The Journal of
Organic
Chemistry

VOLUME 59, NUMBER 26 DECEMBER 30, 1994

Communications

Improved Solid-Phase Synthesis of Long Oligoribonucleotides: Application to tRNA^{phe} and tRNA^{gly}

Jay T. Goodwin,† Wendy A. Stanick,‡ and Gary D. Glick*,†

Department of Chemistry, University of Michigan, Ann Arbor, Michigan 48109-1055, PerSeptive Biosystems Corporation, Cambridge, Massachusetts 02139

Received September 22, 1994®

Summary: We present here the solid-phase synthesis of unmodified phenylalanine transfer RNA (tRNA^{phe}) from yeast, and glycine transfer RNA (tRNA^{gly}) from *Escherichia coli*, using commercially available RNA reagents and standard coupling protocols. Aminoacylation experiments demonstrate that the acceptor activity of the chemically-synthesized tRNA^{phe} is comparable to the activity of the tRNA^{phe} transcript, relative to yeast tRNA^{phe}.

The diverse biological roles of RNA, coupled with the recent discoveries of oligonucleotide sequences that act catalytically,[1] or as protein ligands, enzyme inhibitors,[2] and small-molecule receptors,[3] have driven efforts to improve methods for oligoribonucleotide synthesis. While transcription from DNA templates can yield relatively large amounts of RNA (as much as 3 mg per mL of transcription reaction)[4a] it does not readily allow for the site-specific incorporation of modified nucleosides,[5] which are becoming increasingly important to studies of RNA structure and function.[5-8] Solid-phase chemical synthesis is an attractive alternative to transcription technology. Due to the steric hindrance presented by 2′-hydroxyl protecting groups and the pendant functionality on the phosphorus, stepwise coupling efficiencies are typically ≤98%, thereby limiting the utility of this method to the preparation of relatively short sequences (e.g., ~30-mers).[9] Indeed, synthesis of longer oligoribonucleotides like transfer RNA requires the use of highly reactive RNA monomers, such as ribonucleoside β-cyanoethyl diethyl phosphoramidites.[10a-c] Because these reagents are neither as stable as the corresponding diisopropylphosphoramidite, nor commercially available, they are less attractive for routine, high-volume use.[10a-c]

We have re-evaluated the solid-phase synthesis of longer RNA sequences using nucleoside β-cyanoethyl diisopropyl phosphoramidites. Contrary to previous results[10b] we find that longer oligoribonucleotides can in fact be synthesized in significant quantities with these reagents. We present here a total solid-phase synthesis

* Address correspondence to this author.
† University of Michigan.
‡ PerSeptive Biosystems Corp.
® Abstract published in *Advance ACS Abstracts*, November 15, 1994.

(1) For examples, see: (a) Uhlenbeck, O. C.; Long, D. M. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 6977–6981. (b) Westhof, E.; Altman, S. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 5133–5137. (c) Herschlag, D.; Eckstein, F. Cech, T. R. *Biochemistry* **1993**, *32*, 8299–8311. For a review, see: (d) von Ahsen, U.; Schroeder, R. *BioEssays* **1993**, *15*, 299–307.

(2) (a) Bock, L. C.; Griffin, L. C.; Latham, J. A.; Vermaas, E. H.; Toole, J. J. *Nature* **1992**, *355*, 564–566. (b) Tsai, D. E.; Kenan, D. J.; Keene, J. D. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 8864–8868. (c) Tuerk, C.; Gold, L. *Science* **1990**, *249*, 505–510.

(3) (a) Burgstaller, P.; Famulok, M. *Angew. Chem., Int. Ed. Engl.* **1994**, *33*(10), 1084–1087. (b) Prudent, J. R.; Uno, T.; Schultz, P. G. *Science* **1994**, *264*, 1924–1927. (c) Famulok, M.; Szostak, J. W. *J. Am. Chem. Soc.* **1992**, *114*, 3990–3991. (d) Ellington, A. D.; Szostak, J. W. *Nature* **1990**, *346*, 818–822. For reviews, see: (e) Famulok, M.; Szostak, J. W. *Angew. Chem., Int. Ed. Engl.* **1992**, *31*, 979–988. (f) Szostak, J. W. *Trends Biochem. Sci.* **1992**, *17*, 89–93.

(4) (a) Hoffman, L. M.; Johnson, M. G. *BioTechniques* **1994**, *17*, 372–375. (b) Arnez, J. G.; Steitz, T. A. *Biochemistry* **1994**, *33*, 7560–7567. (c) Sampson, J. R.; DiRenzo, A. B.; Behlen, L. S.; Uhlenbeck, O. C. *Biochemistry* **1990**, *29*, 2523–2532. (d) Sampson, J. R.; Uhlenbeck, O. C. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 1033–1037.

(5) Combined chemical synthesis and enzymatic ligation have recently been utilized to incorporate a modified nucleoside into tRNA. (a) Han, H.; Dervan, P. B. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 4955–4959. See also: (b) Dontsova, O.; Tishkov, V.; Dokudovskaya, S.; Bogdanov, A.; Döring, T.; Rinke-Appel, J.; Thamm, S.; Greuer, B.; Brimacombe, R. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 4125–4129. (c) Usman, N.; Cedergren, K. *Trends Biochem. Sci.* **1992**, *17*, 334–339.

0022-3263/94/1959-7941$04.50/0

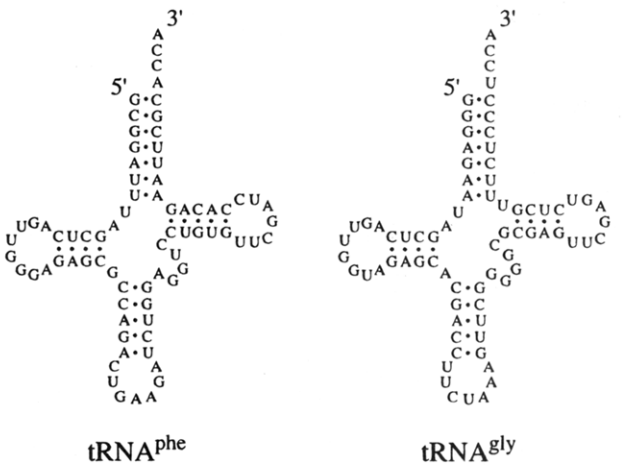

Figure 1. Sequences and secondary structures of unmodified yeast phenylalanine and *Escherichia coli* glycine transfer RNA ($tRNA^{phe}$ and $tRNA^{gly}$, respectively).[12]

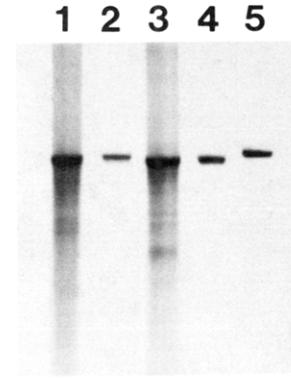

Figure 2. Analytical denaturing PAGE gel of tRNAs.[16] The RNA was electrophoresed on a 15% gel run in Tris–borate–EDTA buffer (pH 8.3) at 250 V and visualized by Stains-All staining. Lane 1: crude fully deprotected $tRNA^{phe}$. Lane 2: purified $tRNA^{phe}$. Lane 3: crude fully deprotected $tRNA^{gly}$. Lane 4: purified $tRNA^{gly}$. Lane 5: $tRNA^{phe}$ from yeast (Sigma).

of the unmodified 76-base-long yeast phenylalanine transfer RNA ($tRNA^{phe}$), along with a synthesis of the 76-base-long glycine transfer RNA ($tRNA^{gly}$) sequence from *Escherichia coli* (Figure 1),[11,12] using commercially available RNA reagents and standard coupling protocols employed in automated nucleic acid synthesis techniques.

Solid-phase synthesis was performed using an Expedite 8909 nucleic acid synthesis system on a 1-μmol scale with β-cyanoethyl diisopropyl RNA phosphoramidites.[9,13,14] Long-chain amino-derivitized controlled-pore glass (CPG; 1000 Å pore size) with protected[13] adenosine attached to the resin via a succinate linker was used for each tRNA synthesis. Average stepwise coupling efficiencies, as determined from the collected trityl cation fractions, were ≥98.0% (corresponding to an overall yield of 22%).[15] Each fully detritylated sequence was treated with anhydrous ethanolic ammonia at 55 °C for 18 h which simultaneously cleaves the oligomer from the support and removes the base- and phosphate-protecting groups. After the RNA solution was decanted from the CPG and the solvent was removed *in vacuo*, the 2′-*O*-silyl protecting groups were cleaved with tetra-*n*-butylammonium fluoride (1 M in THF) at 25 °C. The desilylation reaction was quenched with aqueous sodium acetate (pH 5.5) after 24 h, and the crude RNA (100 OD_{260} units) was isolated by ethanol precipitation.[16–18] Full-length product was separated from failed sequences by denaturing polyacrylamide gel electrophoresis (PAGE) (gel dimensions: 31.0 cm × 38.5 cm × 0.75 mm). The product-containing band was visualized by UV shadowing, and the RNA was electroeluted from the excised gel slice yielding 12 OD_{260} units (~0.5 mg) of pure $tRNA^{phe}$.[16] Purity of the isolated full-length product was >95% as judged by reversed-phase HPLC, denaturing PAGE, and nucleoside composition analysis by enzymatic degradation (Figures 2 and 3).[16,19]

UV thermal denaturation experiments and amino acid acceptor activity were used to verify proper folding and biological activity of the chemically synthesized $tRNA^{phe}$.[4c-d] The melting profile of the chemically synthesized material (T_m = 68 °C) is similar to that observed for the enzymatic transcript.[16,20ab,21] In addition, using a crude synthetase preparation from rabbit reticulocyte lysate, we find that the acceptor activity of the chemically synthesized $tRNA^{phe}$ for [^{14}C]-labeled L-phenylalanine is 80% of that for yeast $tRNA^{phe}$.[16,22–24] Our results compare

(6) (a) Goodwin, J. T.; Glick, G. D. *Tetrahedron Lett.* **1994**, *35*, 1647–1650. (b) Heidenreich, O.; Benseler, F.; Fahrenholz, A.; Eckstein, F. *J. Biol. Chem.* **1994**, *269*, 2131–2138. (c) Fu, D.-J.; Rajur, S. B.; McLaughlin, L. W. *Biochemistry* **1993**, *32*, 10629–10637. (d) Khare, D.; Orban, J. *Nucleic Acids Res.* **1992**, *20*, 5131–5136. See also: (e) Benseler, F.; Fu, D.-J.; Ludwig, J.; McLaughlin, L. W. *J. Am. Chem. Soc.* **1993**, *115*, 8483–8484. (f) Ma, M. Y.-X.; McCallum, K.; Climie, S. C.; Kuperman, R.; Lin, W. C.; Sumner-Smith, M.; Barnett, R. W. *Nucleic Acids Res.* **1993**, *21*, 2585–2589. (g) Cload, S. T.; Schepartz, A. *J. Am. Chem. Soc.* **1991**, *113*, 6324–6326.

(7) For reviews, see: (a) Beaucage, S. L.; Iyer, R. P. *Tetrahedron* **1993**, *49*, 1925–1963. (b) Englisch, U.; Gauss, D. H. *Angew. Chem., Int. Ed. Engl.* **1991**, *30*, 613–629.

(8) For examples of investigations into RNA structure and function, see: (a) Zarrinkar, P. P.; Williamson, J. R. *Science* **1994**, *265*, 918–924. (b) Laggerbauer, B.; Murphy, F. L.; Cech, T. R. *EMBO J.* **1994**, *13*, 2669–2676. (c) Puglisi, J. D.; Chen, L.; Frankel, A. D.; Williamson, J. R. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 3680–3684. (d) Chen, Y.; Sierzputowska-Gracz, H.; Guenther, R.; Everett, K.; Agris, P. F. *Biochemistry* **1993**, *32*, 10249–10253. (e) LeCuyer, K. A.; Crothers, D. M. *Biochemistry* **1993**, *32*, 5301–5311. For recent reviews, see: (f) Draper, D. E. *Acc. Chem. Res.* **1992**, *25*, 201–207. (g) Puglisi, J. D.; Wyatt, J. R.; Tinoco, I., Jr. *Acc. Chem. Res.* **1991**, *24*, 152–158.

(9) Scaringe, S. A.; Francklyn, C.; Usman, N. *Nucleic Acids Res.* **1990**, *18*, 5433–5441.

(10) (a) Gasparutto, D.; Livache, T.; Bazin, H.; Duplaa, A.-M.; Guy, A.; Khorlin, A.; Molko, D.; Roget, A.; Téoule, R. *Nucleic Acids Res.* **1992**, *20*, 5159–5166. (b) Lyttle, M. H.; Wright, P. B.; Sinha, N. D.; Bain, J. D.; Chamberlin, A. R. *J. Org. Chem.* **1991**, *56*, 4608–4615. (c) Ogilvie, K. K.; Usman, N.; Nicoghosian, K.; Cedergren, R. J. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 5764–5768. For a total synthesis of yeast alanine tRNA through combined chemical and enzymatic methods, see: (d) Yu, W. *Acc. Chem. Res.* **1984**, *17*, 393–397.

(11) For reviews of tRNA structure and function, see: (a) *Transfer RNA: Structure, Properties and Recognition*, Schimmel, P. R., Söll, D., Abelson, J. N., Eds.; Cold Spring Harbor Laboratory: Cold Spring Harbor, 1979. (b) *Transfer RNA*; Altman, S., Ed.; MIT Press: Cambridge, MA, 1978.

(12) Because none of the post-transcriptionally modified bases found in native yeast $tRNA^{phe}$ or *Escherichia coli* $tRNA^{gly}$ are incorporated during these syntheses the resulting products are termed *unmodified*.[4d]

(13) *N*-Benzoyladenosine, *N*-benzoylcytidine, *N*-isobutyrylguanosine; the 2′-OH of each nucleoside is protected as a *tert*-butyldimethylsilyl ether.

(14) *Expedite Nucleic Acid Synthesis Operator's Manual*; Millipore Corp.: Milford, MA, 1993.

(15) Brown, T.; Brown, D. J. S. In *Oligonucleotides and Analogues. A Practical Approach;* Eckstein, F., Ed.; IRL Press: New York, 1991; Chapter 1, pp 13–14.

(16) See supplementary material.

(17) The extinction coefficient for single-stranded, unmodified $tRNA^{phe}$ at 25 °C, 7.75 × 10^5 L/mol•cm, was calculated according to: Puglisi, J. D.; Tinoco, I., Jr. *Methods Enzymol.* **1989**, *180*, 304–325.

(18) Hall, K. B.; McLaughlin, L. W. *Nucleic Acids Res.* **1992**, *20*, 1883–1889.

(19) Gehrke, C. W.; Kuo, K. C. *J. Chromatogr.* **1989**, *471*, 3–36.

(20) (a) Hall, K. B.; Sampson, J. R. *Nucleic Acids Res.* **1990**, *18*, 7041–7047. (b) Hall, K. B.; Sampson, J. R.; Uhlenbeck, O. C.; Redfield, A. G. *Biochemistry* **1989**, *28*, 5794–5801.

(21) The melting temperature for the transcript is not explicitly given in ref 20a,b.

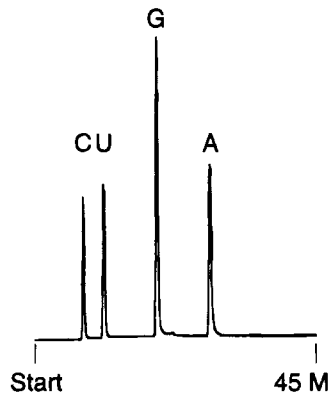

Figure 3. Nucleoside composition analysis of $tRNA^{phe}$. The synthetic tRNA (0.8 OD_{260} units) was treated with nuclease P1 (5 units, pH 5.5, 37 °C, 18 h), followed by calf intestine alkaline phosphatase (240 units, pH 8.2, 37 °C, 6 h), and the nucleosides were resolved by reversed-phase HPLC on a Phenomenex C18 column (0.1 M triethylammonium acetate, pH 6.6, with CH_3CN gradient, monitored at 256 nm). Values obtained by integration of peak areas, normalized against the extinction coefficients of the respective nucleosides, agree with the theoretical values to within <1.9%. Nucleoside composition for $tRNA^{gly}$ was also verified (to within <2.7%) by enzymatic degradation (data not shown).

favorably with those of Uhlenbeck, who showed (using purified yeast phenylalanyl-tRNA synthetase) that the enzymatically transcribed $tRNA^{phe}$ charges to 90% of the activity observed for yeast $tRNA^{phe}$.[4c,d,23] Taken together, these results indicate that our chemically synthesized $tRNA^{phe}$ folds properly and that the chemically and enzymatically synthesized sequences are physically and, *in vitro*, biologically comparable.

Compared with previous efforts to chemically synthesize tRNA molecules we have achieved higher yields of pure full-length product, e.g., 12 OD_{260} units in this work versus ≤2 OD_{260} units elsewhere.[25] These higher yields may in part be attributed to greater consistency in the purity of currently commercially available reagents.[26] The only other previously reported attempt to synthesize a tRNA sequence using RNA β-cyanoethyl diisopropylphosphoramidites, $tRNA^{gly}$ from *Escherichia coli*, was not successful, as noted by both the absence of observable trityl cation intensities in the latter part of the synthesis, and the fact that full-length product was not detected by PAGE.[10b] By contrast, loss in trityl cation intensities was not observed during our synthesis of $tRNA^{gly}$ using these same RNA monomers, and as shown in Figure 2 pure full-length material (4 OD_{260}) was isolated.[27] These results contradict a general perception that longer oligoribonucleotides are not accessible through solid-phase synthesis technology using β-cyanoethyl diisopropylphosphoramidites.[29]

In conclusion, we find that tRNA molecules can readily be synthesized using standard β-cyanoethyl diisopropyl phosphoramidite chemistry in sufficient amounts for biophysical and biochemical analysis. In fact, the combination of several syntheses should yield enough RNA for either NMR or crystallographic studies.[11a,b,20a,b,30] The ability to routinely synthesize RNA sequences in excess of 70 bases in length through chemical means should facilitate a wider range of research into the structure and function of RNA.[31]

Acknowledgment. This work was supported in part by grants from the NIH and NSF. We thank P. L. Toogood and B. V. SirDeshpande for assistance with the aminoacylation assays and D. Mack for use of the ABI 394 synthesizer. J.T.G. was supported by an NIH postdoctoral fellowship. We thank N. Usman for helpful discussions regarding this manuscript.

Supplementary Material Available: Experimental procedures, UV denaturation curve, and HPLC traces of purified tRNA (6 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

(22) PAGE analysis indicates that 85% of the yeast $tRNA^{phe}$ is full-length material, which establishes a lower boundary of 80% for the relative activity of the unmodified sequence.

(23) The acceptor activities for both yeast and unmodified $tRNA^{phe}$ in our assay (690 and 650 pmol/OD_{260} unit ± 10%, respectively) are in close agreement with the plateau values previously observed for yeast $tRNA^{phe}$ assayed with crude rabbit reticulocyte lysate.[24] By comparison, yeast $tRNA^{phe}$ charges to ~1600–1700 pmol/OD_{260} unit with purified yeast phenyalanyl-tRNA synthetase.[4c-d]

(24) Gross, M.; Starn, T. K.; Rundquist, C.; Crow, P.; White, J.; Olin, A.; Wagner, T. *J. Biol. Chem.* **1992**, *267*, 2073–2079. Toogood, P. L. Personal communication.

(25) Purified yields were not reported; estimates were based on the crude yields presented in ref 10b,c.

(26) Identical results have been obtained for syntheses on two separate Expedite instruments (at the University of Michigan and PerSeptive Biosystems), with similar purified yields (11 OD_{260} units) obtained as well from an Applied Biosystems 394 DNA/RNA synthesizer utilizing Millipore reagents, indicating that high-yield RNA synthesis is not unique to one instrument.

(27) The lower yield for this sequence relative to that for $tRNA^{phe}$ may be due to the reduced coupling efficiencies encountered in synthesizing a stretch of five consecutive guanosines in the middle of this sequence.[28]

(28) Murphy, M.; Rieger, M.; Jayaraman, K. *BioTechniques* **1993**, *15*, 1004–1010.

(29) Gait, M. J.; Pritchard, C.; Slim, G. In *Oligonucleotides and Analogues. A Practical Approach;* Eckstein, F., Ed.; IRL Press: New York, 1991; Chapter 2, p 47.

(30) Preliminary results suggest that modifying the synthesis protocol by incorporating CH_2Cl_2 washes prior and subsequent to the detritylation step may also improve coupling efficiencies.[28]

(31) We have recently incorporated a disulfide cross-link site-specifically within the unmodified yeast $tRNA^{phe}$ sequence, results of which will be reported shortly.