = R^3 = R^4 = R^5 = OH$; $R^6 = H$
2f: $R^1 = COCH_3$; $R^2 = R^3 = R^4 = R^5 = OH$; R^6 = a; R = O
2g: $R^1 = COCH_3$; R^2 = b; $R^3 = OH$; R^4-$R^5 = O$; R^6 = a: R = O

Fig. 2. HMBC correlations (→) in the aglycon of the trioxacarcins.

Fig. 3. HMBC correlations (→) in the sugar moieties of the trioxacarcins.





absence of an acetate group. The final structure **2d** was derived by H,H COSY, HMQC and HMBC couplings (Figs. 2 and 3) and the new derivative was named as trioxacarcin D.

Similarly, compound **2e** was obtained as a yellow solid showing the same green fluorescence at 366 nm and a grey colouration with anisaldehyde/sulphuric acid, and the ^1H NMR spectrum was again typical for trioxacarcins. Some sugar signals between δ 5.0~4.0, the CH_2 signal of the epoxide, a methyl singlet at δ 2.4 and a doublet at 1.3, however, were missing. In the ^{13}C NMR spectrum, among others, a ketone signal and anomeric carbon signals of the acetyl sugar were missing. Obviously one sugar residue must have got lost, presumably that at C-13, resulting in the unknown O^{13}-deglyco-trioxacarcin B with a mass of 722.

Table 1. ^{1}H NMR data (δ, J) of trioxacarcins B (**2b**), D (**2d**), E (**2e**), and F (**2f**) in $CDCl_3$.

Atom no.	**2b**	**2d**	**2e**	**2f**	Atom no.	**2b**	**2d**	**2e**	**2f**
2	4.80 (dd, 12.2, 5.8)	4.77 (dd 12.7, 5.4)	4.76 (dd, 12.7, 5.4)	4.78 (dd, 12.7, 5.4)	1'	5.35 (t, 3.7)	5.29 (t 4.0)	5.35 (t, 4.0)	5.35 (d, 3.4)
3_A	2.78 (m)	2.80 ddd 13.7, 5.4, 3.7)	2.78 (ddd, 13.7, 5.4, 3.7)	2.79 (m)	$2'_A$	1.92 (dd, 14.1, 4.1)	1.93 (dd, 14.9, 4.2)	1.94 (dd, 14.9, 4.2)	1.94 (dd, 14.5, 4.2)
3_B	2.21 (td, 13.2, 2.7)	2.17 (td 13.2, 2.7)	2.58 (td, 13.2, 2.7)	2.20 (td, 13.2, 2.7)	$2'_B$	1.60 (d, 14.6)	1.55 (d, 14.9)	1.59 (d, 14.9)	1.63 (d, 14.5)
4	5.43 (t, 2.9)	5.37 (t 2.9)	5.38 (t, 2.9)	5.40 (t, 2.7)	4'	4.74 (d 1.4)	3.18 (s br)	4.72 (s br)	4.74 (s br)
5	7.46 (d, 0.8)	7.49 (d 0.8)	7.44 (d, 0.8)	7.49 (d, 0.8)	5'	4.53 (m)	4.51 (dq 6.4)	4.54 (m)	4.53 (m)
9-OH	14.80 (s)	14.03 (s)	14.06 (s)	14.08 (s)	5'-CH_3	1.22 (d, 6.6)	1.35 (d, 6.6)	1.12 (d, 6.6)	1.22 (d, 6.6)
6-CH_3	2.57 (d, 0.7)	2.59 (d 0.7)	2.48 (d, 0.7)	2.57 (d, 0.7)	4'-$OCOCH_3$	2.11 (s)	-	2.10 (s)	2.12 (s)
10-OCH_3	3.82 (s)	3.83 (s)	3.82 (s)	3.83 (s)	3'-CH_3	1.04 (s)	1.19 (s)	1.02 (s)	1.05 (s)
11	5.14 (d, 4.0)	5.22 (d 4.2)	5.09 (d, 4.2)	5.09 (d, 3.9)	1"	5.65 (d, 2.6)	5.83 (d 2.4)	-	5.89 (d, 2.6)
12	5.21 (d, 4.0)	5.35 (d 3.9)	4.63 (d, 3.9)	5.21 (d, 3.7)	$2"_A$	2.28 (m)	2.42 (dt, 14.9, 5.7)	-	2.41 (dm, 3.6)
16	5.08 (s)	4.75 (s)	4.92 (s)	5.03 (s)	$2"_B$	2.04 (dm, 13.4)	2.10 (dm, 4.4)	-	2.11 (m)
16-OCH_3	3.63 (s)	3.61 (s)	3.61 (s)	3.62 (s)	3"	3.67 (m)	3.69 (m)	-	3.76 (m)
16-OCH_3'	3.56 (s)	3.47 (s)	3.52 (s)	3.61 (s)	5"	4.95 (q, 6.3)	5.01 (q 6.4)	-	4.94 (q, 6.3)
17_A	3.72 (m)	2.97 (d 5.6)	3.52 (m)	3.66 (d, 11.9)	5"-CH_3	1.03 (d, 6.5)	1.07 (d, 6.4)	-	1.03 (d, 6.5)
17_B	3.72 (m)	2.89 (d 5.6)	3.50 (m)	3.53 (d, 12.2)	4"-$COCH_3$	2.44 (s)	2.47 (s)	-	2.45 (s)
OH	**2b**: 6.02 , 4.84, 4.31, 4.08, 4.05, 3.88 **2e**: $CDCl_3$/10% MeOH				OH	**2d**: 4.22, 4.15, 3.58, 3.52 **2f**: 4.56, 4.02, 3.88			

As the observed molecular weight of compound **2e** (*m/z* 740) demands one additional mol of water, the acetal bond between C-11 and C-15 in **2b** must be hydrolysed. In the HMBC spectrum, 12-H and C-15, but not 11-H and C-15, were coupling together, thus confirming the expectation. Also all other correlations were fitting with the new structure **2e**, which we designated as trioxacarcin E.

Due to its chemical characteristics and the ^{1}H NMR and MS data, compound **2f** was a further trioxacarcin derivative. The compound decomposed, however, in chloroform under the influence of light and no ^{13}C and 2D NMR spectra could be measured.

The molecular weight of 912 (ESI MS) was 18 units higher than that of trioxacarcin B (**2b**). The similarity of the proton signals (see Table 1) with those of trioxacarcin E (**2e**) indicated that also here the acetal bond between C-11 and C-15 must have been hydrolysed giving trioxacarcin F (**2f**).

The X-ray structure of gutingimycin[4] (**2g**) delivered the relative stereochemistry of the trioxacarcin skeleton. As the sugar moieties of the trioxacarcins A (**2a**) and B (**2b**) had been identified previously as L-trioxacarcinoses A and B[2], the absolute configuration of trioxacarcin A~F (**2a**~**2f**) is established now as shown in formulae **2a**~**2g**. It should be mentioned again that the trioxacarcins are closely related with the antibiotics of the LL-D49194 group[7], where the

Table 2. Physico-chemical properties of trioxacarcins D (**2d**), E (**2e**) and F (**2f**).

	2d	**2e**	**2f**
Properties	yellow solid	yellow solid	yellow solid
R_f (CH_2Cl_2/10 % MeOH)	0.40	0.45	0.55
(+)-ESI-MS	1691 ([2M+Na]$^+$), 857 ([M+Na]$^+$)	763 ([M+Na]$^+$)	1847 ([2M+Na]$^+$), 935 ([M+Na]$^+$)
(-)-ESI-MS	1667 [2M-H]$^-$, 833 [M-H]$^-$	1479 [2M-H]$^-$, 739 [M-H]$^-$	911 ([M-H]$^-$)
(-)-HRESI-MS	833.2988 (calcd. for [M-H]$^-$ 833.28733)		
Molecular formula	$C_{40}H_{50}O_{19}$	$C_{34}H_{44}O_{18}$	$C_{42}H_{58}O_{22}$
IR (KBr) ν cm^{-1}	3440, 2929, 1717, 1623, 1583, 1463, 1386, 1352, 1225, 1095, 1021, 994	-	-
UV/VIS (MeOH): λ_{max} nm (log ε)	270 (4.54), 396 (4.05)	-	-
$[\alpha]^{20}_D$	+20° (*c* 0.567, MeOH)	-	-

residue at C-13 in the **2** series and one of the methoxy groups at C-16 are exchanged.

Biological Properties

Antibacterial, antifungal and antialgal activities were qualitatively determined using the agar diffusion method. Results using *Bacillus subtilis*, *Streptomyces viridochromogenes* Tü 57, *Staphylococcus aureus* and *Escherichia coli* as test organisms are listed in Table 3. In the agar diffusion tests, trioxacarcins A~E (**2a~e**) and gutingimycin (**2g**) showed comparable activities, trioxacarcins A (**2a**) and D (**2d**) being the most active of all. In tests with the same microorganisms as in Table 3 using liquid medium, the trioxacarcins were found to be highly active with MIC values 0.15~2.5 μg/ml as compared to gutingimycin (**2g**) with MIC values >20 μg/ml. All the compounds were inactive, however, against the fungi *Candida albicans* and *Mucor miehei* and the micro-algae *Chlorella vulgaris*, *Chlorella sorokiniana* and *Scenedesmus subspicatus*.

In addition, the trioxacarcins A~D (**2a~2d**) and gutingimycin (**2g**) were tested against *Plasmodium falciparum*. Trioxacarcin A (**2a**) and D (**2d**) possessed extremely high antiplasmodial activity which was comparable to the most active compound, artemisinin. Trioxacarcin B (**2b**) was about 100 times less active than trioxacarcin A (**2a**) and D (**2d**), and trioxacarcin C (**2c**) and gutingimycin (**2g**) were nearly inactive. The results of the tests are listed in Table 4.

Table 3. Antibacterial activities of **2a~e** and **2g** in the agar diffusion test with 30~40 μg/paper disk (9 mmϕ), diameter of inhibition zones in [mm].

	EC[a]	BS[b]	SV[c]	SA[d]
Crude extract	30	41	26	22
Trioxacarcin A (**2a**)	34	40	25	24
Trioxacarcin B (**2b**)	32	38	27	22
Trioxacarcin C (**2c**)	30	38	24	22
Trioxacarcin D (**2d**)	33	39	28	23
Trioxacarcin E (**2e**)	30	37	24	20
Gutingimycin (**2g**)	28	30	25	21

[a]*Escherichia coli*, [b]*Bacillus subtilis*, [c]*Staphylococcus aureus*, [d]*Staphylococcus aureus*

The known trioxacarcins were already shown to possess anti-cancer activity. In our experiments with permanent human tumor cell lines, the trioxacarcins A~D (**2a~2d**) showed pronounced anti-tumour activity with mean IC_{70}-values ranging from 0.001 μg/ml to 2.161 μg/ml (Table 5).

Table 4. Antiplasmodial activities of **2a**~**d** and **2g** against *Plasmodium falciparum*.

Substances	Test 1*) (ng/ml)		Test 2 (ng/ml		Test 3 (ng/ml)		Test 4 (ng/ml)		Test 5 (ng/ml)		Mean (ng/ml)	
	IC 50 K1	IC50 NF54	IC 50 K1	IC50 NF54	IC 50 K1	IC50 NF54	IC 50 K1	IC50 NF54	IC 50 K1	IC50 NF54	IC 50 K1	IC50 NF54
Trioxacarcin A (**2a**)	<76 ±5.3	<76 ±5.2	1.9 ±0.3	<1.56 ±0.1	1.8 ±0.1	2.0 ±0.1	1.1 ±0.1	1.0 ±0.1	-	-	1.6 ±0.1	1.5 ±0.1
Trioxacarcin B (**2b**)	106 ±6.2	<76 ±4.9	116 ±6.8	94 ±5.7	84 ±4.4	70 ±6.1	-	-	-	-	102 ±4.9	82 ±6.1
Trioxacarcin C (**2c**)	<76 ±5.1	<76 ±4.9	4.1 ±0.2	2.9 ±0.2	-	-	-	-	-	-	>5000 n.d.	>5000 n.d.
Trioxacarcin D (**2d**)	-	-	<78 n.d.	<78 n.d.	<78 n.d.	<78 n.d.	2.0 ±0.1	1.6 ±0.1	2.5 ±0.1	1.7 ±0.1	2.3 ±0.2	1.7 ±0.1
Gutingimycin (**2g**)	3498 ±23.4	4773 ±28.4	4276 ±29.9	>5000 n.d.	-	-	-	-	-	-	3887 ±24.7	>5000 n.d.
Chloroquine	71 ±1.4	4 ±0.1	71 ±5.1	3.5 ±0.1	75 ±6.0	2.5 ±0.1	87 ±5.3	4.3 ±0.1	48 ±2.9	4.1 ±0.1	70 ±5.8	3.7 ±0.1
Artemisinin	0.4 ±0.1	1.1 ±0.2	0.8 ±0.1	1.2 ±0.1	0.94 ±0.1	0.89 ±0.1	1.0 ±0.1	0.7 ±0.1	0.5 ±0.1	1.0 ±0.1	0.7 ±0.1	1.0 ±0.1

*Tests were performed independently in a quadruplet (n.d. = not determined)

Table 5. Antitumor activities of **2a**~**2d** and **2g**.

Substances	Mean IC_{50} (μg/ml)	Mean IC_{70} (μg/ml)	IC_{70} (μg/ml)								
			HT-29	SF-268	H-460	LXFA 526L	LXFL 529L	MCF-7	MEXF 514L	PC3M	RXF 631L
Trioxacarcin A (**2a**)	0.0008	0.001	<0.3	<0.0003	<0.0003	<0.3	<0.0003	<0.0003	0.002	0.009	0.072
Trioxacarcin B (**2b**)	1.107	2.161	6.463	1.477	1.47	2.476	0.609	1.314	5.756	>3.0	2.146
Trioxacarcin C (**2c**)	0.003	0.012	<0.3	0.006	<0.0003	<0.3	0.016	0.002	0.139	0.035	0.118
Trioxacarcin D (**2d**)	0.071	0.34	0.723	0.152	0.033	<0.3	0.646	0.066	1.307	1.553	0.634
Gutingimycin (**2g**)	2.322	3.386	>3.0	>3.0	>3.0	>3.0	3.386	>3.0	>3.0	>3.0	>3.0

Human tumor cell lines of the following tissues were tested: colon cancer HT-29, melanoma MEXF 514L, adeno lung cancer LXFA 526L, large cell lung cancer LXFL 529L and H-460, central nervous system SF-268, mammary cancer MCF-7, prostate cancer PC3M, and renal cancer RXF 631L. Trioxacarcin A (**2a**) was potently active against all tumor cell lines tested. Trioxacarcins B~D (**2b**~**2d**) showed selective antitumor activity against certain tumor cell lines. Gutingimycin (**2g**) was clearly less active than **2a** with a mean IC_{70}-value of 3.386 μg/ml and had no pronounced antitumor selectivity (Table 5).

The inhibiting effect of the test compounds on the

Table 6. Antitumor activity of the trioxacarcins A~D against the large cell lung cancer xenograft LXFL 529 *in vitro*.

	IC_{50} [ng/ml]	IC_{70} [ng/ml]	IC_{90} [ng/ml]
Trioxacarcin A (**2a**)	< 0.1	0.16	0.74
Trioxacarcin B (**2b**)	6.0	25.0	157.0
Trioxacarcin C (**2c**)	< 0.003	0.33	1.0
Trioxacarcin D (**2d**)	0.26	5.0	19.0
Gutingimycin (**2g**)	> 10,000	> 10,000	> 10,000

growth of tumor stem cells to colonies was in addition investigated in a clonogenic assay. Tumor stem cells, which are responsible for the metastatic and infiltrative potential of a tumor, were prepared directly from solid human tumor xenografts growing in nude mice. Hence, the clonogenic assay reflects better the *in vivo* situation than *in vitro* assays using permanent tumor cell lines and has been found to be the most predictive test for further *in vivo* evaluation of anticancer drugs[8~10]. The antitumor activity of the trioxacarcins A~D (**2a**~**d**) on the large cell lung cancer tumor model LXFL 529 could be confirmed in this assay. IC_{70}-values obtained there ranged from 0.16 ng/ml (trioxacarcin A), 25.0 ng/ml (trioxacarcin B), 0.33 ng/ml (trioxacarcin C) to 5.0 ng/ml (trioxacarcin D), whereas gutingimycin (**2g**) did not show pronounced antitumor activity in this assay (IC_{70}>10.0 μg/ml) (Table 6).

Trioxacarcin A (**2a**) also proved to be very toxic in preliminary *in vivo* experiments in tumor bearing nude mice. The maximum tolerated dose (MTD) was found to be between 0.1 and 0.3 mg/kg, given i.p.

In an anti-viral test against the picornavirus EMC[11], parimycin (**1**), trioxacarcin A~D (**2a**~**2d**) and gutingimycin (**2g**) were found to be inactive to protect human A549 cells from virus lysis.

Experimental

Material & methods and antimicrobial tests were used as described earlier[12].

Antitumor Test

Human Tumor Cell Lines

Effects of the test compounds on the proliferation of human tumor cells were determined in an assay where human tumor cell lines, growing as monolayers were treated with the test compounds and surviving cells were stained with a fluorescence dye. Details of the test procedure have been described earlier[13].

Human Tumor Xenografts

Effects of the test compounds on tumor stem cells was investigated in a clonogenic assay, where inhibition of colony formation of tumor stem cells growing in soft agar is examined. Details of the test procedure have been described recently[10].

Anti-plasmodia Test

Antiplasmodial activity was determined using the NF54 strain of *P. falciparum* of unknown origin, first isolated at the Amsterdam airport and the K1 strain from Thailand. While strain NF54 displays normal sensitivity to all known antiplasmodial drugs, strain K1 is resistant to chloroquine and pyrimethamine. A modification of the [^{3}H]-hypoxanthine incorporation assay was used for determining *intra*-erythrocytic inhibition of parasite growth. Briefly, *P. falciparum*-infected human red blood cells in hypoxanthine-deficient culture medium (see above) were exposed to serial drug dilutions in microtiter plates for 48 hours at 37°C in a 4% CO_2-enriched humidified atmosphere before [^{3}H]-hypoxanthine was added for another 24 hours and its incorporation by viable parasites assessed. The drug concentration at which growth was inhibited by 50% (IC_{50}) was calculated by linear interpolation between the two drug concentrations above and below the 50% incorporation line. Chloroquine (10 mg/ml in stock solution) and artemisinin (10 mg/ml in stock solution) were employed as positive references. The values given in Table 4 are means of two independent assays; each assay was run in four individual assays.

Fermentation and Work-up of *Streptomyces* sp. B8652

The strain *Streptomyces* sp. B8652 was fermented using standard parameter and extracted with ethyl acetate to yield 16 g of crude extract A containing *ca.* 10 g Niax PPG 2025 (Union Carbide Belgium N.V. Zwijndrecht) in ethanol. But because of the high solubility of **2g**, it could not be completely extracted with ethyl acetate from the culture filtrate. Incomplete extraction was easily noticed due to the strong fluorescence of gutingimycin (**2g**) under 366 nm. 50 Liters of the culture filtrate were concentrated to about 3 liters under vacuum at 65°C and then lyophilized. The solid was then extracted with methanol and the solution evaporated to dryness (extract B).

Silica gel column chromatography (70×3 cm) of the extract A using a $CHCl_3$: CH_3OH gradient (1300 ml $CHCl_3$, 1000 ml $CHCl_3$/1% MeOH, 1000 ml $CHCl_3$/2% MeOH, 1000 ml $CHCl_3$/3% MeOH, 500 ml $CHCl_3$/5% MeOH, 500 ml $CHCl_3$/7% MeOH, 400 ml $CHCl_3$/10% MeOH, 300 ml $CHCl_3$/13% MeOH, 300 ml $CHCl_3$/20% MeOH, 200 ml MeOH) as eluent delivered eight fractions (I, 3.23 g; II, 1.52 g; III, 1.57 g; IV, 9.65 g (mainly Niax); V, 628 mg; VI, 148 mg; VII, 150 mg; VIII, 660 mg). The antibiotically active fraction II yielded parimycin[3] (**1**).

On TLC, fraction III showed a yellow zone with intensive green fluorescence under 366 nm and dark grey colouration with anisaldehyde/sulphuric acid. Separation on Sephadex LH-20 (4×100 cm, $CHCl_3$/40% MeOH), by PTLC (silica gel, $CHCl_3$/7% MeOH) and again Sephadex LH-20 (2×60 cm, $CHCl_3$/50% MeOH) yielded 257 mg **2a** as light yellow solid. Similarly, fraction V yielded **2b** (18 mg), **2c** (13 mg), and **2d** (8 mg) as yellow solids after separation by PTLC ($CHCl_3$/9% MeOH) followed by preparative HPLC (MeCN/45% H_2O to 100% MeCN in 35 minutes, flow 10 ml/minute) and then finally on Sephadex LH-20 (2×50 cm, $CHCl_3$/40% MeOH). Trioxacarcin E (**2e**) and F (**2f**) were obtained from fraction IV. By chromatography on silica gel (3×60 cm, cyclohexane/25% ethyl acetate) most of the Niax was removed. Preparative HPLC and then PTLC ($CHCl_3$/7% MeOH) yielded two yellow fractions, which delivered on Sephadex LH-20 (1×60 cm, $CHCl_3$/40% MeOH) finally 2.2 mg **2e** and 1.7 mg of **2f**, respectively, as yellow solids. On purification on PTLC (4 plates 20×20 cm, $CHCl_3$/15% MeOH/0.1% AcOH), fraction VIII delivered from the last zone [Rf=0.21 ($CHCl_3$/10% MeOH)] a product, which after chromatography on Sephadex LH-20 (4×100 cm, $CHCl_3$/40% MeOH) gave 60 mg of gutingimycin (**2g**). Purification of extract B in a similar way yielded additional 60 mg of **2g**.

Trioxacarcin D (**2d**)

Rf=0.40 ($CHCl_3$/10% MeOH).—^{1}H-NMR ($CDCl_3$, 500 MHz): δ=14.03 (s, H/D exchangeable, 1H, OH), 7.49 (d, ^4J=0.8 Hz, 1H, 5-H), 5.83 (d, ^3J=2.4 Hz, 1H, 1″-H), 5.37 (t, ^3J=2.9 Hz, 1H, 4-H), 5.35 (d, ^3J=3.9 Hz, 1H, 12-H), 5.29 (t, ^3J=4.0 Hz, 1H, 1′-H), 5.22 (d, ^3J=4.2 Hz, 1H, 11-H), 5.01 (q, ^3J=6.4 Hz, 1H, 5″-H), 4.77 (dd, ^3J=12.7, 5.4 Hz, 1H, 2-H), 4.75 (s, 1H, 16-H), 4.51 (dq, ^3J=6.4 Hz, 1H, 5′-H), 4.22 (s br, H/D exchangeable, 1H, OH), 4.15 (s, H/D exchangeable, 1H, 4″-OH), 3.83 (s, 3H, 10-OCH_3), 3.69 (m, 1H, 3″-H), 3.61 (s, 3H, 16-OCH_3), 3.58 (s br, H/D exchangeable, 1H, OH), 3.52 (s br, H/D exchangeable, 1H, OH), 3.47 (s, 3H, 16-OCH_3), 3.18 (s br, 1H, 4′-H), 2.97 (d, ^2J=5.6 Hz, 17-HA), 2.89 (d, ^2J=5.6 Hz, 17-HB), 2.80 (ddd, ^2J=13.7 Hz, ^3J=5.4, 3.7 Hz, 1H, 3-HA), 2.59 (d, ^2J=0.7 Hz, 3H, 6-CH_3), 2.47 (s, 3H, 4″-$COCH_3$), 2.42 (dt, ^2J=14.9 Hz, ^3J=5.7 Hz, 1H, 2″-HA), 2.17 (td, ^2J=13.2 Hz, ^3J=13.2, 2.7 Hz, 1H, 3-HB), 2.10 (dm, ^2J=14.4 Hz, 1H, 2″-HB), 1.93 (dd, ^2J=14.9 Hz, ^3J=4.2 Hz, 1H, 2′-HA), 1.55 (d, ^2J=14.9 Hz, 1H, 2′-HB), 1.35 (d, ^3J=6.6 Hz, 3H, 5′-CH_3), 1.19 (s, 3H, 3′-CH_3), 1.07 (d, ^3J=6.4 Hz, 3H, 5″-CH_3).—^{13}C-/APT-NMR ($CDCl_3$, 125.7 MHz): δ=210.4 (4″-$COCH_3$), 202.9 (1-C_q), 163.2 (9-C_q), 151.8 (8-C_q), 144.8 (10-C_q), 142.9 (6-C_q), 135.5 (10a-C_q), 126.7 (4a-C_q), 117.0 (5-CH), 114.8 (7-C_q, 8a-C_q), 107.4 (9a-C_q), 104.7 (15-C_q), 102.6 (13-C_q), 99.7 (16-CH), 98.1 (1′-C_q), 94.9 (1″-CH), 68.3 (14-C_q), 79.6 (4″-C_q), 74.4 (4′-CH), 71.4 (12-CH), 70.1 (3″-CH), 69.2 (11-CH), 70.2 (3′-C_q), 68.0 (2-CH), 67.5 (4-CH), 63.8 (5″-CH), 62.7 (6-CH_3), 63.6 (5′-CH), 56.8 (16-OCH_3), 56.2 (16-OCH_3), 48.2 (17-CH_2), 36.6 (3-CH_2), 35.9 (2′-CH_2), 31.5 (2″-CH_2), 27.8 (4″-$COCH_3$), 26.1 (3′-CH_3), 20.4 (6-CH_3), 16.9 (5′-CH_3), 14.5 (5″-CH_3).

Trioxacarcin E (**2e**)

Rf=0.45 ($CHCl_3$/10% MeOH).—^{1}H NMR ($CDCl_3$, 500 MHz): δ see Table 1.—^{13}C/APT NMR ($CDCl_3$, 125.7 MHz): δ 202.7 (C_q-1), 170.4 (4′-$OCOCH_3$), 163.3 (C_q-9), 153.1 (C_q-8), 144.7 (C_q-10), 142.2 (C_q-6), 135.6 (C_q-10a), 126.9 (C_q-4a), 116.6 (CH-5), 114.3 (C_q-8a), 113.0 (C_q-7), 107.4 (C_q-9a), 106.0 (C_q-15), 102.4 (CH-16), 101.6 (C_q-13), 97.8 (C_q-1′), 82.9 (C_q-14), 74.8 (CH-12), 74.4 (CH-4′), 69.4 (CH-11), 68.8 (C_q-3′), 67.9 (CH-2), 62.9 (CH-5′), 62.7 (10-OCH_3), 67.2 (CH-4), 58.6 (16-OCH_3), 56.6 (16-OCH_3), 44.9 (CH_2-17), 36.6 (CH_2-3), 36.6 (CH_2-2′), 25.7 (3′-CH_3), 20.9 (6-CH_3), 20.2 (4′-$OCOCH_3$), 14.6 (5′-CH_3).

Trioxacarcin F (**2f**)

Rf=0.55 ($CHCl_3$/10% MeOH).—^{1}H NMR ($CDCl_3$, 500 MHz) see Table 1.

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