RNA secondary structure design

Bernd Burghardt and Alexander K. Hartmann
Institut für Theoretische Physik, Universität Göttingen,
Friedrich-Hund-Platz 1, D-37077 Göttingen, Germany
(Dated: February 2, 2008)

We consider the inverse-folding problem for RNA secondary structures: for a given (pseudo-knot-free) secondary structure find a sequence that has that structure as its ground state. If such a sequence exists, the structure is called designable. We implemented a branch-and-bound algorithm that is able to do an exhaustive search within the sequence space, i.e., gives an exact answer whether such a sequence exists. The bound required by the branch-and-bound algorithm are calculated by a dynamic programming algorithm. We consider different alphabet sizes and an ensemble of random structures, which we want to design. We find that for two letters almost none of these structures are designable. The designability improves for the three-letter case, but still a significant fraction of structures is undesgnable. This changes when we look at the natural four-letter case with two pairs of complementary bases: undesgnable structures are the exception, although they still exist. Finally, we also study the relation between designability and the algorithmic complexity of the branch-and-bound algorithm. Within the ensemble of structures, a high average degree of undesgnability is correlated to a long time to prove that a given structure is (un-)designable. In the four-letter case, where the designability is high everywhere, the algorithmic complexity is highest in the region of naturally occurring RNA.

PACS numbers: 87.15.Aa, 87.14.Gg, 87.15.Cc

I. INTRODUCTION

RNA plays an important role in the biochemistry of all living systems \[{1,2}\]. Similar to the DNA, it is a linear chain-molecule build from four types of bases—i.e., adenine (A), cytosine (C), guanine (G), and uracil (U). It does not only transmit pure genetic information, but, e.g., works as a catalyst, for example in the ribosome. While for the former only the primary structure—i.e., the sequence of the bases—is relevant, for the latter the kind of higher order structures—i.e., secondary and tertiary structures, is essential for its function. We exemplary mention the following three examples: i) For successful protein synthesis three-dimensional structures of rRNA \[{3,4}\] and tRNA \[{5}\] molecules are inevitable. ii) The catalytic properties of ribozymes depend on their three-dimensional structures \[{6}\]. iii) The function of the internal ribosome entry site (IRES) of picornaviruses which directs binding of ribosomal subunits and cellular proteins in order to accomplish translation initiation, is based on higher order structures \[{7}\].

Like in the double helix of the DNA, complementary bases within RNA molecules can build hydrogen bonds between each other. As opposed to DNA, where the bonds are built between two different strands, in RNA bonds are formed between bases of the same RNA strand. The secondary structure is the information, which bases of the strand are paired, while the spatial structure is called the tertiary structure. The tertiary structure is stabilized by a much weaker interaction than the secondary structure. This leads to a separation of energy scales between secondary and tertiary structure, and gives the justification to neglect the latter in many cases to obtain a first fundamental understanding of the behavior of RNA \[{8}\]. Therefore, although the tertiary structure is important often for an RNA’s functionality, it is sufficient that we deal here with the secondary structure only.

One crucial point for the calculation of the secondary structure is the energy model, which is applied: On the one hand, if one aims to get minimum structures close to the experimentally observed one, one uses energy models that take into account many different structural elements \[{9,10,11,12}\], e.g., hair pin loops or bulges, each being described by a different set of experimentally obtained parameters. On the other hand, if one is interested in the qualitative behavior, one uses models as simple as possible while conserving the general behavior, e.g., in the simplest case a model which exhibits only one kind of base \[{13}\], or models where the energies depend only on the number and on the type of paired bases \[{14,15,16,17}\]. Here we will consider only models with the latter kind of interaction energy.

The standard procedure when dealing with RNA secondary structures is that one starts with a given sequence and calculates, e.g., the ground-state structure in which the RNA will fold for low temperatures. In this paper we look at the inverse problem: For a given secondary structure, does a sequence exist that has the given structure as its ground state? If this is the case, we call the structure designable. We answer this question for different alphabet sizes, i.e., different numbers of complementary bases. As an ensemble of structures we choose a set of random structures of given length and ask how large is the fraction designable structures. In a related study Mukhopadhyay \textit{et al.} \[{18}\] also considered different alphabet sizes, but they determined for a ground-state structure of a given sequence, by using a probabilistic algorithm, i.e., approximately, how many different other sequences have this structure as a ground state. Hence, by definition, all structures encountered are designable. In contrast, we generate structures randomly from scratch, and determine whether there is

\[
\text{Electronic address: burghard@physik.uni-goe.de} \quad \text{Electronic address: hartmann@physik.uni-goe.de}
\]
at least one sequence that has this structure as a ground state. Hence, we can generate structures, which might not be describable at all. The basic idea behind this approach is that nature needs as many different structures as possible to perform many different tasks, and, as it turns out, a minimum number of four letters is necessary for this. Furthermore, we use an exact branch-and-bound algorithm to verify (un-)designability. In another previous work Hofacker et al. [19] looked at the same question whether a given structure is designable. In contrast to our work, they used only a probabilistic approach, hence in some cases solutions may have been missed. Furthermore, they studied a very restricted ensemble of structures, where the structures are assembled from substructures found in nature already, which implies by definition a high degree of designability. Also they did not study the dependence on the alphabet size. Another difference of our work to previous publications is that we also study the relation between the designability and the algorithmic complexity, i.e., the running time of our exact algorithm.

The paper is organized as follows. In section Sec. II, we define our model—i.e., we formally define secondary structures and introduce our energy model and state the design problem. In Sec. III, we explain how to calculate a bound for the ground state with a dynamic programming algorithm and how to solve the design problem with a branch-and-bound algorithm augmented with a randomized algorithm. We also present thoroughly in Sec. III C how we generate the ensemble of random structures. Finally, in Sec. IV, we show the result of our numerical studies.

II. THE SECONDARY STRUCTURE MODEL AND DESIGN PROBLEM

A. RNA secondary structure model

Because RNA molecules are linear chains of bases, they can be described as a (quenched) sequence \( \mathcal{R} = (r_i)_{i=1,\ldots,L} \) of bases \( r_i \in \mathcal{A} \). We denote by \( L \) the length of the sequence and \( \mathcal{A} \) is the alphabet, which contains the underlying base types that build the RNA sequence. Typically \( \mathcal{A} = \{A, C, G, U\} \) is used, but we also consider here alphabets with two and three letters. Within this single stranded molecule some bases can pair and build a secondary structure. The Watson-Crick base pairs—i.e., A-U and C-G—have the strongest affinity to each other, they are also called complementary base pairs. Each base can be paired at most once. For a given sequence \( \mathcal{R} \) of bases the secondary structure can be described by a set \( S \) of pairs \( (i, j) \) (with the convention \( 1 \leq i < j \leq L \)), meaning that bases \( r_i \) and \( r_j \) are paired. For convenience of notation we further define a Matrix \( (S_{i,j})_{i,j=1,\ldots,L} \) with \( S_{i,j} = 1 \) if \((i, j) \in S\), and \( S_{i,j} = 0 \) otherwise. Two restriction are used:

1. [non-crossing condition] Here we exclude so called pseudo knots, that means, for any \((i, j), (i', j') \in S\), either \( i < j < i' < j' \) or \( i < i' < j' < j \) must hold—i.e., we follow the notion of pseudo knots being more an element of the tertiary structure [20].

2. [min-distance condition] Between two paired bases a minimum distance is required: \( |j - i| \geq h_{\text{min}} \) is required, due to the bending rigidity of the molecule. Our main results below will be for \( h_{\text{min}} = 2 \), but for comparison we discuss the unphysical case \( h_{\text{min}} = 1 \) as well. Larger—and more realistic—\( h_{\text{min}} \) values do not change the qualitative results compared to the \( h_{\text{min}} = 2 \) case, but are computationally more demanding.

In the following we assume that each structure \( S \) 'fits' to all considered sequences \( \mathcal{R} \)—i.e., for all pairs \((i, j) \in S\) the indices \( i \) and \( j \) are smaller or equal to the length \( L \) of the sequence \((1 \leq i, j \leq L)\). By \( S^{m,n} \) we denote a substructure of \( S \) between the \( m \)'th and \( n \)'th letter, i.e., \( S^{m,n} := \{(i, j) \in S | m \leq i < j \leq n\} \). Similar, a subsequence between the \( m \)'th and \( n \)'th letter is denoted by \( \mathcal{R}^{m,n} = (r_i)_{i=m,\ldots,n} \).

B. Energy models

In this section we define an energy model, which assigns every secondary structure \( S \) belonging to a sequence \( \mathcal{R} \) an energy \( E(S, \mathcal{R}) \). For a given sequence \( \mathcal{R} \) the minimum \( E(\mathcal{R}) = \min_S E(S, \mathcal{R}) \) is the ground-state energy of the sequence \( \mathcal{R} \).

Motivated by the observation that the secondary structure is due to building of numerous base pairs where every pair of bases is formed via hydrogen bonds, one assigns each pair \((i, j)\) a certain energy \( e(r_i, r_j) \) depending only on the kind of bases. The total energy is the sum over all pairs

\[
E_p(S, \mathcal{R}) = \sum_{(i,j) \in S} e(r_i, r_j),
\]
e.g., by choosing \( e(r, r') = +\infty \) for non-complementary bases \( r, r' \) pairings of this kind are suppressed. In our numerical studies we restrict our self to the energy model

\[
e(r, r') = \begin{cases} E_p & \text{if } r \text{ and } r' \text{ are compl. bases} \\ +\infty & \text{otherwise} \end{cases}
\]

with a pair energy \( E_p \leq 0 \) independent of the kind of bases. Another possible model is to assign an energy \( E_s \) to a pair \((i, j) \in S \) iff also \((i + 1, j - 1) \in S \). This stacking energy can be motivated by the fact that a single pairing gives some gain in the binding energy, but also reduces the entropy of the molecule, because through this additional binding it looses some flexibility. Formally the total energy of a structure can be written as

\[
E_s(S, \mathcal{R}) = \begin{cases} \sum_{(i,j) \in S} E_s S_{i+1,j-1} & \text{if all } (i, j) \in S: \\ +\infty & \text{if } r_i \text{ and } r_j \text{ are compl. bases} \end{cases}
\]

Real RNAs cannot be described by just one energy parameter, because the free energy depends on the type and the size of
the structural elements, e.g., hair pin loops. Here, we examine the sum of both models—stacking energy and pair energy—

\[ E(S, R) := E_p(S, R) + E_s(S, R), \]

where the parameters \( E_p \) and \( E_s \) can be freely adjusted, including both models discussed above. For real RNA both parameters, \( E_p \) and \( E_s \), are of the same order of magnitude, namely about 1 ... 10 kcal mol\(^{-1}\) \cite{2, 21, 22}, therefore we choose \( E_p = -2 \) and \( E_s = -1 \) in our simulations.

A sequence \( R \) is said to be compatible with a structure \( S \), if \( e(r_i, r_j) \leq 0 \) for all \((i, j) \in S \).

Further, we define for a structure \( S \) (independent of \( R \)) the energy

\[ E(S) := E_{\text{min}} |S| + \sum_{(i,j) \in S} E_s i, j - 1, \]

with \( E_{\text{min}} = \min_{r, r' \in A} e(r, r') \). For the energy model of Eq. \( \ref{eq:energy_model} \) it is \( E_{\text{min}} = E_p \). Thus, \( E(S) \) is a lower bound of \( E(S, R) \) for any \( R \).

### C. Designing RNA Secondary Structure

The energy model \( \ref{eq:energy_model} \) has been previously studied \cite{23}, in the standard way, i.e., by calculating ground states for given sequences. In this paper we take, as already mentioned in the introduction, a different point of view: we choose a random structure \( S \) and ask, whether there exists any sequence \( R \) that has this structure as its ground state.

The design problem can be more formally stated as following: For a given structure \( S \) find a sequence \( R \) such that \( E(S, R) = E(R) \) holds. If such a sequence exists, the structure \( S \) is called designable. However, we do not require that \( S \) is the unique ground state of this sequence, since this issue has been addressed previously \cite{13}.

The design problem for an energy model without stacking energy, i.e., which exhibits only a pair energy according to Eq. \( \ref{eq:energy_model} \), can be solved easily as follows (Fig. 1: assign to any pair \((i, j) \in S \) the letters \( A \) at position \( i \) and \( U \) at position \( j \), and for every unpaired position a base of type \( G \) (in the two letter case use \( A \) again). There are exactly \( |S| \) pairs of bases therefore the ground-state energy can not be below \( E_p |S| \), which is just the ground-state energy of the structure \( S \).

For the case \( E_s \leq 0 \) this construction scheme might fail as one can see in the example shown in Fig. 1 re-grouping of the enclosed base pairs leads to the formation of two adjacent pairs, i.e., a stack of size two. This results in an energy of the re-grouped structure below the energy of the given structure, hence the given structure is not a ground state of the given sequence. Nevertheless, the structure shown in the example is in fact designable, the slightly modified sequence—position 2 and 4 are swapped—\( \text{AUGAGAGUUAGU} \) has the given structure as a ground state.

The case \( h_{\text{min}} = 1 \), i.e., neighboring bases can be paired, is of little interest: both, from the physical point of view—the RNA molecule cannot be bent arbitrarily strong—as well as from the design problems point of view. As an undesignable example look at the structure sketched in figure Fig. 2: for any alphabet size there is only a finite number of different 2-tuples \((r_1, r_2)\), whenever there are more than this number of neighboring pairs paired in a structure, at least two of them must be of the same kind—e.g., \((A, U)\)—this two can be re-paired and gaining some stacking energy, rendering the structure undesignable.

### III. Algorithms

In principle the design problem can be solved by calculating the ground state energy \( E(R) \) of every compatible sequence \( R \) and testing whether this is equal to \( E(S, R) \), but, because the number of sequences growth exponentially with the sequence size \( L \) (roughly as \(|A|^{|L|-|S|}\)), this is impractical.

Therefore we use a branch-and-bound algorithm, where one tries to find an upper bound \( E^B(Q) := \max_{R \in Q} E(R) \) for the ground-state energies for a (large) set \( Q \) of sequences compatible with the structure \( S \). If this bound is below the energy \( E(S) \) of the structure—i.e., \( E^B(Q) < E(S) \)—then none of the sequences in \( Q \) can be a solution of the design problem.

Here, we consider in particular sets of sequences, where at some positions all sequences of the set have the same letter (but possible different ones for the different positions), and where for all other positions all possible combinations of letters occur, which are compatible with the sequence. Hence, these positions can be described by a joker letter. For a more formal definition of \( Q \), see below. In Sec. III A an algorithm

\[ \begin{array}{ccccccc}
A & A & G & U & G & A & U \\
\end{array} \]

FIG. 1: In the case \( E_s = 0 \) the structure can be easily designed, e.g., by building \((A, U)\)-pairs for the paired bases, and assigning \( c \) to the unpaired bases. However, this is not necessarily a solution for the \( E_s < 0 \) case: in this example two pairs could be re-paired (dashed lines) giving a lower overall energy.

\[ \begin{array}{cccccccc}
A & A & G & U & G & A & U & A \\
\end{array} \]

FIG. 2: In the case of \( h_{\text{min}} = 1 \) and \( E_s < 0 \) this is an example of an undesignable structure. There is only a finite number of different 2-tuples \((r_1, r_2)\), whenever there are more than this number of neighboring pairs paired in a structure, at least two of them must be of the same kind—e.g., \((A, U)\)—this two can be re-paired (dashed lines) gaining some stacking energy, rendering the structure undesignable.
A. Calculating a bound for the ground-state energy

In this section we introduce a modification of the algorithm presented in Ref. [23] which allows us to calculate an upper bound for the ground-state energy of sequence, where some bases are still unassigned, i.e., represented by the joker letter.

Thus, for a formal description of the algorithm we extend the alphabet by the joker-letter *, where * represents any letter in the original alphabet. Note that * is complementary to any \( r \in A \). The new alphabet is denoted by \( A^* := A \cup \{ \ast \} \). Sequences \( R^* = (r_i^*), i = 1 \ldots L \), \( r_i^* \in A^* \), over this extended alphabet \( A^* \), we call \( R^* \) a generalized sequence, represent a set \( Q \) of sequences over the original alphabet \( A \): \( Q = \{(r_i), i = 1 \ldots L | r_i \in A, r_i = r_i^* \text{ if } r_i^* \in A \} \). For a given structure \( S \) and a generalized sequence \( R^* \), the scheme explained in the following can be used to calculate the bound for the ground-state energy. Note that for a sequence without a *-letter this bound is equal to the ground-state energy.

We start the explanation of the algorithm by considering the contribution to the bound arising from a single pair \((i,j)\). If the letters in the sequence are fixed, i.e., \( r_i, r_j \in A \), then the energy contribution is simply \( e(r_i, r_j) \), since there is no choice. If at least one of the two letters is the joker letter *, then we have different choices. First, if \((i,j) \in S\), then the energy contribution must be negative, because otherwise, since we are considering ground states, bases \( i \) and \( j \) would not be paired leading to an energy contribution zero. On the other hand, we are looking for an maximum over all sequences described by the generalized \( R^* \), hence we have to take the maximum over all possible negative contributions, either over all possible combinations of two letters (two * symbols), or, over all possible letters at the one position with a * symbol. Second, if \((i,j) \notin S\), then the energy contribution should be positive if bases \( i,j \) are paired nevertheless, such that within the ground-state calculation, automatically the case is selected where bases \( i,j \) are not paired. We assume that for all possible cases with one or two * symbols, always combinations of letters are available, such that the pair energy is positive. Since in this case, the ground-state requirement will automatically disregard the pair \((i,j)\), instead of maximizing over all energies, we can simply assume the energy contribution \( r_\infty \) here. This leads to the energy contribution \( e^*_{R^*,S}(i,j) \) for a pair \((i,j)\) which depends on the given generalized sequence \( R^* \) and the given structure \( S \):

\[
e^*_{R^*,S}(i,j) = \begin{cases} 
e(r_i, r_j) & \text{if } r_i, r_j \in A \land |i-j| \geq h_{\min} \\ e^*_{\max} & \text{if } r_i = *, r_j = *, (i,j) \in S \\ e^*_{\max} & \text{if } r_i = *, r_j \in A, (i,j) \in S \\ +\infty & \text{else} \end{cases}
\]

(6)

with the largest possible negative pair energies

\[
E^*_{\max} := \max \{ e(r, r') < 0 | r, r' \in A \} \\
E^*_{\max} := \max \{ e(r, r') < 0 | r' \in A \} \\
E^*_{\max} := \max \{ e(r, r') < 0 | r \in A \}
\]

(7)

and for the maximum of the empty set: \( \max \emptyset := -\infty \). For alphabets, where each base has a complementary base, e.g., the two- and four-letter cases discussed below, with the energy \( e(r, r') \) from Eq. (2) \( e^*_{R^*,S} \) has the form

\[
e^*_{R^*,S}(i, j) = \begin{cases} e(r_i, r_j) & \text{if } r_i, r_j \in A \\ E_p & \text{if } r_i = * \land r_j = *, (i,j) \in S \\ +\infty & \text{else} \end{cases}
\]

(8)

For alphabets with letters that have no complementary counterpart, e.g., letter \( G \) in the three-letter alphabet of Sec. IV B the sets in Eq. (7) might be empty leading to an energy contribution \( -\infty \), i.e., resulting in an upper bound \( E^B(R^*) = -\infty \). In our implementation of the algorithm we do not consider (generalized) sequences, where at a position of a paired base such a letter appears, because this would lead do non-compatible sequences. Note that for the case that also the pair \((i-1, j+1)\) is present, additionally to \( e^*_{R^*,S}(i,j) \) a stacking-energy contribution \( E_s \) arises. This is handled by the following recursive equations, which perform the ground-state calculation. They are slightly modified compared to Ref. [23]. We denote by \( \hat{N}_{i,j} \) the maximum ground-state energy over the set of compatible subsequences given by the generalized subsequence \( r_i^*, r_{i+1}^*, \ldots, r_j^*, \). \( \hat{N}_{i,j} \) is defined in the same way, only that additionally it is assumed that letters \( r_i^* \) and \( r_{j+1}^* \) are paired, which leads simply to an additional stacking-energy contribution. The basic idea is that for the ground state of subsequence \( r_i^*, r_{i+1}^*, \ldots, r_j^* \) either the last letter \( j \) is not paired, or it is paired to another letter \( k \in \{i, i+1, \ldots, j-1\} \) (the requirement \( j-i \geq h_{\min} \) is treated through energy \( e^*_{R^*,S}(i,j) \)).

The ground state is the minimum over all these cases, where in each case, due to the exclusion of pseudo knots, the ground-state calculation decomposed into the calculation for shorter subsequences. The recursion equations for \( N_{i,j} \) and \( \hat{N}_{i,j} \) read as follows:

\[
N_{i,j} = \min_{k=i}^{j-1} \left[ N_{i,k-1} + e^*_{R^*,S}(k,j) + \hat{N}_{k+1,j-1} \right] \\
\hat{N}_{i,j} = \min_{k=i}^{j-1} \left[ N_{i,k-1} + e^*_{R^*,S}(k,j) + \hat{N}_{k+1,j-1} \right]
\]

(9)

for \( j-i > 0 \)

The values of \( N_{i,j} \) and \( \hat{N}_{i,j} \) are calculated “bottom up”, i.e., in a dynamic programming fashion, starting at small values of
to calculate the ground-state energy and the upper bound for two functions appear, $G$ sequences represented by this node. Within the algorithm, traverses the tree from the root towards the leafs calculating yet. Initially $T$ are generated from existing nodes, by selecting a node, i.e., each node represents a generalized sequence state. In the case that the problem has a solution a random-

In Fig. 3, a pseudo code of the algorithm is shown. There, the idea of the algorithm is that it constructs a tree, where each node represents a generalized sequence $R^*$, i.e., a set $Q$ of sequences, and all children of a node represent a partition of $Q$. The root node stands for the set of all sequences length $L$, i.e., which is described by the generalized sequence $(r^*_i)_{i=1,...,L}$, $r^*_i = \ast$. For every node $(r^*_i)$ in the tree with at least one $r^*_j = \ast$ its children are constructed by replacing $r^*_j$ with one letter from $A$. Sequences with no $\ast$-letters are the leaf nodes of the tree (sets with exactly one element/sequence).

In Fig. 3 a pseudo code of the algorithm is shown. There, $T$ contains all nodes of the tree which have not been treated yet. Initially $T$ contains only the root node. New nodes are generated from existing nodes, by selecting a node, i.e., a generalized sequence, selecting one position where a $\ast$ appears, and generating $|A|$ new nodes by replacing this $\ast$ by all possible letters $\alpha \in A$. In this way algorithm traverses the tree from the root towards the leaves calculating an upper bound of the ground state energies of the sequences represented by this node. Within the algorithm, two functions appear, $\text{GROUND-STATE-ENERGY}(R^*_a)$ and $\text{GROUND-STATE-BOUND}(R^*_a)$, which essentially use Eq. (46) to calculate the ground-state energy and the upper bound for it, respectively. If this upper bound is below the energy $E(S)$ of the structure $S$, none of the sequences represented by this node has this structure as a ground state, and the descend towards the children of this node can be stopped here: the algorithm ignores this node by not putting it into $T$. On the other hand, if a leaf node is reached and its ground state energy is equal to the energy of the structure, a solution is found and the algorithm terminates successfully.

The selection steps in line 4 and 5 require further explanations: We use a stack-like data structure, so the last inserted sequence in line 13 is used first here (depth-first search). The selection step of a joker-letter in line 5 is more difficult: we tried some strategies in which the next inserted base can be chosen. All this strategies were static ones, that means the order of insertion was chosen based on the concrete structure given, but the order was fixed before starting with the algorithm. At the end we found the following strategy to be the best: We first insert paired bases, and we choose the base pair $(i,j)$ first that encloses the most other bases—i.e., $S_{i:j}$ is the largest substructure of any $(i,j) \in S$. The procedure continues with the substructure $S_{i+1:j-1}$, if it is not empty, or continues with a pair $(i',j') \notin S_{i+1:j-1}$ enclosing the next largest substructure. At the end we insert the unpaired bases.

### 1. Branch-and-Bound algorithm

Our deterministic algorithm follows the Branch-and-Bound approach (e.g., in Ref. 25, pp. 499). Here, it finds a sequence $R^*$—if such a sequence exists—that has the $S$ as one ground-state.

The idea of the algorithm is that it constructs a tree, where each node represents a generalized sequence $R^*$, i.e., a set $Q$ of sequences, and all children of a node represent a partition of $Q$. The root node contains the set of all sequences length $L$, i.e., which is described by the generalized sequence $(r^*_i)_{i=1,...,L}$, $r^*_i = \ast$. For every node $(r^*_i)$ in the tree with at least one $r^*_j = \ast$ its children are constructed by replacing $r^*_j$ with one letter from $A$. Sequences with no $\ast$-letters are the leaf nodes of the tree (sets with exactly one element/sequence).

In Fig. 3 a pseudo code of the algorithm is shown. There, $T$ contains all nodes of the tree which have not been treated yet. Initially $T$ contains only the root node. New nodes are generated from existing nodes, by selecting a node, i.e., a generalized sequence, selecting one position where a $\ast$ appears, and generating $|A|$ new nodes by replacing this $\ast$ by all possible letters $\alpha \in A$. In this way algorithm traverses the tree from the root towards the leaves calculating an upper bound of the ground state energies of the sequences represented by this node. Within the algorithm, two functions appear, $\text{GROUND-STATE-ENERGY}(R^*_a)$ and $\text{GROUND-STATE-BOUND}(R^*_a)$, which essentially use Eq. (46) to calculate the ground-state energy and the upper bound for it, respectively. If this upper bound is below the energy $E(S)$ of the structure $S$, none of the sequences represented by this node has this structure as a ground state, and the descend towards the children of this node can be stopped here: the algorithm ignores this node by not putting it into $T$. On the other hand, if a leaf node is reached and its ground state energy is equal to the energy of the structure, a solution is found and the algorithm terminates successfully.

The selection steps in line 4 and 5 require further explanations: We use a stack-like data structure, so the last inserted sequence in line 13 is used first here (depth-first search). The selection step of a joker-letter in line 5 is more difficult: we tried some strategies in which the next inserted base can be chosen. All this strategies were static ones, that means the order of insertion was chosen based on the concrete structure given, but the order was fixed before starting with the algorithm. At the end we found the following strategy to be the best: We first insert paired bases, and we choose the base pair $(i,j)$ first that encloses the most other bases—i.e., $S_{i:j}$ is the largest substructure of any $(i,j) \in S$. The procedure continues with the substructure $S_{i+1:j-1}$, if it is not empty, or continues with a pair $(i',j') \notin S_{i+1:j-1}$ enclosing the next largest substructure. At the end we insert the unpaired bases.

### 2. Randomized steepest-descent Algorithm

We further implemented a randomized algorithm for finding a solution of the design problem for a given structure $S$ similar to Ref. 24 while in Ref. 19 a much more sophisticated method is explained. We start with a compatible sequence, e.g., every pair of the structure is assigned a A-U pair and all unpaired bases are assigned to G (again A if the al-
phabnet contains only two letters). Either this already solves
the design problem or we modify the sequence at one place as
following: for the given sequence we calculate a ground-state
structure \( S_0 \), then we choose a pair \( \varphi \), which is in exactly one
of the structures \( S \) and \( S_0 \) — i.e., \( \varphi \in S \cap S_0 \) — and randomly
modify one of these two bases — if \( \varphi \in S \) we keep the other
base complementary. We accept this step, if the ground-state
energy is not below that of the previous sequence. The pro-
duced is repeated until a sequence is found that solves the
design problem, or until a certain number of random steps has
been executed, in this case, the algorithm stops unsuccess-
fully.

Of course, this method can never prove that a certain struc-
ture is undesirable. However, we combined this strategy
with the branch-and-bound algorithm above: whenever a re-
jection step takes place — i.e., the condition in line 14 of algo-
rithm in Fig. 3 is reached — one random step with an indepen-
dently stored sequence is done. This can be quite efficient in
the designable case, because on average it requires much less
steps than the deterministic branch-and-bound algorithm. On
the other hand it doubles the efforts in the undesirable case.
This pays off in particular for the four-letter case discussed in
Sec. [IVC] because there almost all structures are designable.
Especially, for design times much larger than the sequence
length — i.e., \( T \geq 10L \) — the random-method is almost always
faster than the deterministic algorithm. This is different in the
two- and three-letter case, where the deterministic algorithm
requires less steps.

C. Generating random secondary structures

Later on we examine the designability of randomly gen-
erated secondary structures for a given sequence length \( L \).
We parametrize our ensemble by the probability \( p \) that a cer-
tain base in the sequence is paired (for rRNA \( p \) is typical in
the range 0.6 . . . 0.8 [24]). We construct each sample in two
steps: First, we draw the number of pairs \( P \) of the structure
from a binomial distribution between 0 and \( \lfloor L/2 \rfloor \) centered at
\( pL/2 \). Then, among all possible structures of length \( L \) having
\( P \) pairs, we select one randomly, such that each structure has
the same probability of being chosen. The achieve this, we
have to perform a preprocessing step first:

In the preprocessing step, we calculate the number \( S(P, L) \)
of possible structures of a sequence of length \( L \) and with \( P \)
pairs. The number \( S(P, L) \) is the number of possible struc-
tures \( S(P, L-1) \) of the smaller sequence plus the number of
possible structures, where base \( L \) is paired with base \( L-k \).
Hence, the value \( S(P, L) \) can be calculated by the following
recursion relation [27]:

\[
S(P, L) = S(P, L-1) + \sum_{k=1}^{L-1} \sum_{q=0}^{L-1-P} S(q, k-1) S(P-q-1, L-k-1),
\]

(10)

\[
S(P = 0, L) = 1, \quad S(P < 0, L) = S(P > L/2, L) = 0
\]

The first sum is over all possible distances between this two
bases; the second sum is over the number of pairs enclosed
by the pair \( (L-k, L) \). The product is the number of possible
structures having \( q \) pairs enclosed by \( (L-k, L) \) and the
remaining \( P-q-1 \) pairs in the range from 1 to \( L-k-1 \).

The construction of the matrix \( S(P, L) \) requires \( O(L^4) \) calculation
steps, but this is required only once for all lengths up to a ma-
ximum length \( L \). Note that for \( h_{\min} = 1 \) the number of struc-
tures can be calculated explicitly \( S(P, L) = \frac{1}{L+1} \binom{2L}{P} \binom{L}{2P} \).

Now, for each sample to be generated, where the number
of pairs \( P \) of pairs has been randomly chosen as explained above, the
actual structure is selected in the following way. First, note
that depending on \( h_{\min} \) there are values of \( P \) and \( L \), where
no structures exist, i.e., \( S(P, L) = 0 \), these cases are re-
jected immediately. Otherwise, the random structure is con-
structed with a backtracing algorithm: starting at \( S(P, L) \)
choose one of the summations according to its weight, in-
sert the corresponding pair to the structure and recurs into the
sub-sequences. As an example we show the random
construction of a structure of length \( L = 8 \) with \( P = 3 \)
pairs (see Fig. 4). The non-zero contributions to \( S(3, 8) = S(3, 7) + S(0, 1) S(2, 5) + S(1, 3) S(1, 3) + S(2, 5) S(0, 1) + S(2, 6) S(0, 0) \), each of the sumsmands represents a possible
pairing of base number 8 with another base — with the excep-
tion of the first summation, which counts the number of pos-
sible structure, where base number 8 is not paired at all.

We choose the last summation, meaning that base 8 is paired
with base 1. Leaving two pairs which must be distributed between
the bases from 2 to 7; here we choose to pair base 7 with base
5, leaving only one possibility for the remaining pair, namely
base 4 paired with base 2.

Finally, note that the average number of structures available
for given $p$ and $L$ is given by

$$s(p, L) = \sum_{P=0}^{[L/2]} \binom{[L/2]}{P} p^P (1-p)^{[L/2]-P} S(P, L).$$

$$\textbf{IV. NUMERICAL RESULTS}$$

For an ensemble of randomly chosen structures of given sequence length $L$ we examined, whether these structures are designable or not. We used different alphabets with two, three and four letters. All calculations for the results presented below were performed with the parameters $E_p = -2$, $E_s = -1$, and $h_{\min} = 2$. Note that increasing the stacking energy $E_s$ in comparison to the pair energy makes the design problem more difficult: in the limit $E_s \to -\infty$ it would be favorable to remove all non-stacked pairs from the structure, if this allows only one additional stacked pair. Considering the minimum distance $h_{\min}$ between two paired bases of natural RNA, it seems to be more appropriated to use a larger value for $h_{\min}$, e.g., $h_{\min} = 5$ would be more appropriate, but this increases the computational effort without changing the qualitative results: only $h_{\min} = 1$ has a different qualitative behavior (see Fig. 4).

A. Two-letter alphabet

The alphabet consists of two complementary letter, e.g., A and U, only. In Fig. 5 the fraction $U$ of the undesignable structures is shown as a function of the probability $p$ that a base is paired. Even for small sequences and low probabilities of bases being paired, almost all structures are undesignable. Missing error bars are of the size of the symbols or smaller, and omitted for legibility. (Parameter used: $E_p = -2$, $E_s = -1$, $h_{\min} = 2$, 1000 samples.)

![Fig. 5: The undesignability $U$ of random structures for an underlying two-letter alphabet is shown as function of the probability $p$ that a base is paired. Even for small sequences and low probabilities of bases being paired, almost all structures are undesignable. Missing error bars are of the size of the symbols or smaller, and omitted for legibility. (Parameter used: $E_p = -2$, $E_s = -1$, $h_{\min} = 2$, 1000 samples.)](image)

The maxima of this curve is at smaller $p$-value than the maximum of $U(p, L = 90)$. (Parameter used: $E_p = -2$, $E_s = -1$, $h_{\min} = 2$, 1000 samples.)

We also looked at the “time” $T$ required to find a solution—if any exists. “Time” means here, how often either of the two functions $\text{GROUND-STATE-ENERGY}(R_s^*)$ or $\text{GROUND-STATE-BOUND}(R_s^*)$ (see Fig. 3) is called; because this two function are called at least $L$-times, $T$ is at least $L$. In Fig. 4 the average of $\ln(T/L)$ is shown as a function of $p$. Because $T \geq L$ a value close to zero indicates, that a solution is found (on the average) almost immediately.

![Fig. 6: The undesignability $U$ of random structures for an underlying three-letter alphabet is shown as function of the probability $p$ that a base is paired. In comparison to the two-letter case (Fig. 5) many more of structures are designable, but still a reasonable fraction of structures is undesignable. In light gray the average number of structures is undesignable.](image)
\[ E_p = -2, \ E_s = -1, \ h_{\text{min}} = 2, \ 1000 \text{ samples.} \]

C. Four-letter alphabet

The alphabet consists of two pairs of complementary letters, e.g., A, U and C, G. In this case we observe that for all lengths up to \( L = 90 \) the undesignability \( U \) is essentially zero—i.e., so far we have not found any random structure that is undesignable. This means that four letters are sufficient, at least for moderate system lengths, to design all possible structures maybe needed in cell processes. Nevertheless, as shown in Sec. [IVD] structures exist, that are undesignable even in the four-letter case, but such structures must be quite rare for lengths up to \( L = 90 \). This means that in the limit of infinite RNA lengths, which is only of abstract academic interest, almost all random structures become undesignable, because the probability that somewhere in the infinite sequence there is an undesignable subsequence of finite length is one, as explained in the next section. Since, as already pointed out above, naturally occurring RNA have to be only of rather restricted length to perform their functions, this effect has no influence and a four-letter alphabet seems to be sufficient.

In Fig. [9] we show the average “time” \( T \) to find a solution as a function of \( p \), but here we used a combined deterministic-randomized algorithm, which is quite fast for low pairing probabilities—i.e., \( p < 0.4 \)—where on the average less than \( L \) ground-state calculations are necessary to find a solution. On the other hand for values \( p \approx 0.6 \) the design time \( T \) seems to grow faster than exponentially in the sequence length \( L \). This strong increase of the running time is not accompa-

FIG. 7: For the three-letter alphabet the design time \( T \) for designable structures is shown as a function of the pairing probability \( p \). The positions of the maxima are at similar positions as the corresponding maxima in Fig. [6]. Missing error bars are of the size of the symbols or smaller, and omitted for legibility. (Parameter used: \( E_p = -2, \ E_s = -1, \ h_{\text{min}} = 2, \ 1000 \text{ samples.} \))
FIG. 9: For the four-letter alphabet the design time \( T \) for designable structures is shown as a function of the pairing probability \( p \). The positions of the maxima are at similar positions as the corresponding maxima in Fig. 7. Missing error bars are of the size of the symbols or smaller, and omitted for legibility. (Parameter used: \( E_p = -2 \), \( E_s = -1 \), \( h_{\min} = 2 \), 1000 samples.)

This implies that structures \( S \) which contain a subchain \( C \) of length \( l \geq 16 \) are also designable. In the limit \( L \to \infty \) with pair probability \( p > 0 \) we expect that almost all random structures contain a subchain of size \( l \geq 16 \), thus making this structures designable. However, for native RNA this limit is not relevant: For an ensemble of 10,000 random structures of length \( L = 1024 \) and pair probability \( p = 0.7 \) we looked for each structure the subchain of the longest length \( l \) and found none longer than 11. Assuming that all designable structures in the four-letter case are designable because they contain a subchain longer than \( l = 15 \), such structures are very rare even for biological lengths.

Finally, we shortly want to mention the five-letter case: two pairs of two complementary bases (A-U, C-G) and an unpairable fifth letter (e.g., X). In this case it is easy to see that even structures as explained in Fig. 10 are designable: Start with a sequence of type ACUGACUGACUGACUG ACUGACUGACUGACUG... replace the bases at positions 2,5,8,11,14... with \( h_{\min} - 1 \) letters of type X, e.g., yielding in the case \( h_{\min} = 2 \): AXUGCUXACGXGAXUG... First, in this sequence stacked-pairs are impossible, because for non pair \( r_i r_{i+1} \) there is a required complementary pair \( r_{i+1} r_i \). Further, this sequence is compatible to the structure and there are exactly as many complementary bases pairs as there are pairs in the structure. Of course, this does not prove that with five letters all structures are designable, but designable structures are at least expected to be even much less frequently than in the four-letter case.

V. SUMMARY

We numerically investigated the RNA secondary structure design problem for different alphabet sizes. We used a deterministic branch-and-bound algorithm to get definite answers, whether a given structure is designable or not. Due to efficiency reasons in the designable cases, we combined this al-
algorithm with an acceptable one, gaining significantly performance improvements in the four-letter case.

We examined the designability for an ensemble of random structures as a function of the probability that a base of sequence is paired. Our findings for the two-letter case are that it is almost impossible to design most of the structures. In the three-letter case already for small sequence sizes ($L \approx 90$) about 10% of the structures are undesignable for biological relevant pairing probabilities, leading to the conclusion that for biological sequence sizes ($L \approx 1000$) again most structures are undesignable.

Interestingly, this changes when going to the (natural) four-letter alphabet: within our studies we have not found a single random structure that we could prove to be undesignable. Although, there are structures that are undesignable, they occur with very low frequencies.

We further studied the computational time required to design a structure. Although, this for sure depends strongly on the algorithm, we found in three-letter case that required time is maximal in the regime where the undesignability is largest. In the four-letter case the design times look similar to that of the three-letter case: again we observed a maximum of the design times in for $p \approx 0.6$, close to the region where naturally occurring RNA can be found. Although, (almost) all structures are designable, it is sometimes difficult to design them.

**Acknowledgments**

The authors have obtained financial support from the Volkswagenstiftung (Germany) within the program “Nachwuchsgruppen an Universitäten”. The simulations were performed at the Paderborn Center for Parallel Computing in Germany and on a workstation cluster at the Institut für Theoretische Physik, Universität Göttingen, Germany. We thank M. Jungsbluth for helpful remarks.

[1] R. F. Gesteland, T. R. Cech, and J. F. Atkins, eds., The RNA World (Cold Spring Harbor Laboratory Press, New York, 1999), 2nd ed.
[2] P. G. Higgs, Quarterly Reviews of Biophysics 33, 199 (2000).
[3] H. F. Noller, Annual Review of Biochemistry 53, 119 (1984).
[4] R. Green and H. F. Noller, Annual Review of Biochemistry 66, 679 (1997).
[5] S. H. Kim, F. L. Suddath, G. J. Quigley, A. McPherson, J. L. Susman, A. M. J. Wang, N. C. Seeman, and A. Rich, Science 285, 435 (1974).
[6] K. Kruger, P. J. Grabowski, A. J. Zaug, J. Sands, D. E. Gotschling, and T. R. Cech, Cell 31, 147 (1982).
[7] J. Schmidt-Brauns, Acta virologica 47, 65 (2003), URL www.aepress.sk/acta/acta02_2003.htm
[8] R. Bundschuh and T. Hwa, Phys. Rev. Lett. 83, 1479 (1999).
[9] M. Zuker, Science 244, 48 (1989).
[10] J. S. McCaskill, Biopolymers 29, 1105 (1990).
[11] I. L. Hofacker, W. Fontana, P. F. Studler, L. S. Bonhoeffer, M. Tacker, and P. Schuster, Monatsh. Chemie 125, 167 (1994).
[12] R. Lyngsø, M. Zuker, and C. N. S. Pedersen, Bioinformatics 15, 440 (1999).
[13] T. Liu and R. Bundschuh, Phys. Rev. E 69, 61912 (pages 10) (2004), URL http://link.aps.org/abstract/PRE/v69/e061912
[14] P. G. Higgs, Phys. Rev. Lett. 76, 704 (1996).
[15] R. Bundschuh and T. Hwa, Phys. Rev. E 65, 31903 (2002), URL link.aps.org/abstract/PRE/v65/e031903
[16] E. Marinari, A. Pagnani, and F. Ricci-Tersenghi, Phys. Rev. E 65, 041919 (pages 7) (2002), URL http://link.aps.org/abstract/PRE/v65/e041919
[17] A. Pagnani, G. Parisi, and F. Ricci-Tersenghi, Phys. Rev. Lett. 84, 2026 (2000).
[18] R. Mukhopadhyay, E. Emberly, C. Tang, and N. S. Wingreen, Phys. Rev. E 68, 41904 (2003).
[19] M. Andronescu, A. P. Fejes, F. Hutter, H. H. Hoos, and A. Condon, J. Mol. Biol. 336, 607 (2004), URL http://www.sciencedirect.com/science/article/B6NK7-4B9D3/2/0af6bce0c5c933f700d0f38f730a904f
[20] I. Tinoco, Jr and C. Bustamante, J. Mol. Biol. 293, 271 (1999).
[21] M. E. Burkard, D. H. Turner, and J. Tinoco, Ignacio, The RNA World, chap. The Interactions That Shape RNA Structure, pp. 233–264, in [1] (1999), 2nd ed.
[22] P. G. Higgs, Journal de Physique I 3, 43 (1993), URL www.edpsciences.org/articles/jpI/article全文�的第一节 jika.png
[23] B. Burghardt and A. K. Hartmann, Phys. Rev. E 71, 021913 (pages 9) (2005), URL http://link.aps.org/abstract/PRE/v71/e021913
[24] P. Schuster, W. Fontana, P. F. Studler, and I. L. Hofacker, Proc. R. Soc. Lond. B. 285, 279 (1994), URL www.tbi.univie.ac.at/ivo/pub/shap-rsb.pdf
[25] B. Korte and J. Vygen, Combinatorial Optimization, vol. 21 of Algorithms and Combinatorics (Springer, Berlin, 2002), 2nd ed.
[26] J. J. Gillespie, M. J. Yoder, and R. A. Wharton, J. Mol. Evol. 61, 114 (2005), URL http://www.springerlink.com/openurl.asp?genre=article
[27] I. L. Hofacker, P. Schuster, and P. F. Studler, Discrete Applied Mathematics 88, 207 (1998), URL http://www.sciencedirect.com/science/article/B6TYW-0E9/2/2935f9a392f8995f77b600007f020070
[28] A. Hartmann and M. Weigt, Phase Transitions in Combinatorial Optimization Problems (Wiley-VCH, Berlin, 2005).
[29] M. R. Garey and D. S. Johnson, Computers and Intractability (W. H. Freeman and Company, San Francisco, 1979).
[30] D. Mitchell, B. Selman, and H. Levesque, in Proceedings of the 10th National Conference on Artificial Intelligence (AAAI’92) (AAAI Press/MIT Press, Menlo Park, California, 1992), pp. 440–446.
[31] M. Weigt and A. K. Hartmann, Phys. Rev. Lett. 84, 6118 (2000), URL link.aps.org/abstract/PRL/v84/p6118

[32] Surprisingly, we found that the insertion in plain order from 1 to $L$ is better than many other complicated strategies.