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Delineation of gilvocarcin, jadomycin, and landomycin pathways through combinatorial biosynthetic enzymology

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The exact sequence of events in biosyntheses of natural products is essential not only to understand and learn from nature's strategies and tricks to assemble complex natural products, but also for yield optimization of desired natural products, and for pathway engineering and muta-synthetic preparation of analogues of bioactive natural products. Biosyntheses of natural products were classically studied applying *in vivo* experiments, usually by combining incorporation experiments with stable-isotope labeled precursors with cross-feeding experiments of putative intermediates. Later genetic studies were dominant, which consist of gene cluster determination and analysis of gene inactivation experiments. From such studies various biosynthetic pathways were proposed, to a large extent just through *in silico* analyses of the biosynthetic gene clusters after DNA sequencing. Investigations of the complex biosyntheses of the angucycline group anticancer drugs landomycin, jadomycin and gilvocarcin revealed that *in vivo* and *in silico* studies were insufficient to delineate the true biosynthetic sequence of events. Neither was it possible to unambiguously assign enzyme activities, especially where multiple functional enzymes were involved. However, many of the intriguing ambiguities could be solved after *in vitro* reconstitution of major segments of these pathways, and subsequent systematic variations of the used enzyme mixtures. This method has been recently termed 'combinatorial biosynthetic enzymology'.

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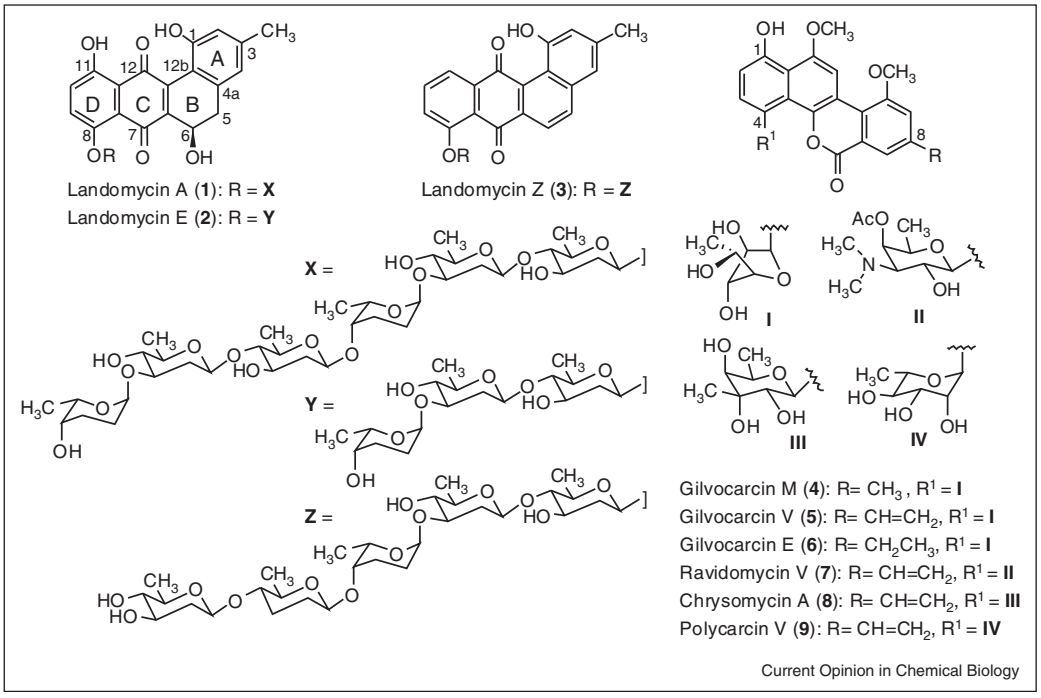
Introduction

Landomycins and gilvocarcins belong to the angucycline group of natural products, the largest group of polyketide-derived natural products, rich in biological activities and intriguing chemical scaffolds. The unusual repetitive saccharide decoration pattern and high degree of deoxygenation in the aglycone moiety stimulated biosynthetic inquisitiveness for the landomycins, while particularly the oxidative framework rearrangements by post-polyketide synthase tailoring oxidoreductases triggered extensive biosynthetic studies of the kinamycin, jadomycin and gilvocarcin biosyntheses [1•].

With so far 26 members, the landomycins [2–5], produced by *Streptomyces cyanogenus* S-136 and *Streptomyces globisporus* 1912, with the principal products landomycin A (**1**, Figure 1) and landomycin E (**2**), respectively, are one of the largest and most studied families among the typical angucyclines. Their structures differ from each other in their saccharidal length and composition as well as oxygenation pattern of the aglycone moiety, for example, landomycin Z (**3**) [1,6,7]. The landomycins have received much attention for their structures [8–14,15••] and anticancer activities, partly because of the fact that they are not substrates of multi-drug resistance efflux pumps. They interfere with DNA synthesis, but do not bind directly to DNA. Their exact cell target and mechanisms-of-action remain elusive [16–21].

Although already discovered in the mid-1950s [22], the chemical structures of the gilvocarcin group of potent anticancer agents remained elusive until 1981, when an X-ray structure [23] revealed the relative configuration of gilvocarcin M (**4**), which in turn led to the structure determination of various other members of this group of [24–26]. Gilvocarcin V (**5** = toromycin [27,28], anandimycin [24,29]), the major and most active metabolite of *Streptomyces griseoflavus* Gö 3592 as well as of various other *Streptomyces* species, is usually produced along with the minor congeners gilvocarcin M (**4**) and E (**6**) that vary with respect to their 8-substitution [24,27,30,31]. Several gilvocarcin analogues (e.g. **7**–**9**, Figure 1), now collectively called the gilvocarcin group of natural products, have been isolated from different *Streptomyces* species all containing the characteristic polyketide-derived benzo[*d*]naphtho[1,2-*b*]pyran-6-one chromophore and different *C*-glycosidically linked sugar units [32–36]. The group is known for their strong antitumor activities, unique mode of action and low toxicity [25,34,37]. The 8-vinyl side chain of the benzo[*d*]naphtho[1,2-*b*]pyran-6-one moiety undergoes photoactivated [2+2]-cycloaddition with thymine residues of DNA under irradiating conditions with low energy UV or visible light [29,38,39], and the sugar moiety appears to be essential for the

Figure 1



Representative chemical structures of the landomycin and gilvocarcin groups of anticancer drugs.

observed gilvocarcin-mediated cross-linking of histone H3 or heat shock protein GRP78 with DNA resulting in the disruption of DNA replication and transcription [40••]. Gilvocarcins also exhibit strong antibacterial [22] and antiviral properties [41]; however, the inherent poor solubility of these molecules appeared to be a major obstacle toward their development as therapeutics [1,42–44].

Incorporation experiments with stable-isotope labeled precursors

The landomycin pathway was investigated by incorporation studies and genetic experiments. The carbon backbone of landomycinone (**10**) is derived from 10 acetate and malonate units. Experiments involving ^{18}O-labeled molecular oxygen ($^{18}O_2$) and $CH_3C^{18}O^{18}OH$ indicated that only two of the six oxygen atoms of **1** (Figure 2), namely those 1-position and 8-position, originate from the polyketide building blocks [3], however, the ^{18}O-incorporation experiments failed to further solve the intriguing biosynthesis of the aglycone [45].

Incorporation studies with isotope-labeled precursors [46–49] suggested that the unique benzo[*d*]naphtho[1,2-*b*]pyran-6-one chromophore of the gilvocarcins emerges from a polyketide-derived angucyclinone intermediate through a complex oxidative rearrangement process; however, the details and exact sequence of events and involvement of enzymes remained elusive (Figure 2) [50].

Gene cluster analysis and conclusions from gene inactivation and gene complementation experiments

Two gene clusters of landomycin producers (*lan* from the 1-producer *S. cyanogenus*, and *lnd* from the 2-producer *S. globisporus*) were cloned (Figure 3) [51–55,56•,57•]. The clusters are almost identically organized, with only three biosynthetic genes missing in the *lnd* cluster, namely equivalents of *lanK*, *lanGT3*, and *lanZ2*. Many of the functions of the gene-products could be unambiguously assigned thorough gene inactivation/complementation studies. The entire glycosylation sequence was solved, many of the regulatory aspects of the landomycin biosynthesis could be deduced [4,53–55,56•,57•,58•,59–67], and a couple of new genetically engineered landomycins were generated [19,68–71]. However, the deduction of the post-PKS tailoring oxidoreductase catalyzed reactions of the aglycone biosynthesis remained ambiguous, although some information was gained from accumulated products upon gene inactivation (see Figure 4 for examples) [69,72,73]. However, the exact substrates of the involved enzymes and the exact sequence of their actions remained ambiguous.

The gilvocarcin biosynthetic gene cluster (*gil*) was cloned and heterologously expressed [30], and the clusters of chrysomycin (*chry*) and ravidomycin (*rav*) were cloned and analyzed (Figure 3) [74•,75•,76,77•,78•]. The functions of the post-PKS gene products were