Plasticity in gilvocarcin-type *C*-glycoside pathways: discovery and antitumoral evaluation of polycarcin V from *Streptomyces polyformus*†‡

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Gilvocarcin-type polyketide glycosides represent some of the most powerful antitumor therapeutics. Bioactivity-guided fractionation of a culture extract of *Streptomyces polyformus* sp. nov. (YIM 33176) yielded the known gilvocarcin V (**2**) and a novel related compound, polycarcin V (**1**). Structure elucidation by NMR and chemical derivatization revealed that the congener (**1**) features a *C*-glycosidically linked α-L-rhamnopyranosyl moiety in lieu of the D-fucofuranose. The concomitant production of two distinct furanosyl and pyranosyl *C*-glycosides that share the same aglycone is unprecedented in bacteria. A conversion of both isoforms *via* a quinone methide intermediate can be ruled out, thus pointing to two individual *C*-glycosylation pathways. Cytotoxicity profiling of polycarcin V in a panel of 37 tumor cell lines indicated significant antitumoral activity with a pronounced selectivity for non-small-cell lung cancer, breast cancer and melanoma cells. As the antiproliferative fingerprint is identical to that of actinomycin D, the known DNA interaction of gilvocarcins was established as a general principle of antitumorigenic activity.

Introduction

Bacterial polyketide metabolites represent a major source for novel chemotherapeutics. A particularly promising lead structure is gilvocarcin V (**2**) (Fig. 1),[1–6] the prototype of a family of benzo[*d*]naphtha[1,2-*b*]pyran-6-one glycosides from *Streptomyces* spp. Members of this family generally exhibit a high antitumoral activity with a low overall toxicity. Structurally these compounds differ in the nature of the sugar moiety and the C_2 substituent at C-8. Gilvocarcin V (**2**), the most intensively studied congener, is active against murine tumors (*e.g.* sarcoma 180 and lymphocytic leukemia P388 in mice).[1,3,4] The antitumoral mode of action of the gilvocarcin-type antibiotics seems to depend on several mechanisms. Most importantly, gilvocarcin V efficiently intercalates into DNA, allowing a UV-induced DNA-cleavage by [2 + 2]-cycloaddition of the vinyl moiety and a thymine residue.[7–9] It is also capable of cross-linking between DNA and histone H3, a major component of the histone complex.[10,11] Ravidomycin (**3**) and deacetylravidomycin (**4**) are potent photosensitizers and thus DNA-damaging agents.[12,13] Ravidomycin analogues FE35A (**5**) and B (**6**) are cytotoxic against U937 histiocytic lymphoma cells in the low nanomolar range and induce caspase-3-like apopotosis above 200 nM.[14] Deacetylravidomycin M (**7**) strongly inhibits IL-4-induced signal transduction in U937 cells while being only moderately cytotoxic.[15,16] Likewise, analogues such as gilvocarcins M (**8**) and E (**9**), and chrysomycin M (**10**), which all carry an aliphatic residue instead of the vinyl group, are not cytotoxic, thus pointing out the pivotal role of the vinyl group.[3,17,18] On the other hand, the potent inhibition of topoisomerase II by both chrysomycin V (**11**) and M, as well as gilvocarcin V and ravidomycin M (**12**), seems to be independent of the vinyl group.[17,19] The *O*-glycosidic analogues, BE12406-A (**13**) and -B (**14**), which both also carry a methyl group, are 1000-fold less active than gilvocarcin V in *in vitro* cytotoxicity studies.[20,21]

The nature of the sugar moiety seems to significantly contribute to cell-type specificity, potency, transport and pharmacokinetics. Thus, the fucofuranose moiety of gilvocarcin V (**2**) is considered important in the interaction with histone H3. The *N*-substituted 3,6-dideoxy-3-(*N*-amino)altropyranose sugar as in the ravidomycins significantly enhances antitumor activity as compared to other sugars,[12,13] with even stronger potency in deacetylravidomycin (**4**).[12] Deacetylravidomycin *N*-oxide shows significantly less acute toxicity in mice than its parent compound, while retaining its strong antitumor properties.[22] On the other hand, defucogilvocarcin V, which lacks any sugar moiety, has an antitumor activity comparable to that of gilvocarcin V, albeit with a shifted antimicrobial pattern.[23] Thus, the role of the sugar moiety in the bioactivity of benzo[*d*]naphtha[1,2-*b*]pyran-6-one needs further investigation.

The biosynthesis of the benzo[*d*]naphtha[1,2-*b*]pyran-6-one pharmacophore was recently unraveled, when J. Rohr and

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‡ Dedicated to Professor Dr Axel Zeeck on the occasion of his 70th birthday.

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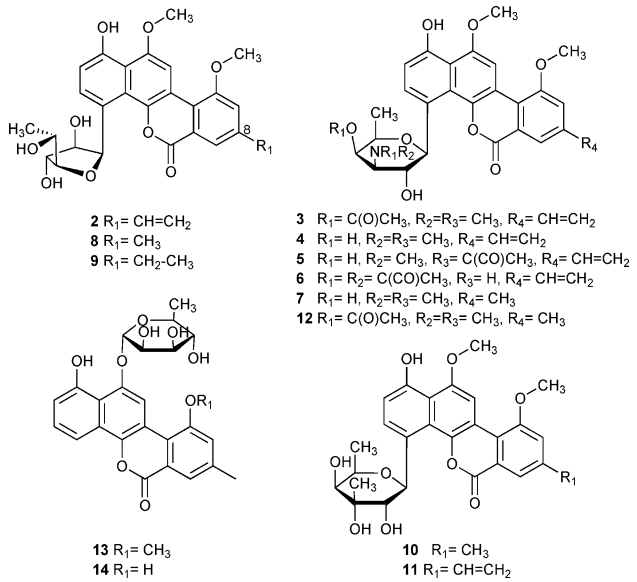


Fig. 1 Chemical structures of gilvocarcin-type glycosides.

co-workers cloned, sequenced and characterised the entire gilvocarcin V biosynthesis gene cluster.[24–28] Yet, little is known on the biosynthetic mechanism of the *C*-glycoside formation, which is a prerequisite for engineering new secondary metabolites with altered sugar moieties. Here, we report the "natural" plasticity of the glycoside biosynthesis as evidenced by polycarcin V (**1**) (Fig. 2), a novel member of the gilvocarcin family, co-occurring with gilvocarcin V (**2**). **1** carries a L-rhamnopyranose, instead of the D-fucofuranose moiety that is present in **2**. An in-depth characterization of the cytotoxic potential of polycarcin V highlights its therapeutic potential.

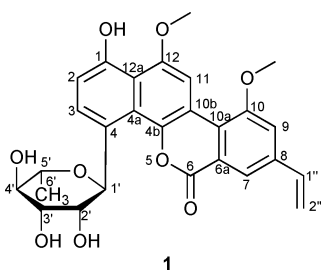


Fig. 2 Chemical structure of polycarcin V (**1**).

Results and discussion

Identification and structure elucidation of polycarcin V

In the search for novel chemotherapeutic agents from microbial sources we have explored extreme and rare habitats in southeast Asia.[29–31] The screening programme comprised over 500 microbial isolates including alkalophilic and halophilic actinomycetes, as well as epi- and endophytes from plants related to traditional Chinese medicine. Six tumor cell lines that differ in chemosensitivity towards standard chemotherapeutic agents were applied to identify cytotoxic activity in microbial extracts and for bioactivity-guided fractionation. The extracts of *Streptomyces polyformus* sp. nov. (YIM 33176),[32] a strain isolated from a soil sample collected in the north of Vietnam, showed strong cytotoxic activity against all six tumor cell lines of the screening panel (T/C >70%). TLC analysis on silica gel ($CHCl_3$–MeOH = 9 : 1) revealed two intensely yellow spots that were also UV-absorbing and fluorescent (R_f: **1** 0.4, **2** 0.5). HPLC/UV calibration curves of the initial crude products indicated that the strain produced 7.2 mg L^{-1} of **1** and 11.5 mg L^{-1} of **2**.

The identical molecular masses and UV spectra of the two major products suggested that **1** and **2** are isomers. For a full characterization of the metabolites, we scaled up the fermentation (100 L) of the producing strain. Extracts of the harvested mycelium were subjected to open column chromatography on silica gel, yielding pure **1** and **2** as yellow amorphous powders. We found that compound **2** was identical with gilvocarcin V on the basis of ^{1}H and ^{13}C NMR data, MS data, and optical rotation.[2]

The molecular formula of compound **1** was established as $C_{27}H_{26}O_9$ by HRESI-MS at m/z 493.1470 $[M - H]^-$ (calcd 493.1499). The IR spectrum indicated the presence of hydroxy groups (3363 cm^{-1}) and a carbonyl group (1727 cm^{-1}). ^{1}H NMR data of **1** showed two pairs of coupled aromatic proton signals, δ_H 6.96 (d, 8.3 Hz) and 7.78 (d, 8.4 Hz) and δ_H 7.97 (d, 1.5 Hz) and 7.73 (d, 1.5 Hz), one singlet aromatic proton signal at δ_H 8.45 (s); three proton signals on a mono-substituted double bond at δ_H 5.50 (d, 11.0 Hz), 6.13 (d, 17.6 Hz) and 6.94 (dd, 17.6, 11.0 Hz) (supplementary material†). In addition, two methoxy groups at δ_H 4.16 (3H, s) and 4.11 (3H, s), and one methyl group at δ_H 1.28 (3H, d, 6.4 Hz) were detected. Finally, a sugar moiety was indicated by signals at δ_H 3.36–5.84 (5H). Signals of the ^{13}C NMR and DEPT spectra of **1** showed all 15 carbon atoms corresponding to the ^{1}H NMR data and, additionally, 12 quaternary carbons. The aglycone of **1** proved to be identical to that of gilvocarcin V (**2**) on the basis of similar ^{1}H and ^{13}C NMR data.[2]

According to HMBC correlations of H-1′ with C-4, C-4a and C-3, the sugar moiety is *C*-glycosidically attached to C-4 like the fucofuranose in gilvocarcin V (**2**). As for the sugar moiety, the absence of relevant $^3J_{1'\text{-H},2'\text{-H}}$ coupling of the anomeric proton, and the coupling constants of $^3J_{2'\text{-H},3'\text{-H}}$ = 3.2 Hz, $^3J_{3'\text{-H},4'\text{-H}}$ = 9.1 Hz and $^3J_{4'\text{-H},5'\text{-H}}$ = 9.1 Hz clearly indicated the substitution pattern of an α-rhamnopyranoside; see Fig. 3.[33] The presence of a pyranose was further confirmed by an HMBC correlation between C-5′ and H-1′.

The absolute stereochemistry of the rhamnose was elucidated using the method reported by Tanaka *et al.*[34] For chromatographical comparison (HPLC–MS), L-rhamnose was derivatised by D- and L-cysteine methyl ester followed by phenylisothiocyanate (PITC) (Fig. 4). The liberated and derivatised sugar moiety of the acid hydrolysate (MeOH/HCl) of polycarcin V (**1**)[2,19] was detected at the same retention time as the L-cysteine methyl ester derivative.

In summary, **1** is an analogue of gilvocarcin V with a novel *C*-glycosidically tethered α-L-rhamnopyranoside sugar moiety, and was named polycarcin V.

C-Glycoside plasticity is independent of chemical furanosyl–pyranosyl interconversion

The concomitant production of two distinct *C*-glycosides that share the same aglycone but feature different sugar moieties is to the best of our knowledge unprecedented for bacterial metabolites. The gross structures of **1** and **2** suggested that the *p*-hydroxy

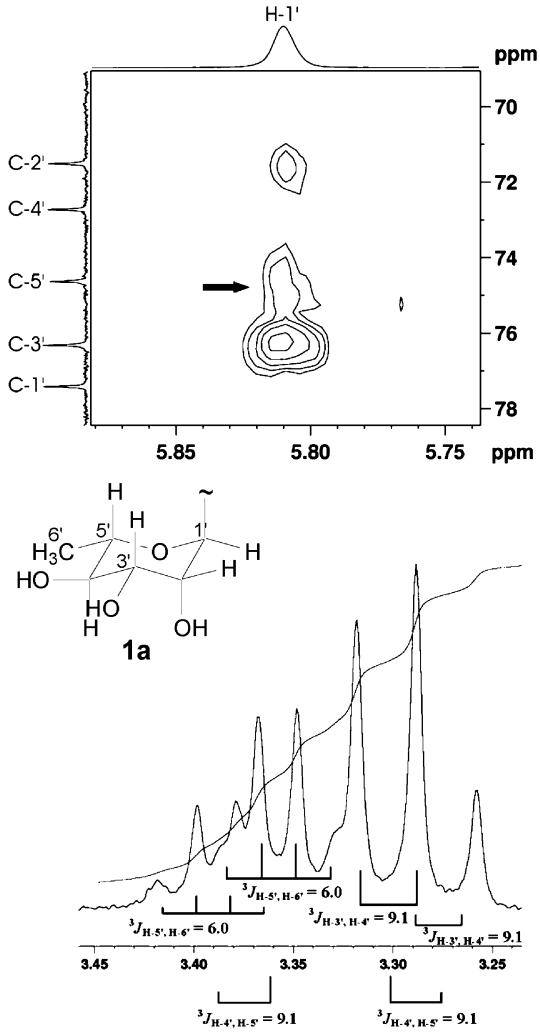


Fig. 3 NMR data in support of the rhamnopyranosyl moiety (**1a**) in polycarcin V (**1**). Top: HMBC correlation between C-5 and H-1′ (DMSO-d_6); bottom: ^{1}H coupling pattern of H-5′ (δ_H 3.37) and H-4′ (δ_H 3.28; 2% MeOH-d_4 in DMSO-d_6).

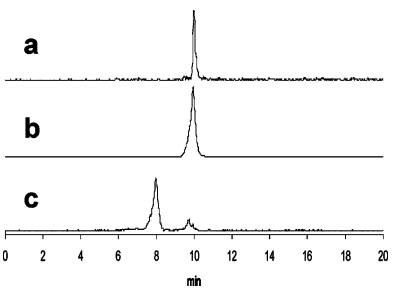


Fig. 4 HPLC–MS profiles showing extracted single ion peaks of $[M + H]^+$ at m/z 417. (a) Rhamnose-L-cysteine methyl ester-PITC derivative of polycarcin V (**1**) hydrolysate; (b) authentic L-rhamnose-L-cysteine methyl ester-PITC derivative; (c) authentic L-rhamnose-D-cysteine methyl ester-PITC derivative.

furanosyl and pyranosyl *C*-glycosides of the respective sugars could be converted *via* a quinone methide isoform (Scheme 1). To test this hypothesis we examined the stability of both compounds under moderately acidic and basic conditions (r.t., DMSO with 0.1 N HCl and 0.1 N NaOH, respectively). HPLC–MS/UV analysis of all four reaction mixtures did not show any sign of decomposition, rearrangement or formation of a related intermediate for **1** and **2**.

We thus concluded that **1** and **2** result from individual biosynthetic assembly, during which the rhamnopyranosyl and fucofuranosyl moieties are attached to the aglycone, most likely by means of a single glycosyl transferase (GT) with a relaxed substrate specificity. Despite the chemical stability, it is also possible that rhamnose is initially introduced prior to an enzymatic ring contraction. Such a rearrangement might be incomplete in case of the present producer. As rhamnose is an ubiquitous sugar moiety in microbial secondary metabolism, it might be a precursor of the rare fucofuranose, possibly prior to attachment. A ring contraction from a pyranose to a furanose was discussed by Rohr and co-workers for gilvocarcin V (**2**) assembly.[24,28,35] In the biosynthesis of **2**, *C*-glycosylation is catalysed by GilGT, the only glycosyltransferase identified in the gilvocarcin V biosynthesis gene cluster in *Streptomyces griseoflavus*.[24–28] However, the enzymatic mechanism of *C*-glycoside formation is still not fully understood and might involve a Fries-type rearrangement or a transient *O*-glycoside. The *O*-glycosidic analogues BE-12406A (**12**) and BE-12406B (**13**), and derivatives thereof, may be intermediates or shunt products of such a pathway.[20,21,36]

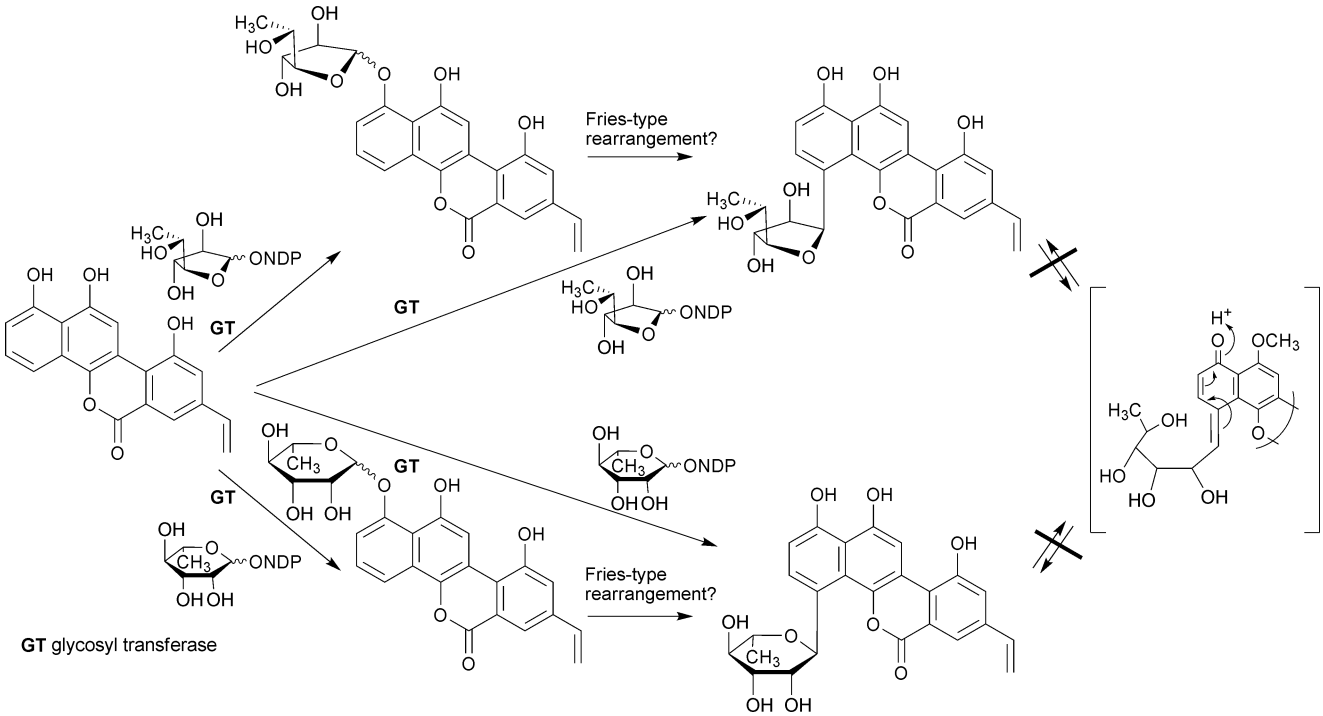
Antitumor efficacy of polycarcin V

We determined the antitumoral efficacy of polycarcin V in monolayer cultures of 37 different human tumor cell lines representing 14 different solid tumor types.[31,39] **1** led to concentration-dependent inhibition of tumor cell growth with a mean IC_{70}-value of 8.0 ng mL^{-1}. IC_{70}-values ranged from 0.3 ng mL^{-1} to 431.0 ng mL^{-1}, indicating a pronounced antitumor specificity. An above-average activity of **1** was observed towards 3 out of 4 breast cancer cell lines (MCF7, MDAMB231, MDAMB468: IC_{70}-values ranging from <0.3 ng mL^{-1} to 4.0 ng mL^{-1}), 3 out of 5 melanoma cell lines (MEXF 462NL, MEXF 514 L, MEXF 520L: IC_{70}-values ranging from <0.3 ng mL^{-1} to 0.4 ng mL^{-1}), 2 out of 6 cell lines of non-small-cell lung cancer (LXF 1211 L, LXFL 529 L: IC_{70}-values of <0.3 ng mL^{-1} and 0.3 ng mL^{-1}, respectively), as well as individual cell lines of colon cancer (HT29: IC_{70} = 3.0 ng mL^{-1}), gastric cancer (GXF 251 L: IC_{70} = 1.0 ng mL^{-1}), prostate cancer (DU145: IC_{70} <0.3 ng mL^{-1}), renal cancer (RXF 1781 L: IC_{70} <0.3 ng mL^{-1}), and uterine cancer (UXF 1138 L: IC_{70} = 0.9 ng mL^{-1}).

By comparison with more than 100 reference compounds in the COMPARE algorithm, the antiproliferative fingerprint of **1** correlated specifically with that of actinomycin D ($\rho = 0.6$).[37,38] As actinomycin D intercalates into DNA, this finding is in agreement with the known interaction of gilvocarcin V (**2**) with DNA.[6,8]

Conclusions

We have observed the unusual concomitant production of two distinct bacterial *C*-glycosides that share the same aglycone but feature sugar moieties of differing constitution and configuration. Polycarcin V (**1**), a new member of the benzo[*d*]naphtha[1,2-*b*]pyran-6-one glycoside metabolites, carries a *C*-glycosidially linked α-L-rhamnopyranose. Together with the known gilvocarcin V (**2**), it is produced as major metabolite of *Streptomyces polyformus* sp. nov. (YIM 33176). We have suggested possible



Scheme 1 Possible routes for glycosyl attachment on putative aglycone precursor in polycarcin V (**1**) and gilvocarcin V (**2**) biosynthesis.

routes for the biosynthetic formation of the two metabolites and can rule out an acid- or base-mediated interconversion of furanosyl and pyranosyl isoforms *via* a quinone methide.

In cytotoxicity profiling on a panel of 37 tumor cell lines, polycarcin V showed significant cytotoxicicity with a pronounced selectivity for non-small-cell lung cancer, breast cancer and melanoma cells. As the antiproliferative fingerprint in this broad cell panel is identical to that of actinomycin D, this finding provides proof for the DNA interaction of gilvocarcins as a general principle of antitumorigenic activity.

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References

1 H. Nakano, Y. Matsuda, K. Ito, S. Ohkubo, M. Morimoto and F. Tomita, *J. Antibiot.*, 1981, **34**, 266.
2 K. Takahashi, M. Yoshida, F. Tomita and K. Shirahata, *J. Antibiot.*, 1981, **34**, 271.
3 D. M. Balitz, F. A. O'Herron, J. Bush, D. M. Vyas, D. E. Nettleton, R. E. Grulich, W. T. Bradner, T. W. Doyle, E. Arnold and J. Clardy,, *J. Antibiot.*, 1981, **34**, 1544.
4 M. Morimoto, S. Okubo, F. Tomita and H. Marumo, *J. Antibiot.*, 1981, **34**, 701.
5 F. Tomita, K. Takahashi and T. Tamaoki, *J. Antibiot.*, 1982, **35**, 1038.
6 T. T. Wei, J. A. Chan, P. P. Roller, U. Weiss, R. M. Stroshane, R. J. White and K. M. Byrne, *J. Antibiot.*, 1982, **35**, 529.
7 R. K. Elespuru and S. K. Gonda, *Science*, 1984, **223**, 69.
8 T. T. Wei, K. M. Byrne, D. Warnick-Pickle and M. Greenstein, *J. Antibiot.*, 1982, **35**, 545.
9 Y. C. Tse-Dinh and L. R. McGee, *Biochem. Biophys. Res. Commun.*, 1987, **143**, 808.
10 M. J. Peak, J. G. Peak, C. M. Blaumueller and R. K. Elespuru, *Chem. Biol. Interact.*, 1988, **67**, 267.
11 R. Arce, R. Oyola and A. E. Alegría, *Photochem. Photobiol.*, 1998, **68**, 25.
12 M. Greenstein, T. Monji, R. Yeung, W. M. Maiese and R. J. White, *Antimicrob. Agents Chemother.*, 1986, **29**, 861.
13 K. Singh, *J. Antibiot.*, 1984, **37**, 71.
14 N. Yamashita, K. Shin-Ya, K. Furihata, Y. Hayakawa and H. Seto, *J. Antibiot.*, 1998, **51**, 1105.
15 M. Arai, H. Tomoda, A. Matsumoto, Y. Takahashi, B. H. Woodruff, N. Ishiguro, S. Kobayashi and S. Omura, *J. Antibiot.*, 2001, **54**, 554.
16 M. Arai, H. Tomoda, N. Tabata, N. Ishiguro, S. Kobayashi and S. Omura, *J. Antibiot.*, 2001, **54**, 562.
17 S. Rakhit, C. Eng, H. Baker and K. Singh, *J. Antibiot.*, 1983, **36**, 1490.
18 A. Lorico and B. H. Long, *Eur. J. Cancer*, 1993, **29A**, 1985.
19 U. Weiss, K. Yoshihira, R. J. Highet, R. J. White and T. T. Wei, *J. Antibiot.*, 1982, **35**, 1194.
20 S. Nakajima, K. Kojiri, H. Suda and M. Okanishi, *J. Antibiot.*, 1991, **44**, 1061.
21 K. Kojiri, H. Arakawa, F. Satoh, K. Kawamura, A. Okura, H. Suda and M. Okanishi, *J. Antibiot.*, 1991, **44**, 1054.
22 T. Narita, M. Matsumoto, K. Mogi, K. Kukita, R. Kawahara and T. Nakashima, *J. Antibiot.*, 1989, **42**, 347.
23 R. Misra, H. R. Tritch III and R. C. Pandey, *J. Antibiot.*, 1985, **38**, 1280.
24 T. Liu, C. Fischer, C. Beninga and J. Rohr, *J. Am. Chem. Soc.*, 2004, **126**, 12262.
25 Kharel L. Zhu, T. Liu and J. Rohr, *J. Am. Chem. Soc.*, 2007, **129**, 3780.
26 K. Takahashi and F. Tomita, *J. Antibiot.*, 1983, **36**, 1531.
27 S. J. Gould, *Chem. Rev.*, 1997, **97**, 2499.
28 C. Fischer, F. Lipata and J. Rohr, *J. Am. Chem. Soc.*, 2003, **125**, 7818.
29 D. J. Newman, G. M. Cragg and K. M. Snader, *J. Nat. Prod.*, 2003, **66**, 1022.
30 L. Reddy, B. Odhav and K. D. Bhoola, *Pharmacol. Ther.*, 2003, **99**, 1.
31 J. He, E. Roemer, C. Lange, X. Huang, A. Maier, G. Kelter, Y. Jiang, L. H. Xu, K.-D. Menzel, S. Grabley, H.-H. Fiebig, C.-L. Jiang and I. Sattler, *J. Med. Chem.*, 2007, **50**, 5168.

32 Y. Jiang, W. J. Li, W. L. Wu, Y. X. Peng, L. F. Li, H. M. Yang, L. H. Xu and C. L. Jiang, *J. Yunnan Univ.*, 2004, **26**, 215.
33 S. Grond, H.-J. Langer, P. Henne, I. Sattler, R. Thiericke, S. Grabley, H. Zähner and A. Zeeck, *Eur. J. Org. Chem.*, 2000, 929.
34 T. Tanaka, T. Nakashima, T. Ueda, K. Tomii and I. Kouno, *Chem. Pharm. Bull.*, 2007, **55**, 889.
35 T. Liu, M. K. Kharel, C. Fischer, A. McCormick and J. Rohr, *ChemBioChem*, 2006, **7**, 1070.
36 H. Suda, K. Kojiri, A. Okura, K. Funaishi, K. Kawamura, M. Okanishi and B. Hansen, *Eur. Pat.* BE12406 EP0381138 (30 January 1990).
37 H. H. Fiebig, T. Metz, E. Tetling, R. Vollmer, A. Korrat, N. Bausch and G. Kelter, *Proc. Am. Assoc. Cancer Res.*, 2005, **46**, 3967.
38 L. F. Cavalieri and R. G. Nemchin, *Biochim. Biophys. Acta*, 1964, **87**, 641.
39 In the six-cell-line panel of the screening routine, compounds **1** and **2** showed identical selectivity and potency (data not shown).