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Synthetic fermentation of bioactive molecules

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The concept of *synthetic fermentation* is to 'grow' complex organic molecules in a controlled and predictable manner by combining small molecule building blocks in water — without the need for reagents, enzymes, or organisms. This approach mimics the production of small mixtures of structurally related natural products by living organisms, particularly microbes, under conditions compatible with direct screening of the cultures for biological activity. This review discusses the development and implementation of this concept, its use for the discovery of protease inhibitors, its basis as a chemistry outreach program allowing non-specialists to make and discover new antibiotics, and highlights of related approaches.

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Introduction

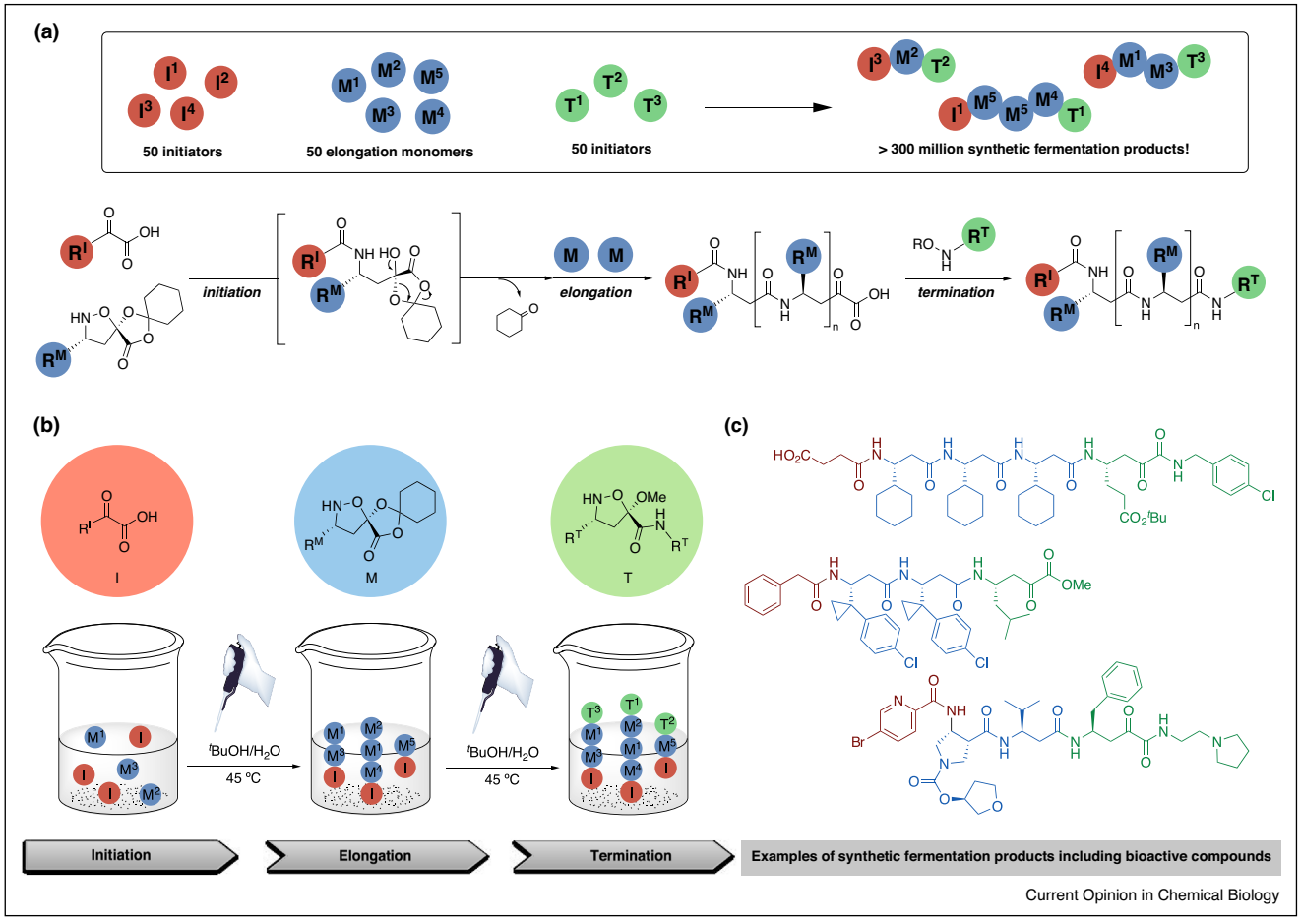
In 2014, we introduced the concept of *synthetic fermentation* — 'growing' complex organic molecules in a controlled and predictable manner by combining small molecule building blocks in water — without the need for reagents, enzymes, or organisms [1••]. Since no byproducts that interfere with compound screening are produced, the 'cultures' of molecules can be directly screened in many types of assays, including direct phenotypic screens on plants, animals, or cells. In direct analogy to natural products isolation, the active components can be identified by simple deconvolution strategies. Larger scale preparations of the bioactive molecules can be prepared from constituent building blocks without the need for traditional organic synthesis.

To achieve this, we took advantage of the unique chemoselectivity of the α-ketoacid–hydroxylamine amide-forming (KAHA) ligation, a process that does not require any catalysts or activating agents [2,3]. Through the design of a monomer scaffold bearing both a hydroxylamine moiety and a masked α-ketoacid, a cascade oligomerization process was realized [4–7]. The α-ketoacid initiator reacts with the hydroxylamine of the monomer, freeing the previously masked α-ketoacid to react with further monomers. The reaction can subsequently be stopped by the addition of a hydroxylamine terminator, leading to a mixture of β−peptides of varying sequence and length (Figure 1) [1••].

Our unusual use of the word 'fermentation' attracted some criticism. However, we feel this nomenclature is apt due to the widespread use of the term fermentation for the production of bioactive natural products including vancomycin, cyclosporine and tetracycline [8,9]. These complex, stereochemically rich molecules are produced from simple feedstocks by truly impressive biosynthetic transformations. These pathways usually involve an initiation step, starting from a specific small molecule, an elongation phase of iterative couplings of structurally related components, and a termination step — often featuring cyclization or the introduction of new functional groups [10,11]. Synthetic fermentation can be regarded as an attempt to emulate these biosynthetic pathways in a simple reaction flask or 96-well plate, without the need for complex enzymatic machinery.

The analogy to natural products biosynthesis extends beyond the term 'fermentation'. The implementations of synthetic fermentation reported to date deliver small mixtures of organic molecules. These mixtures — similar to extracts from natural sources such as plants or bacterial cultures — can be directly screened for biological activity. Any trained natural products chemist would be familiar with the screening, deconvolution, and isolation strategies employed to identify an active component. In the case of a positive result, the mixtures can be easily fractionated and the active molecules identified by standard analytical methods. In comparison to other approaches to the synthesis of bioactive compound libraries, synthetic fermentation boasts several distinct and attractive features. Unlike split pool combinatorial chemistry and related techniques, producing a mixture of molecules by synthetic fermentation requires very little work and just a few hours of reaction time. Any hits can be immediately resynthesized from the constituent building blocks, thereby reducing or eliminating false positives. Changes to the 'fermentation culture' by restricting the number of building blocks or exploring structure activity relationships can be accomplished even before the active

Figure 1



(a) The *synthetic fermentation* of natural product-like molecules relies on the unique chemistry of elongation monomers (M), which undergo a controlled oligomerization after reaction with an initiator (I). The reaction is stopped by addition of a terminator (T). **(b)** Large numbers of compounds can be quickly prepared from a small number of building blocks. **(c)** Examples of synthetic fermentation products prepared simply by mixing initiators, elongation monomers, and terminators under aqueous conditions. The resulting product mixtures can be diluted and directly screened in a biological assay [1••].

compound is conclusively identified. This represents an advantage of synthetic fermentation over conventional methods for the phenotypic screening of mixtures, where multistep syntheses and purification steps are often necessary to resynthesize compounds after obtaining an initial hit.

Synthetic fermentation of non-ribosomal peptide HCV protease inhibitors

For a first attempt at identifying bioactive molecules with synthetic fermentation, we targeted the discovery of HCV NS3/4A protease inhibitors. To achieve this, we designed and prepared a terminator scaffold that results in α-ketoamides [1••], which are known to be excellent protease inhibitors [12,13]. As a proof-of-concept, we used just 23 building blocks (6 initiators, 8 elongation monomers, and 9 terminators) to prepare a small library of about 6000 α-ketoamides in two 96-well plates (about 30 compounds per well). The 'cultures' were pre-formed with 120 μg of building blocks in 5 μL ^{t}BuOH/buffer in each well; these preliminary cultures collectively required about 50 mg of precursor organic compounds and produced enough material for more than 20 assays under standard conditions. After appropriate dilution, less than 1% ^{t}BuOH remained in the assay. The 'cultures' could be directly screened with a biochemical HCV protease assay in a 96-well plate reader and a simple combinatorial approach allowed us to identify the most active components. By making two additional focused cultures using the most active components in the preliminary assay, we identified, 'cultured' on large scale, isolated and characterized a new β-peptide based HCV NS3/4A protease inhibitor with an IC_{50} of 1.0 μM.