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Proximicins A, B, and C—Antitumor Furan Analogues of Netropsin from the Marine Actinomycete *Verrucosispora* Induce Upregulation of p53 and the Cyclin Kinase Inhibitor p21**

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Dedicated to Professor François Diederich

Netropsin (**1**) and distamycin (**2**) are naturally occurring γ-peptides with antiviral and antibacterial activity (Figure 1).[1] Netropsin, formerly named congocidine, was isolated from *S. netropsis* in 1951,[2] while distamycin was isolated from *S. distallicus* in 1964.[3,4] Both compounds bind in the minor groove of DNA, and they were arguably the first compounds for which AT-selective DNA binding was demonstrated.[5–8] The design of synthetic derivatives capable of addressing a specific sequence of DNA would allow the selective inhibition of gene expression and thus result in compounds that act as antitumor agents.[5] As a consequence, netropsin and distamycin were used as the basic structures for the synthesis of numerous analogues to enable the investigation and modulation of their minor-groove binding. This was also achieved by using combinatorial approaches.[5–11] A particular challenge was to generate selective binders for GC base pairs.[5–8] One approach was based on the assumption that the introduction of a hydrogen-bond acceptor in the pyrrole rings of netropsin might allow the drug to bind to GC-rich sequences.[5–8] By following this strategy, a large number of molecules were synthesized in which the *N*-methylpyrrole ring of netropsin was substituted by other heterocycles, for example, imidazole, thiazole, triazole, pyrazole, and furan.[5,8] Since netropsin and distamycin lack selectivity and toxicity[8,9] their use as drugs was prevented, except as an antiviral agent in a topical application.[12] Recent efforts have aimed to remedy these disadvantages.[9] A further important bioactivity of netropsin and distamycin is their inhibitory activities against the malaria-causing parasite *Plasmodium falciparum*.[7]

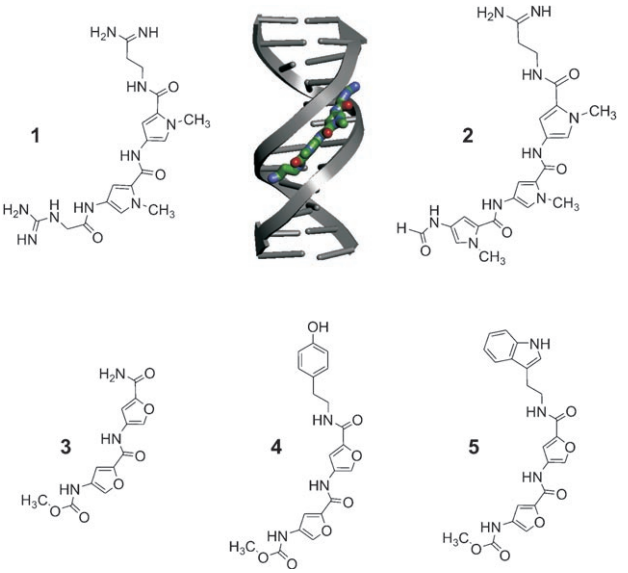

Figure 1. Structures of netropsin (**1**), distamycin (**2**), and of the compounds from the marine *Verrucosispora*: proximicin A (**3**), proximicin B (**4**), and proximicin C (**5**). The double helix illustrates the binding of netropsin (**1**) in the minor groove of DNA to form a 1:1 complex.[13]

Herein we report the structure elucidation of new netropsin analogues, named proximicin A (**3**), B (**4**), and C (**5**), from marine actinomycetes of the genus *Verrucosispora* (Figure 1). The proximicins bear the hitherto unknown γ-amino acid 4-aminofuran-2-carboxylic acid, which adds a new element of structural diversity to the previously described heterocyclic antibiotics. Furthermore, the antitumor activities

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of netropsin and distamycin were compared to those of the proximicins and synthetic netropsin–proximicin hybrids (**6**, **7**, **8**; Figure 2), with the latter found to be considerably more cytotoxic in the investigated cell lines.

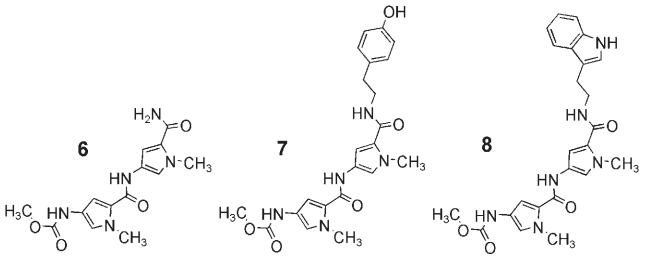

Figure 2. Structures of the synthetic netropsin–proximicin hybrids A (**6**), B (**7**), and C (**8**).

Recently, we reported on the fermentation, isolation, and structure elucidation of the polyketide antibiotics abyssomicins B–D from the marine actinomycete *Verrucosispora* sp. AB-18-032, which was isolated from sediment collected from the Sea of Japan.[14,15] Careful evaluation of the HPLC chromatograms of extracts of this strain revealed significant amounts of another compound (**3**) that was not assignable to any other known compound of our HPLC-DAD (HPLC analysis with a diode array detector) database.[16] Remarkably, the extract from another marine member of the rare genus *Verrucosispora*, strain MG-37, which was isolated from sediment collected in the Raune Fjord (Norway) at a depth of 250 m, showed an HPLC-DAD signal identical to that of compound **3**. Furthermore, two further signals with similar UV spectra as that of **3** were also seen, thus indicating the presence of a family of structure-related peptide metabolites, which were named proximicin A (**3**), B (**4**), and C (**5**).[17] The compounds were purified by size-exclusion and adsorption chromatography as well as by preparative reversed-phase HPLC, and structure elucidation was carried out by means of mass spectrometry (HPLC-ESI-MS, FT-ICR-MS, GC-MS) and two-dimensional NMR spectroscopy.

The exact masses determined by high-resolution ESI-FT-ICR-MS for proximicin A, B, and C were 294.0722 Da $[(M+H)^+]$, 436.1116 Da $[(M+Na)^+]$, and 437.14566 Da $[(M+H)^+]$, respectively. These values correspond to the molecular formulas $C_{12}H_{11}N_3O_6$ (**3**) $[(M+H)^+_{theor} = 294.07206;\ \Delta m = 0.477\ ppm]$, $C_{20}H_{19}N_3O_7$ (**4**) $[(M+Na)^+_{theor} = 436.11152;\ \Delta m = 0.165\ ppm]$, and $C_{22}H_{20}N_4O_6$ (**5**) $[(M+H)^+_{theor} = 437.14556;\ \Delta m = 0.215\ ppm]$. After hydrolysis and derivatization of the compounds, analysis by GC-MS revealed the presence of tyramine in the case of proximicin B (**4**) and of tryptamine in the case of proximicin C (**5**).

The subsequent 1D and 2D NMR analyses provided the missing structural fragments. The chemical shifts and the coupling constants in combination with the HMBC correlations suggested that all the proximicins contained a furan ring system with a 2,4-disubstitution pattern, and led to the assignment of the 2-position as the carbonyl function and the 4-position as the amino function. The HMBC experiment provided crucial evidence for a dipeptide of 4-aminofuran-2-carboxylic acid, which represents the core structure of the proximicins. The ^1H-^{15}N HSQC spectrum of **4** revealed three signals: two corresponding to secondary amides and one corresponding to a carbamate functionality. The N-terminal methyl carbamate is present in all three proximicins, and with the structural difference between the compounds lying in the C-terminal modifications. Proximicin A (**3**) contains a C-terminal amide, whereas proximicin B (**4**) and proximicin C (**5**) have tyramine and tryptamine modifications, respectively. Interestingly, the proximicins show a high structural analogy to netropsin (**1**) and distamycin (**2**). The characteristic fragment of these latter two compounds, the 2,4-disubstituted *N*-methylpyrrole, is replaced by a 2,4-disubstituted furan ring, which constitutes a hitherto unknown γ-amino acid. Although a considerable number of netropsin analogues have been synthesized by exchanging the *N*-methylpyrrole ring with other heterocycles, this structural motif was until now unknown. Furthermore, the γ-amino acid 4-aminofuran-2-carboxylic acid has some structural analogy to GABA mimetics.[18]

To evaluate the structural and functional relationships between netropsin and the proximicins we synthesized the netropsin-proximicin-hybrids **6**–**8** (Figure 2).[19] In these hybrids the *N*-methylpyrrole core of netropsin is combined with the corresponding N- and C-terminal modifications of proximicins A, B, and C. Testing the proximicins **3**–**8** for antitumor activity against three carcinoma cell lines showed proximicins **3**–**5** had a significantly higher antitumor activity than **1** and **2** (Table 1), while **7** and **8** had a moderately higher activity. The DNA binding activity of the proximicins was assessed by analyzing the DNA melting curves. In contrast to netropsin and distamycin, no shift in the DNA melting temperature T_m was detected for proximicin A, B, and C (**3**–**5**) from *Verrucosispora* or for the synthetic hybrid molecules (**6**–**8**).

Table 1: Antitumor activity of compounds **1**–**8** against three carcinoma cell lines.[b]

Compound	AGS[a] GI_{50}[b] [μM]	HepG2[a] GI_{50}[b] [μM]	MCF7[a] GI_{50}[b] [μM]
netropsin (**1**)	>116.2	60.4	74.3
distamycin (**2**)	>103.8	54.0	14.3
proximicin A (**3**)	2.0	2.8	24.6
proximicin B (**4**)	3.6	23.0	12.1
proximicin C (**5**)	0.6	1.8	20.6
6	93.9	119.0	131.5
7	28.4	34.1	41.0
8	17.7	21.6	24.9

[a] Cell lines: AGS: gastric adenocarcinoma; HepG2: hepatocellular carcinoma, MCF7: breast adenocarcinoma. [b] GI_{50}: 50% growth inhibition.

A cell-cycle analysis in AGS cells revealed that **1** (116.2 μM) and **2** (103.8 μM) produce an accumulation of cells in G2/M after incubation for 24 h (**1**: +6.9%; **2**: +5.4%) and 40 h (**1**: +11.3%; **2**: +28.9%), and reduce the ratio of cells in the G0/G1 phase (**1**: −9.2% and −9.5%; **2**: −7% and −20.5%). Proximicin C (**5**; 1.1 μM) and compound **8** (21.6 μM)

produced cell arrest in the G0/G1 phase after incubation for 24 h (**5**: +5.6%; **8**: +21.4%). After 40 h, there was an increase in the number of cells in the sub-G1 phase, that is, apoptotic cells (**5**: +2.9%; **8**: +9.8%).

On the basis of these data we evaluated whether **5** and **8** activate cell-cycle regulatory proteins involved in the transition of cells from the G1 to the S phase (p53, p21, cyclin E). It was found that proximicin C (**5**; data not shown) and hybrid C (**8**) induce upregulation of p53 and of the cyclin kinase inhibitor p21 in AGS cells (Figure 3). However,

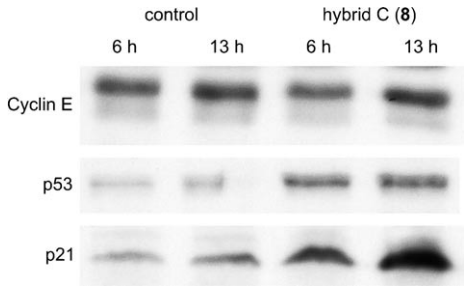

Figure 3. Western blot analysis showing the effect of netropsin–proximicin hybrid C (**8**) on the upregulated expression of p21, p53, and cyclin E in AGS cells after 6 h and 13 h incubation.

distamycin (**2**; 103.8 μM) did not induce the expression of p53 and p21 (see the Supporting Information). To further compare the effects of **1**, **2**, **5**, and **8**, growth inhibition by the compounds was tested in hepatoma cells with mutated p53 (Huh7).[20] The data obtained showed that **1** and **2** had similar cytotoxicity as in the HepG2 cells (GI_{50}: **1**: 57.7 μM; **2**: 62.3 μM), while **5** and **8** did not inhibit cell growth.

Netropsin (**1**; 58.1 μM) arrested Huh7 cells in G2/M (+4.4%), while distamycin (**2**; 51.9 μM) arrested Huh7 cells in G2/M (+8.7%) and in the S phase (+10.6%). Proximicin C (**5**; 4.4 μM) and compound **8** (21.6 μM) did not alter the cell-cycle distribution in the Huh7 cells.

Remarkably, the experiments show that the antitumor activities of the proximicins bearing an N-terminal methyl carbamate are significantly higher than those of netropsin (**1**) and distamycin (**2**), which have guanidinylglycine and formyl functions, respectively. Analyses of the DNA melting curves show that the antitumor activity of proximicins **3**–**5** as well as of the hybrids **6**–**8** cannot be based on DNA binding. The structural moieties which constitute the structural differences of the proximicins compared to netropsin are modifications at 1) the N terminus, 2) the C terminus, and 3) the furan–*N*-methylpyrrole exchange. The different C-terminal amide modifications of proximicin A (**3**) and C (**5**) does not have a great influence on the antitumor activity. This effect is more pronounced in the synthetic hybrids **6**–**8**; however, these *N*-methylpyrrole derivatives display a generally lowered antitumor activity compared to the furan analogues. The exchange of the furan oxygen atom by an *N*-methyl group leads to a 1.2- to ca. 30-fold decrease in antitumor activity. This decrease may be based on a significant change in the electronic properties of the proximicins, since the two furan oxygen atoms may act as additional hydrogen-bond acceptors and furthermore increase the overall polarity of the molecule. Although hybrid C (**8**) possesses a different molecular structure and shows a lower antitumor activity, it seems to address the same cellular target as the corresponding proximicin C (**5**), thereby suggesting that the C-terminal tryptamine residue can exert a significant influence on the upregulation of p53 and p21. However, it seems as if the methyl carbamate is the main contributor to the antitumor activity. Boger et al. reported on a similar effect for distamycins in which the formyl function was exchanged for a *tert*-butyl carbamate moiety, while keeping a positive charge at the C terminus.[11]

Overall, in regard to the effect of the herein-described proximicins on the cell cycle, we found an arrest of AGS cells in G0/G1 and an increase in the levels of p53 and p21, whereas distamycin, as reported by Poot et al.,[20] arrested the cells in G2/M. Distamycin, in contrast to the proximicins, inhibited cell growth in p53-mutated cells (Huh7). These data strongly confirm that the proximicins act on a different cellular target, that is, the transition of cells from the G1 to the S phase.

In summary, we have presented the structures of three new netropsin-type antibiotics from marine actinomycete strains that have interesting antitumor activity. Current work is directed towards the total synthesis of proximicins as well as to the generation of furan-based proximicin–netropsin hybrids, to evaluate the antitumor activity of these compounds in more detail.

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[1] J. W. Lown, *J. Mol. Recognit.* **1994**, *7*, 79–88.
[2] A. C. Finlay, F. A. Hochstein, B. A. Sobin, F. X. Murphy, *J. Am. Chem. Soc.* **1951**, *73*, 341–343.
[3] F. Arcamone, S. Penco, P. Orezzi, V. Nicolella, A. Pirelli, *Nature* **1964**, *203*, 1064–1065.
[4] P. G. Baraldi, D. Preti, F. Fruttarolo, M. A. Tabrizi, R. Romagnoli, *Bioorg. Med. Chem.* **2007**, *15*, 17–35.
[5] C. Bailly, B. J. Chaires, *Bioconjugate Chem.* **1998**, *9*, 513–538.
[6] P. B. Dervan, B. S. Edelson, *Curr. Opin. Struct. Biol.* **2003**, *13*, 284–299.
[7] B. S. P. Reddy, S. K. Sharma, J. W. Lown, *Curr. Med. Chem.* **2001**, *8*, 475–508.
[8] D. E. Wemmer, *Biopolymers* **1999**, *52*, 197–211.
[9] C. J. Suckling, *Expert Opin. Ther. Pat.* **2004**, *14*, 1693–1724.
[10] D. L. Boger, M. A. Dechantsreiter, T. Ishii, B. E. Fink, M. P. Hedrick, *Bioorg. Med. Chem.* **2000**, *8*, 2049–2057.
[11] D. L. Boger, B. E. Fink, M. P. Hedrick, *J. Am. Chem. Soc.* **2000**, *122*, 6382–6394.
[12] L. De Angelis, *Drugs Today* **1977**, *13*, 319–326.
[13] M. L. Kopka, C. Yoon, D. Goodsell, P. Pjura, R. E. Dickerson, *J. Mol. Biol.* **1985**, *183*, 553–563.
[14] J. Riedlinger, A. Reicke, H. Zähner, B. Krismer, A. T. Bull, L. A. Maldonado, A. C. Ward, M. Goodfellow, B. Bister, D. Bischoff, R. D. Süssmuth, H.-P. Fiedler, *J. Antibiot.* **2004**, *57*, 271–279.
[15] B. Bister, D. Bischoff, M. Ströbele, J. Riedlinger, A. Reicke, F. Wolter, A. T. Bull, H. Zähner, H.-P. Fiedler, R. D. Süssmuth,

Angew. Chem. **2004**, *116*, 2628 – 2630; *Angew. Chem. Int. Ed.* **2004**, *43*, 2574 – 2576.
[16] H.-P. Fiedler, *Nat. Prod. Lett.* **1993**, *2*, 119 – 128.
[17] H.-P. Fiedler, C. Bruntner, J. Riedlinger, A. T. Bull, G. Knutsen, A. L. Jones, M. Goodfellow, W. Beil, K. Schneider, S. Keller, R. D. Süssmuth, *J. Antibiot.* **2008**, in press.
[18] a) J. P. Burkhart, G. W. Holbert, B. W. Metcalf, *Tetrahedron Lett.* **1984**, *25*, 5267 – 5270; b) M. Fu, R. B. Silverman, *Bioorg. Med. Chem. Lett.* **2004**, *14*, 203 – 206; c) Z. Wang, H. Yuan, D. Nikolic, R. B. Van Breemen, R. B. Silverman, *Biochemistry* **2006**, *45*, 14513 – 14522.
[19] For the synthesis of the netropsin–proximicin hybrids **6**–**8** see the Supporting Information.
[20] M. Poot, K. H. Hiller, S. Heimpel, H. Hoehn, *Exp. Cell Res.* **1995**, *218*, 326 – 330.

