# Engineered Biosynthesis of a Complete Macrolactone in a Heterologous Host

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Macrocyclic polyketides have been subjects of great interest in synthetic and biosynthetic chemistry because of their structural complexity and medicinal activities. With expression of the entire 6-deoxyerythronolide B synthase (DEBS) (10,283 amino acids) in a heterologous host, substantial quantities of 6-deoxyerythronolide B (6dEB), the aglycone of the macrolide antibiotic erythromycin, and 8,8a-deoxyoleandolide, a 14-membered lactone ring identical to 6dEB except for a methyl group side chain in place of an ethyl unit, were synthesized in *Streptomyces coelicolor*. The biosynthetic strategy utilizes a genetic approach that facilitates rapid structural manipulation of DEBS or other modular polyketide synthases (PKSs), including those found in actinomycetes with poorly developed genetic methods. From a technological viewpoint, this approach should allow the rational design of biosynthetic products and may eventually lead to the generation of diverse polyketide libraries by means of combinatorial cloning of naturally occurring and mutant PKS modules.

Polyketides are natural products found in most groups of organisms and are especially abundant in the actinomycetes, a class of mycelial bacteria. These compounds have generated much interest in synthetic and biosynthetic chemistry because of their structural complexity and antibiotic, anticancer, and immunosuppressive activities. In a reaction sequence analogous to fatty acid biosynthesis (*1*), a polyketide synthase (PKS) catalyzes the formation of a polyketide through repeated decarboxylative condensations between enzyme-bound acyl carrier protein (ACP)–thioesters. After each condensation, the β-keto group of the growing chain remains unchanged or is reduced to a hydroxyl, enoyl, or methylene group. After the final condensation cycle, the polyketide chain is cyclized appropriately and released from the PKS by thiolysis or acyltransfer (*2*). Thus, the diversity of naturally occurring polyketides stems from the controlled variation of chain length, chain-building extender units, and the degree of β-keto reduction during polyketide biosynthesis (*2*).

During the past decade, cloning and sequencing of PKS genes have defined two classes of PKSs. Those that catalyze the biosynthesis of aromatic polyketides exemplify one class and have a single set of iteratively used active sites, carried either on one polypeptide (type I) or a series of separate polypeptides (type II) (*3*). In contrast, complex or "modular" PKSs, which include synthases for the aglycones of erythromycin and avermectin, are assemblies of large multifunctional proteins that harbor a distinct active site for every catalyzed step in polyketide biosynthesis (*4*, *5*) (for an example, see Fig. 1). Consequently, structural diversity in these complex polyketides occurs through variations in the number and type of active sites in modular PKSs. The discovery of this one-to-one correspondence between active sites and catalytic steps provides a potentially powerful model for the rational design and engineered biosynthesis of polyketides through genetic manipulation. Indeed, two examples in which chromosomal mutants were constructed in *Saccharopolyspora erythraea*, the natural producer of erythromycin, have shown the feasibility of this approach (*4*, *6*). Although in principle this strategy for constructing chromosomal mutants could be extended to other macrolide-producing actinomycetes, the development of genetic methods in hitherto uncharacterized microorganisms remains a major technological barrier. Even in strains for which there are well-developed genetic methodologies, the construction of chromosomal mutants is often a time-consuming process.

We have reported the development of an actinomycete host-vector system that allows efficient construction and expression of recombinant aromatic (type II) PKSs (*7*). The use of this expression system has led to insights into structure-function relations for this class of PKSs, as well as to the generation of numerous aromatic polyketides (*7*, *8*). However, extrapolation of the same approach to study modular PKSs poses several significant conceptual and technical challenges. First, the large sizes of modular PKS gene clusters (>30 kb) make their manipulation on plasmids difficult. Modular PKSs also often utilize substrates other than acetyl–coenzyme A (CoA) and malonyl-CoA monomers; these may be absent in a heterologous host. Finally, proper folding, assembly, and posttranslational modification of very large foreign polypeptides are not guaranteed. We report here the development of a potentially generally applicable genetic strategy for convenient mutagenesis and functional expression of complex PKSs. We used this approach for the heterologous expression of a complete macrolactone PKS and isolated substantial quantities of two structurally related macrolide aglycones, thereby obtaining mechanistic insights into structure-function relations in this enzyme.

The model PKS in our study is the 6-deoxyerythronolide B synthase (DEBS) (Fig. 1), a modular PKS that catalyzes the biosynthesis of 6-deoxyerythronolide B (6dEB) (**1**) (*4*). DEBS comprises three large polypeptides (DEBS1, DEBS2, and DEBS3) that together have at least 28 distinct active sites. The genes that encode these three proteins (*eryAI*, *eryAII*, and *eryAIII*) span 32 kb in the *ery* gene cluster of the *S. erythraea* genome and are organized in six repeated units, each designated a "module." In a model proposed from

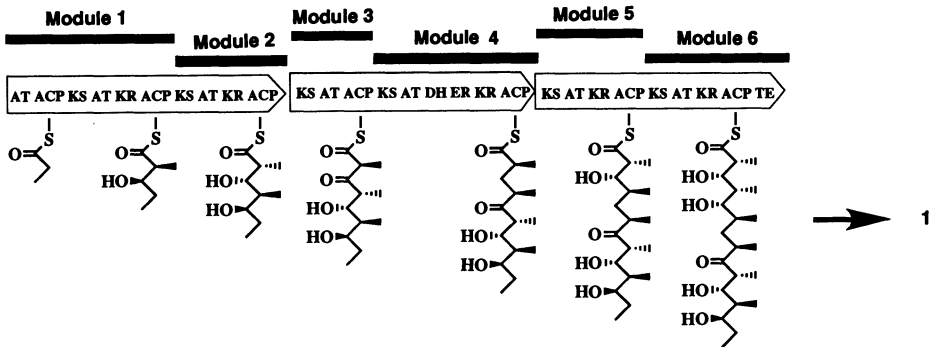


**Fig. 1.** Genetic model for the 6-deoxyerythronolide B synthase (DEBS) [adapted from (*4*) with permission]. Each module includes an acyltransferase (AT), a β-ketoacyl carrier protein synthase (KS), and an acyl carrier protein (ACP) as well as a subset of reductive active sites [β-ketoreductase (KR), dehydratase (DH), and enoyl reductase (ER)]. The number of reductive sites within a module corresponds to the extent of β-keto reduction in each condensation cycle. Our study shows that the thioesterase (TE) encoded at the end of module 6, which was earlier proposed to cleave the full-length polyketide chain from the enzyme (*4*), catalyzes lactone formation.

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genetic analysis, each module encodes a functional synthase unit that participates in one of the elongation steps of 6dEB formation (Fig. 1).

Our approach for the heterologous expression of modular PKSs expands on the host-vector system developed to study aromatic PKSs. The heterologous host, *Streptomyces coelicolor* CH999 (*7*), contains a chromosomal deletion of the entire *act* gene cluster, which encodes the biosynthesis of the aromatic polyketide actinorhodin. Because of the large sizes of the *eryA*I, *eryA*II, and *eryA*III genes, and the presence of multiple active sites, we developed an in vivo recombination technique to clone these genes into a plasmid suitable for their expression in CH999 (9). The genetic strategy (Fig. 2) utilizes derivatives of pMAK705 (*10*) to permit in vivo recombination between a temperature-sensitive and recipient plasmid in *Escherichia coli*. The three *ery*A genes thus cloned gave pCK7, a derivative of pRM5 (*7*). A control plasmid, pCK7f, which contains a frameshift mutation in *eryA*I, was also constructed. Both pCK7 and pCK7f possess a *ColE*I replicon for genetic manipulation in *E. coli* as well as a truncated SCP2* (low copy number) *Streptomyces* replicon. These plasmids also contain the *act*I promoter and *act*II-ORF4, an activator gene that is required for transcription from this promoter and activates expression during the transition from growth to stationary phase in the vegetative mycelium (*11*). High-level expression of PKS genes occurs at the onset of the stationary phase of mycelial growth; therefore, the recombinant strains produce polyketides as secondary metabolites in a quasi-natural manner.

With growth on R2YE medium (*12*), CH999/pCK7 produced abundant quantities of two polyketides (Fig. 3). The addition of propionate to the growth medium increased the yield of polyketide product (*13*). Mass spectrometry and proton and $^{13}C$ nuclear magnetic resonance (NMR) spectroscopy, in conjunction with propionic-1-$^{13}C$ acid feeding experiments, confirmed the major product as **1** (>40 mg/liter). The minor product was identified as 8,8a-deoxyoleandolide (**2**) (>10 mg/liter), which apparently originates from an acetate starter unit instead of propionate in 6dEB biosynthesis. Feeding experiments with $^{13}C_2$ sodium acetate confirmed the incorporation of acetate into **2**. Three large proteins (>200 kD), presumably DEBS1, DEBS2, and DEBS3 (*14*), were also observed in crude extracts of CH999/pCK7 by means of SDS–polyacrylamide gel electrophoresis (*15*). No polyketide products were isolated from CH999/pCK7f.

The production of 6dEB by CH999/pCK7 demonstrates that the three polypeptides encoded by the *ery*A genes are sufficient for polyketide biosynthesis and that all auxiliary activities required for in vivo PKS activity are present in *S. coelicolor*, even though it is not known to produce macrolides. For example, the incorporation of isotopically labeled propionic acid into **1** and **2** demonstrates the uptake of the exogenous precursor by *S. coelicolor* as well as the presence of a propionyl-CoA synthetase (or an appropriate CoA transferase) and a propionyl-CoA carboxylase. Because (2*S*)-methylmalonyl-CoA is the sole substrate for DEBS (*16*), our finding also indicates that either the *S. coelicolor* propionyl-CoA carboxylase gives rise to the (2*S*) enantiomer, like the *S. erythraea* enzyme (*17*), or a methylmalonyl-CoA epimerase (*18*) is present in *S. coelicolor*. Moreover, the methylmalonyl-CoA mutase (*19*) and isobutyryl-CoA mutase (*20*) activities that have been observed in other *Streptomyces* strains may also be present in *S. coelicolor* to provide a source of methylmalonyl-CoA for 6dEB biosynthesis.

Our results also show that unlike in *E. coli* (*21*), proper folding, assembly, and post-translational modification of the multifunctional DEBS occur in the intracellular environment of the heterologous host. In particular, the high amounts of macrolactone produced suggest that all of the ACP domains of a large fraction of the expressed enzyme are phosphopantothenylated, implying that *S. coelicolor* contains an appropriate holoACP synthetase. It remains to be determined whether the same enzyme also phosphopantothenylates the monofunctional ACPs involved in the biosynthesis of fatty acids or actinorhodin or both.

The successful cyclization of the aglycone illustrates that DEBS can lactonize the full-length polyketide chain with correct re-

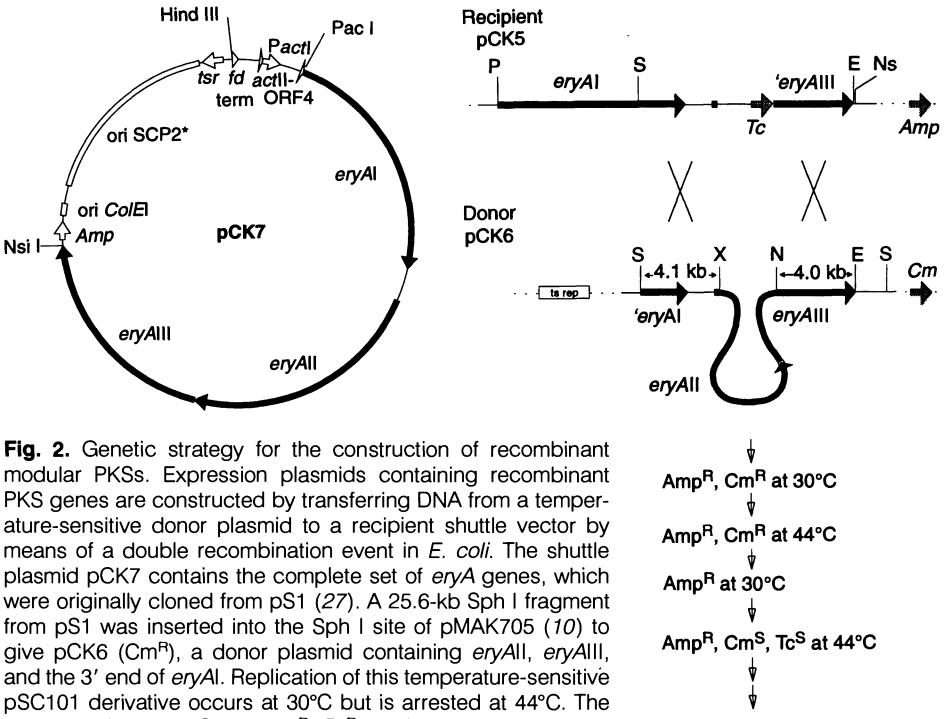


**Fig. 2.** Genetic strategy for the construction of recombinant modular PKSs. Expression plasmids containing recombinant PKS genes are constructed by transferring DNA from a temperature-sensitive donor plasmid to a recipient shuttle vector by means of a double recombination event in *E. coli*. The shuttle plasmid pCK7 contains the complete set of *eryA* genes, which were originally cloned from pS1 (*27*). A 25.6-kb Sph I fragment from pS1 was inserted into the Sph I site of pMAK705 (*10*) to give pCK6 ($Cm^R$), a donor plasmid containing *eryA*II, *eryA*III, and the 3′ end of *eryA*I. Replication of this temperature-sensitive pSC101 derivative occurs at 30°C but is arrested at 44°C. The recipient plasmid, pCK5 ($Amp^R$, $Tc^R$), includes a 12.2-kb *eryA* fragment from the *eryA*I start codon (*14*) to the Xcm I site near the beginning of *eryA*II, a 1.4-kb Eco RI–Bsm I pBR322 fragment encoding the tetracycline resistance gene (*Tc*), and a 4.0-kb Not I–Eco RI fragment from the end of *eryA*III (*28*). pCK5 is a derivative of pRM5 (*7*). The upstream and downstream regions of homology are 4.1 kb and 4.0 kb, respectively. We transformed (*29*) *E. coli* MC1061 with pCK5 and pCK6 and subjected it to carbenicillin and chloramphenicol selection at 30°C. Colonies harboring both plasmids ($Amp^R$, $Cm^R$) were then streaked at 44°C on carbenicillin and chloramphenicol plates. Only cointegrates formed by a single recombination event between the two plasmids were viable. Surviving colonies were propagated at 30°C on carbenicillin plates to select for recombinant plasmids formed by the resolution of cointegrates through a second recombination event. To enrich for pCK7 recombinants, we streaked the colonies again on carbenicillin plates at 44°C. Approximately 20% of the resulting colonies displayed the desired phenotype ($Amp^R$, $Tc^S$, $Cm^S$). The final pCK7 candidates were checked by restriction mapping. A control plasmid, pCK7f, which contains a frameshift error in *eryA*I, was constructed in a similar manner. Unmethylated pCK7 and pCK7f DNA was obtained by means of transformation into *E. coli* ET12567 (*30*) and subsequently introduced into *S. coelicolor* CH999 with standard protocols (*12*). In principle, even larger plasmids could be constructed by incrementally adding DNA through the iterative use of the recombination strategy described here. Abbreviations for restriction sites are as follows: E, Eco RI; N, Not I; Ns, Nsi I; P, Pac I; S, Sph I; and X, Xcm I.

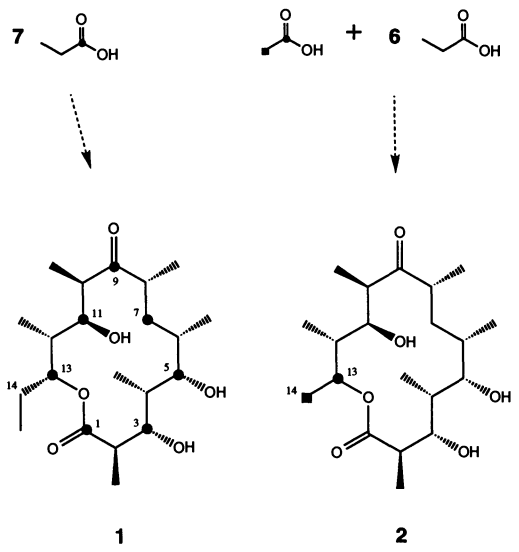

**Fig. 3.** Structures of macrolactones produced by recombinant strains. The CH999/pCK7 colonies were grown on R2YE medium (*12*) at 30°C under thiostrepton selection (50 μg/liter). Two macrolactones were isolated from this strain. The structures of 6-deoxyerythronolide B (6dEB) (**1**) and 8,8a-deoxyoleandolide (**2**) were confirmed by NMR spectroscopy and mass spectrometry. In separate experiments, the growth medium was supplemented with propionic acid (300 mg/liter, Aldrich) (PA), propionic-1-$^{13}C$ acid (200 mg/liter, Aldrich) (PA13C), and acetic-$^{13}C_2$ acid (sodium salt, 300 mg/liter, Aldrich) (AA13C) (*31*). After 5 days, the mycelia were extracted with ethyl acetate (pH 7). The extract was concentrated to dryness, resuspended in 3:1 hexane:ethyl acetate, and purified isocratically on a silica gel column (Baker) with the same solvent system. Yields of **1** and **2** from PA were >40 mg/liter and >10 mg/liter, respectively. No polyketide products were isolated from CH999/pCK7f. The proton NMR spectrum of **1** from PA was identical to that of an authentic sample (*32*), and $^{13}C$ NMR spectral peaks matched those previously reported (*33*). The chemical shifts of C-13 and C-14 in **2** from PA, in addition to the absence of a side chain methylene peak, were consistent with $^{13}C$ NMR data for oleandomycin (*33*) and 5-nor erythromycin C (*24*). The $^{13}C$ NMR data of **1** and **2** from PA13C revealed the incorporation of seven and six $^{13}C$-enriched carbons, respectively. The $^{13}C$ NMR spectrum of **2** from AA13C displayed triplet peaks at δ 18.1 and δ 70.3 ($J$ = 40 Hz). The molecular weights of **1** and **2** determined by mass spectrometry were 386 and 372, respectively.

giospecificity. Although a separate open reading frame in the *ery* gene cluster of *S. erythraea* shows significant homology to fatty acid synthase thioesterases (*22*), our results demonstrate that its gene product is not required for aglycone cyclization in *S. coelicolor*. Instead, we propose that the thioesterase domain at the COOH-terminal end of DEBS3 is solely responsible for lactone formation through an acyl enzyme intermediate.

The deoxyaglycone of oleandomycin, 8,8a-deoxyoleandolide, was previously isolated from a blocked *S. erythraea* mutant that also produced **1** (*23*). Trace quantities of its erythromycin analog, 15-nor erythromycin C, have also been found in the mother liquors of an erythromycin-producing strain (*24*). The production of **2** from *S. erythraea* strains maintaining presumably intact PKSs, as well as from our heterologous host, reveals a relaxed specificity of DEBS for the starter unit moiety. These results are reminiscent of the starter unit variability found in the avermectin and milbemycin macrolides (*25*) and also illustrate the ability of downstream active sites to utilize growing acyl chains with different starter units. The high yields of **2** relative to **1** (1:4) from CH999/pCK7 probably reflect lower intracellular concentrations of propionyl-CoA in *S. coelicolor* as compared with *S. erythraea* because the ratio of **1** to **2** can be increased by adding propionate to the growth medium. This observation might also indicate the absence in *S. coelicolor* of a methylmalonyl-CoA decarboxylase, an enzyme proposed to provide propionyl-CoA starter units for 6dEB biosynthesis in *S. erythraea* (*26*). Moreover, the observation that 5-nor erythromycin C has antibiotic activity (*23*, *24*) hints at the possibility of rapidly generating and assaying antibiotic candidates by feeding aglycones obtained through genetic engineering to *eryA* mutants of *S. erythraea*.

Finally, our results provide a conceptual framework for the generation of polyketides through the genetic manipulation of modular PKSs. The ability to directly manipulate large PKS genes in *E. coli* expands the repertoire of genetic tools available for mutagenesis, whereas the substantial yields of secondary metabolites in *S. coelicolor* greatly facilitate polyketide characterization. These advantages should enable rapid construction and analysis of a variety of targeted PKS mutants aimed at exploring the catalytic degrees of freedom in complex polyketide biosynthesis. In addition to guiding the rational design of polyketides, these insights may also lead to the development of combinatorial genetic strategies that exploit the modular organization of these enzymes, thereby generating chemical diversity.

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# Creation of Liquid Crystal Waveguides with Scanning Force Microscopy

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**The rubbing of a polymer layer, a commonly applied process, leads to an anisotropic surface morphology, aligning liquid crystal molecules. Scanning force microscopy can be used to intentionally create areas with a similar anisotropy by operating the instrument at loads in the range of $10^{-7}$ to $10^{-5}$ newtons. These areas have an orientation effect on liquid crystals indistinguishable from the rubbing process, which allows a systematic investigation of the orientation properties of an alignment layer as a function of its nanometer-scale morphology. Refractive index patterns can be tailored with this method by scratching a suitable area, as demonstrated by fabrication of an optical waveguide 6 micrometers wide and 5 millimeters long.**

Light-guiding structures play an important role in modern optics-based communication systems. Today, most of these devices are fabricated from glassy materials or from single crystals. Few attempts have been made to use liquid crystals (LCs), mainly because their switching speeds (microsecond to millisecond) are many orders of magnitude too slow to compete with conventional electrooptic materials. Recently, however, LCs with large optical nonlinearity have been found (*1*) that are extremely fast, so fast that they even act as diodes for light frequencies, leading to frequency-doubled light (second harmonic generation). The construction of an ultrafast nonlinear optical LC switching device (for example, a Mach-Zehnder interferometer) would require the development of methods for the production of light-guiding structures in LC layers. As an approach to this problem, we used a scanning force microscope (SFM) (*2*) as a tool to produce orientation patterns for light guiding in LCs.

The basic structure of a light-guiding LC layer is very similar to the familiar LC display (LCD): two glass plates separated by several micrometers with the LCs sandwiched in between. The glass plates have transparent electrodes and are, in addition, covered by thin polymer layers, which align the LC material. This alignment property is achieved by unidirectionally rubbing the polymer layer with a velvet roller. This technique is widely used in LCD fabrication and leads to a uniform orientation of the LC molecules near that surface. The mechanism responsible for the orientation is still uncertain (*3*).

A lot of work has been done on the modification of polymer surfaces with an SFM. The fabrication of grooves on various polymer surfaces with an SFM has been reported by a number of authors (*4*). These grooves look very similar to the grooves created by rubbing (*5*). We used an SFM to modify the polymer surface in a highly controlled manner to investigate how these modifications affect LC orientation. We found that by varying parameters such as loading, scan speed, and line density, different degrees of orientation can be achieved. This allows us to systematically investigate the LC orientation properties of a substrate as a function of morphology. The refractive index of the LC layer can also be manipulated on the micrometer scale by selecting a suitable SFM scratching pattern.

The SFM measurements were carried out with a commercially available instrument (*6*) operated in the constant-force mode under ambient conditions at room temperature. Microfabricated force sensors with spring constants of 0.12 and 3 N/m were used. The SFM was operated in two different modes: an imaging mode (loading force of about $10^{-9}$ N) and a surface modification mode (loading force, $10^{-7}$ to $10^{-5}$ N), which we will term scratching. Scratching on a larger scale was performed with a home-built instrument capable of scanning a force sensor over a 80 μm by 10 mm area. Movements in the small direction are achieved with a piezoelectric bimorph. This scanner is attached to a commercial motorized translation stage, enabling movements in the large direction at uniform scan speeds from 3.6 to 50 μm/s.

As samples, we used glass plates covered with a 25-nm thin layer of sputtered InSnO (ITO) onto which a 5- to 25-nm thin nylon layer was spin-coated. After deposition, the nylon layer was rubbed with a velvet roller and additionally scratched. Subsequently, an LC cell was made by mounting this plate together with a regularly rubbed nylon-coated glass plate. The rubbing directions on both plates were selected to be parallel. The spacing of the glass plates was fixed at about 6 μm by glass fiber cuts of the same diameter. The cell was filled with a nematic LC mixture with a refractive index parallel and perpendicular to the nematic director of 1.65 and 1.5, respectively. Additionally, optical properties of LC cells were investigated by polarizing microscopy to study the LC orientation.

Figure 1 shows an SFM image of a rubbed nylon orientation layer; the area in the top left part of the figure was additionally scratched by the SFM. The bright areas can be identified as nylon. The striations (or grooves) visible parallel to the rubbing direction *R* are caused by the rubbing pro-

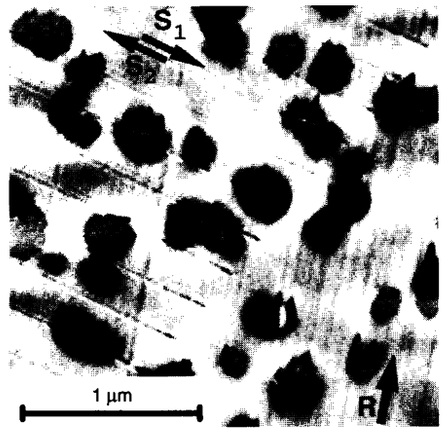


**Fig. 1.** An SFM image of a rubbed nylon layer that was also scratched. The picture was acquired at a tip loading of about $10^{-9}$ N. The rubbing direction is marked by arrow R. The area in the top left corner shows scratches made by the SFM at a load of $10^{-7}$ N. The scratches made in forward and backward direction are marked as arrows $S_1$ and $S_2$, respectively.

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