Synthese von Trinukleotidsynthons an fester Phase und löslichem Träger

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Inhaltsverzeichnis

Abkürzungsverzeichnis

Ac	Acetyl
ACN	Acetonitril
Äq.	Äquivalente
AS	Aminosäuren
Bz	Benzoyl
CPG	*controlled pore glass*
CV	*column volume*
dA	Desoxyadenosin
dC	Desoxycytidin
dG	Desoxyguanosin
DC	Dünnschichtchromatographie
DCE	Dichlorethan
DCM	Dichlormethan
DEPT	*distortionless enhancement by polarization transfer*
DMAc	Dimethylacetamid
DMF	Dimethylformamid
DMT	Dimethoxytrityl
DMSO	Dimethylsulfoxid
DNA	*deoxyribonucleic acid*
DTT	Dithiothreitol
DQF-COSY	*double quantum filtered correlation spectroscopy*
HEPES	2-(4-(2-Hydroxyethyl)-1-piperazinyl)-ethansulfonsäure
HSQC	*heteronuclear single quantum coherence*
Ibu	Isobutyryl
LB	*lysogeny broth*
MeOH	Methanol
MTM	Methylthiomethyl
MS	Massenspektrometrie
NMR	*nuclear magnetic resonance*
PCR	*polymerase chain reaction*
PEG	Polyethylenglycol
PG	*protecting group*

PS	Polystyrol
RP-HPLC	*reversed phase high performance liquid chromatography*
RT	Raumtemperatur
T	Thymidin
TBDMS	*tert*-Butyldimethylsilyl
TCEP	Tris(2-carboxyethyl)phosphin
TEA	Triethylamin
TEAAc	Triethylammoniumacetat
TSTU	*N,N,N′,N′*-Tetramethyl-*O*-(*N*-succinimidyl)uroniumtetrafluorborat
ü.N.	über Nacht
UV	Ultraviolettes Licht

1. Zusammenfassung der kumulativen Dissertation

1.1 Einleitung

Seit es 1979 Khorana et al. erstmals gelang, ein Gen synthetisch herzustellen, hat sich die Oligonukleotidsynthese rasch entwickelt.[1] Von den Anfängen der Oligonukleotidsynthese mittels Phosphordiesterchemie fand die Entwicklung über die Phosphortriestermethode und Phosphittriesterchemie bis zur heutigen, standardisierten Phosphoramiditmethode statt.[2-6] Die Festphasensynthese stammt ursprünglich aus der Peptidchemie.[7] Diese Verbindung mit der Phosphoramiditmethode, wie sie in Abbildung 1 gezeigt ist, ermöglicht die Oligonukleotidsynthese von bis zu 200 Nukleotiden und kann nicht nur mit geringem Zeitaufwand, sondern durch Entwicklung des Oligonukleotid-Synthesizers auch automatisiert stattfinden.[8-10]

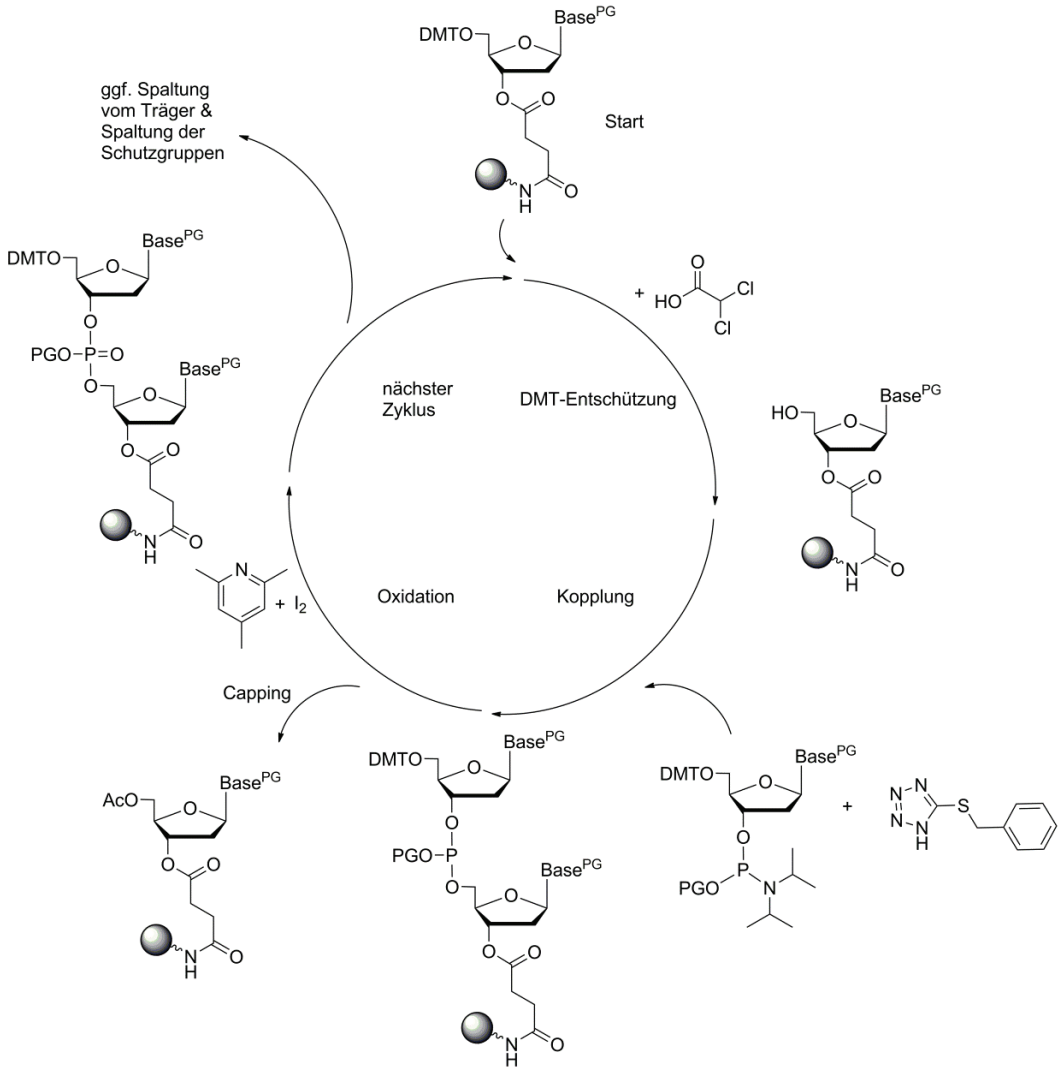

Abbildung 1: Schema der DNA-Synthese mittels Oligonukleotid-Synthesizer, PG = *protecting group*.

Eine Anwendung der automatisierten Oligonukleotidsynthese ist – im Vergleich zur Synthese festgelegter Sequenzen – der gezielte Einbau von Mutationen bzw. randomisierter Oligonukleotidabschnitte zur Generierung von Oligonukleotidbibliotheken, welche zur Erstellung von Genbibliotheken genutzt werden können.[11,12] In diesem Zusammenhang bezeichnet eine Genbibliothek eine Sammlung von Varianten genetischen Materials, welche sich durch Mutationen in einem definierten Bereich unterscheiden.[13] Diese Anwendung ist vorwiegend für den Bereich des *Protein Engineerings* von Relevanz. Hierbei steht die Generierung von Proteinen mit neuen, für die Medizin bzw. Industrie relevanten Eigenschaften im Mittelpunkt.[14-17]

Die *in vivo* vorkommenden Enzyme besitzen eine hohe Selektivität und Effizienz für ihre natürlichen Substrate. Jedoch sind die Mengen sowie die Beschränkung der Katalyse auf natürliche Substrate für eine industrielle Anwendung meist nicht ausreichend.[18] Zur Optimierung natürlich vorkommender Proteine zu Proteinen mit definierten Eigenschaften gibt es zwei grundlegende Strategien: Das rationale Proteindesign und die gerichtete Evolution.[15] Beim rationalen Proteindesign werden gezielte Mutationen nach *in silico*-Berechnungen eingeführt, welche zu den vorteilhaften Eigenschaften des Proteins führen sollen. Die gerichtete Evolution hingegen beruht auf der möglichst randomisierten Einführung von Mutationen und dem Screening der daraufhin erhaltenen Genbibliothek nach erwünschten Proteinvarianten.[19]

Die Strategie des rationalen Proteindesigns weist eine Vielzahl von Limitationen und Nachteilen auf. So benötigt das rationale Design eine Vielzahl an Informationen über Struktur und Mechanismus des Proteins, um vorhersagen zu können, welche Mutation die gewünschte Veränderung der Katalyse bewirkt. Insbesondere wenn diese Informationen nicht gegeben sind oder wenn Lösungsmittelstabilität, Temperaturstabilität, Enantioselektivität, Chemoselektivität oder Substratspezifität eines Proteins optimiert werden sollen, ist die gerichtete Evolution bis heute das Mittel der Wahl.[20] Genbibliotheken, welche das Kernstück der gerichteten Evolution darstellen, können auf unterschiedlichste Weise erzeugt werden. Übliche Beispiele hierfür sind PCR-basierte Methoden wie die *error-prone* PCR oder rekombinierende Methoden wie das DNA *Shuffling*.[21,22] Jedoch führt dies nicht zwangsläufig zu vollständig randomisierten Genbibliotheken, da es beispielsweise durch die verwendete Polymerase bei der *error-prone* PCR zu Selektionsverzerrung kommen kann.[23] Zusätzlich sollte die Genbibliothek nicht zu groß werden, damit das anschließende Screening

handhabbar bleibt.[24] Weitere Schwierigkeiten der Genbibliotheken liegen in der Natur des degenerierten genetischen Codes, welcher Aminosäuren (AS), die von mehreren Triplets codiert werden, gegenüber jenen bevorzugt, die nur durch einen Triplettcode erfasst werden.[23] Der zufällige Einbau von Stoppcodons ist für die Erstellung von Genbibliotheken ebenfalls problematisch, da dies die Synthese von Abbruchfragmenten anstelle von funktionsfähigen Proteinen zur Folge hat.[25]

Bei den Herausforderungen, die sich bei der Erstellung von Genbibliotheken ergeben, zeigt die Verwendung von Trinukleotiden im Rahmen der Oligonukleotidsynthese großes Potential (Abb. 2). Die Verwendung von Trinukleotiden (welche den Codons der 20 kanonischen Aminosäuren entsprechen) zur oligonukleotidbasierten Generierung einer Genbibliothek führt zu deren Optimierung und erlaubt vollständige Kontrolle über die Randomisierung ohne den Einbau von Stoppcodons (Abb. 2).[26,27] Weiterhin ist es möglich – je nach Anwendung – statt einer Trinukleotidmischung, die für alle 20 Aminosäuren codiert, nur jene Trinukleotide einzusetzen, welche die basischen, sauren oder hydrophoben Aminosäuren berücksichtigen. Ein zusätzlicher Vorteil ist die Möglichkeit, die Sequenzen codon-optimiert zu erhalten, sodass die *codon usage* je nach Zielorganismus berücksichtigt werden kann.

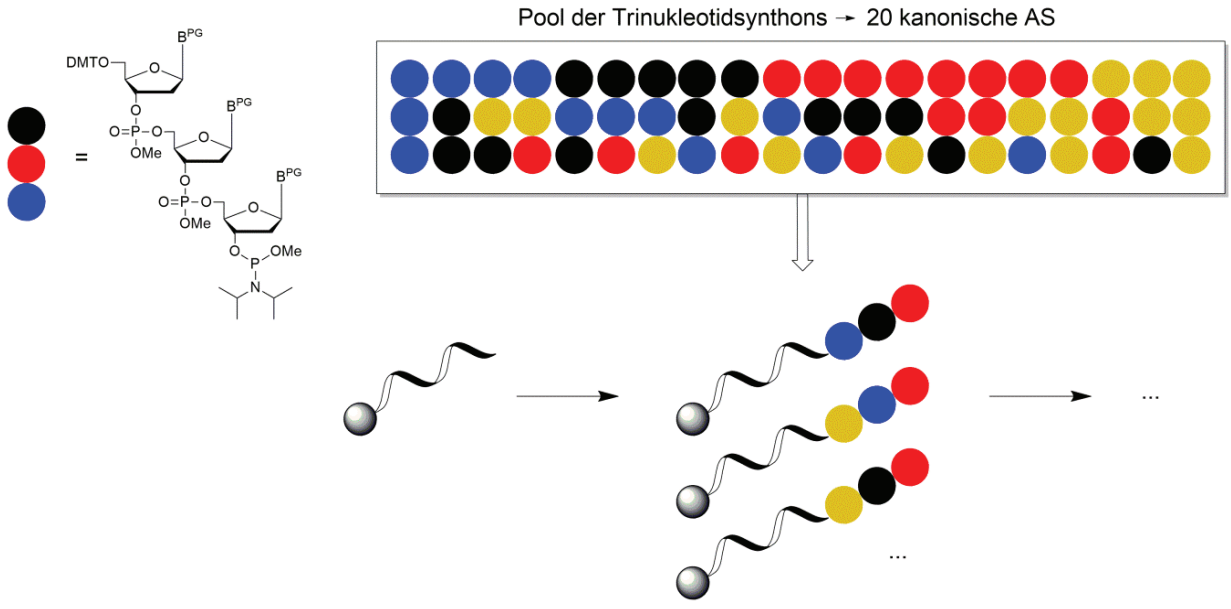

Abbildung 2: Erstellung einer randomisierten Oligonukleotidbibliothek mittels Trinukleotidphorsphoramidten als Synthons, blau ≙ dA, schwarz ≙ dC, gelb ≙ T, rot ≙ dG (Abbildung adaptiert nach Publikation 2.3).

Eine weitere Verwendung für Trinukleotidblöcke (bzw. auch Di-, Tetra-, oder Pentanukleotidblöcke) liegt in der Nutzung dieser als sogenannte Blockmere und der daraus

resultierenden Möglichkeit einer effizienteren Oligonukleotidsynthese. Die bekannten Oligonukleotidsequenzen können in eine Abfolge von Di- bis Pentameren unterteilt werden, welche anstelle der Monomere am Oligonukleotid-Synthesizer eingebaut werden. Auf diese Weise werden einzelne Kopplungsschritte, welche auch immer mit Verlusten einhergehen, eingespart. Insbesondere wenn sich Blockmersequenzen wiederholen, kann diese Strategie lohnend sein. In diesem Fall können einmal in größerem Umfang synthetisierte Blockmere mehrfach in einer Sequenz eingesetzt werden, sodass weniger verschiedene Blockmere synthetisiert werden müssen und der Syntheseaufwand entsprechend geringer wird. Weiterhin wird die Aufreinigung der Oligonukleotidsequenz erleichtert, da Abbruchfragmente mit n-1/n-2 fehlen.

Über diese gesteigerte Effizienz in der Oligonukleotidsynthese schließt sich der Rahmen zur Anwendbarkeit in Industrie und Medizin. Gerade die Untersuchung von Oligonukleotiden als Therapeutika hat in den letzten 40 Jahren immer mehr an Bedeutung gewonnen.[28] Mittlerweile wurde eine Vielzahl an Oligonukleotidtherapeutika auf den Markt gebracht, welche auf der Modulation der Genexpression beruhen. Mehrere weitere Anwendungen befinden sich in klinischen Entwicklungsphasen.[29] Davon stammt ein Großteil aus der *Antisense*-Technologie bzw. nutzt *small interfering* RNAs, sodass die Wirkung vor allem auf der komplementären Wechselwirkung mit natürlich vorkommenden Oligonukleotiden beruht.[30,31] Dementsprechend ist eine möglichst effiziente Oligonukleotidsynthese wünschenswert, um Oligonukleotidtherapeutika auch wirtschaftlich attraktiv zu machen.

Die Synthese von Trinukleotiden, wie sie schematisch in Abbildung 3 dargestellt ist, findet hauptsächlich *in Lösung*, seltener aber auch an der *Festphase* und in neueren Publikationen mittels *löslicher Träger* statt.[32-36] (und Publikationen 2.1-2.5) Insbesondere die Wahl geeigneter orthogonaler Schutzgruppen spielt eine entscheidende Rolle, damit einerseits die Möglichkeit der selektiven Entschützung und andererseits eine ausreichende Stabilität während der Trinukleotidsynthese, Phosphitylierung sowie Oligonukleotidsynthese gegeben sind.

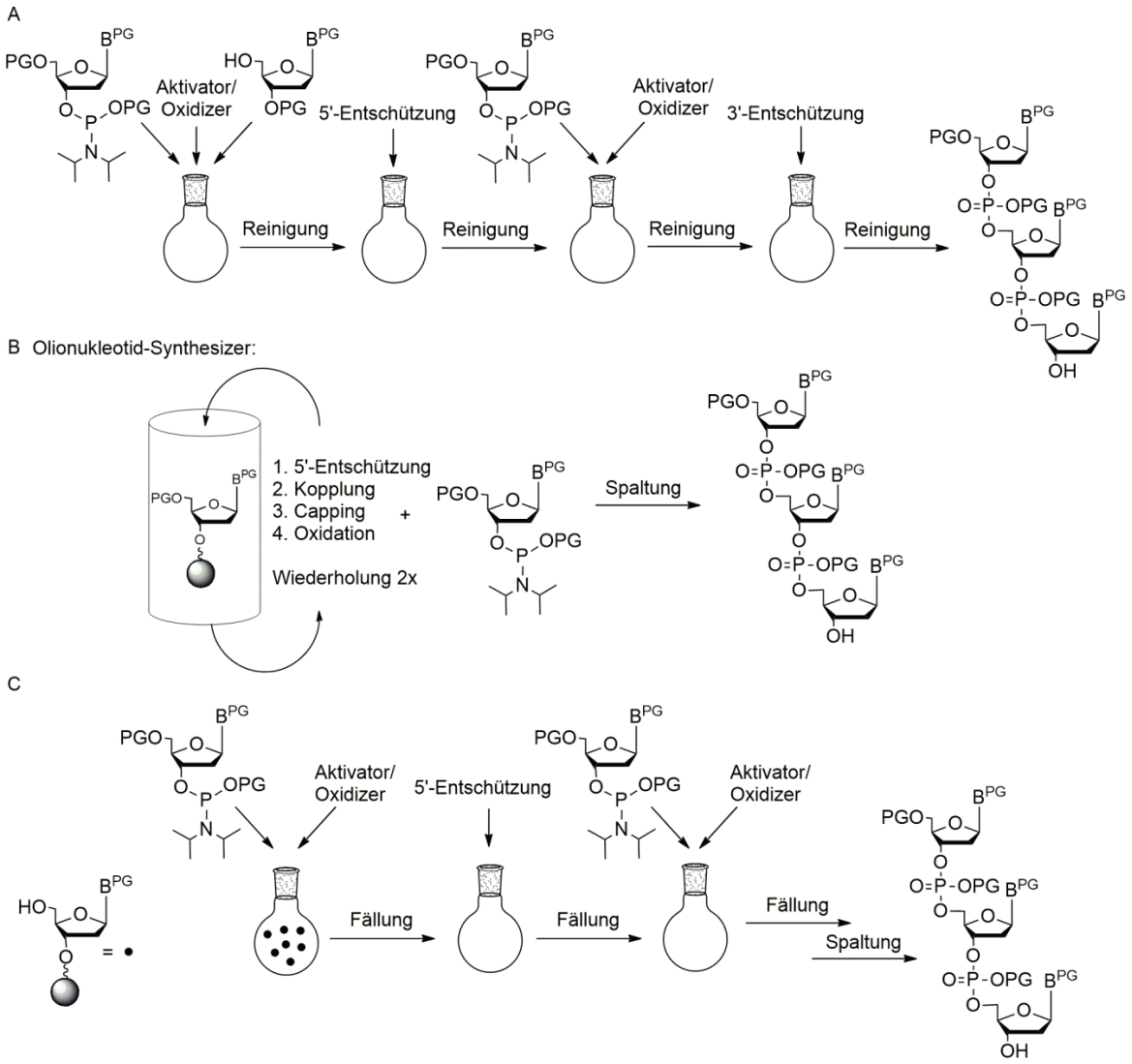

Abbildung 3: Schema der Synthese von Trinukleotiden *in Lösung* (A), an der *Festphase* mit Hilfe des Oligonukleotid-Synthesizers (B) und mittels *löslichem Träger* (C) (Abbildung modifiziert nach Publikation 2.2).

Die ersten Trinukleotide wurden 1994 *in Lösung* synthetisiert.[37] Die verschiedenen Synthesestrategien von Trinukleotiden *in Lösung* variieren sowohl in der Wahl der Schutzgruppen als auch in der Verwendung der Phosphotriester- bzw. Phosphittriester-Chemie.[32] (und Publikation 2.1) Die Darstellung von Trinukleotiden beginnt mit der Synthese eines geschützten Dinukleotids, welches nach Entschützung der entsprechenden Funktionalität in 3'- oder 5'-Richtung verlängert wird. Folglich ist die Selektivität bei den Entschützungsbedingungen entscheidend, sodass die 3'- bzw. 5'-Schutzgruppe gespalten werden kann und entsprechend die anderen Schutzgruppen wie für die exozyklische Aminogruppe der Nukleobase, das Phosphat und die 3'- bzw. 5'-OH-Gruppe erhalten bleiben. Die Phosphittriester-Chemie hat sowohl in den Anfängen der Trinukleotidsynthese als auch in der späteren Anwendung eine große Rolle gespielt.[38-40] Eine alternative Herangehensweise über die Phosphotriestermethode führte zu Beginn unter anderem zu Nebenprodukten, da das

3'-OH ungeschützt blieb und entsprechend 3'-3' Verbindungen entstehen konnten.[41,33] Zehl et al. entwickelten daraufhin eine weitere phosphotriesterbasierte Methode. Dieses Verfahren konnte die Nebenproduktbildung durch die Wahl der Levulinoyl-Schutzgruppe für die 3'-OH-Gruppe umgehen. Zusätzlich wurde die Synthese dadurch verbessert, dass die Möglichkeit bestand die Synthese der Trimere vom Dimer ausgehend sowohl in 5'- als auch in 3'-Richtung auszuführen, sodass weniger Dimervarianten synthetisiert werden mussten, um eine Vielzahl an Trinukleotiden zu erhalten.[42] (und Publikation 2.1) Im Anschluss an die Trinukleotidsynthese folgte die Phosphitylierung, um die Trinukleotidphosphoramidite als Synthons am Oligonukleotid-Synthesizer einsetzen zu können. Hierfür muss die 3'-OH-Gruppe zur Verfügung stehen und, wenn 3'-OH Schutzgruppen zur Verminderung von Nebenprodukten verwendet wurden, zuvor selektiv entschützt werden. Eine Vielzahl an Syntheserouten für Trinukleotide mit unterschiedlichen Schutzgruppenstrategien wurde entwickelt, sodass die synthetisierten Trinukleotide mit Kopplungsausbeuten von 70-98 % am Oligonukleotid-Synthesizer eingesetzt werden konnten.[43,44] Grundsätzlich vereint die verschiedenen Syntheseansätze die Schwierigkeit der Orthogonalität und Stabilität der verwendeten Schutzgruppen.[37,39-42,45,46] Die vielversprechendsten Ansätze sind die Verwendung der *tert*-Butyldimethylsilyl- (TBDMS) und der 2'-Azidomethylbenzoyl-schutzgruppe am 3'-OH, welche sich als äußerst stabil unter den Synthesebedingungen erwiesen haben.[38,43] Wie bei Zehl et al. verminderte die stabile Schützung der 3'-OH-Gruppe die Bildung von Nebenprodukten. Weiterhin ermöglichen die orthogonalen Spaltungsbedingungen dieser Schutzgruppen die Synthese von vollständig geschützten Trinukleotiden mit freier 3'-OH-Gruppe, welche zur Umwandlung in Trinukleotidphosphoramidite bereitstehen. Mittlerweile steht eine Vielzahl an qualitativ unterschiedlichen Möglichkeiten zur Synthese von Trinukleotidsynthons *in Lösung* zur Verfügung, die jedoch alle den Nachteil aufweisen, dass nach jedem Syntheseschritt aufwendige Reinigungs- bzw. Isolationsschritte erforderlich sind.

Die Synthese von Trinukleotidsythons an der *Festphase* hat den Vorteil, dass keine aufwendigen Reinigungsschritte nötig sind, da Nebenprodukte und Reagenzien einfach von der *Festphase* gewaschen werden, während das Produkt an der *Festphase* verbleibt. Hinzu kommt jedoch die Notwendigkeit der Spaltung der Trinukleotide von der *Festphase* unter Erhaltung der verwendeten Schutzgruppen. Folglich sind die kommerziell für die Immobilisierung der Startnukleoside an die *Festphase* verwendeten Succinatlinker ungeeignet. (Publikation 2.2) Die nukleophil bzw. basisch spaltbaren Schutzgruppen würden

bei der Spaltung des Trimers von der Festphase verloren gehen. Eine Variante zur Trinukleotidsynthese an der *Festphase* ist hierbei die Verwendung eines Oxalyllinkers, der nur sehr leichte basische Bedingungen bei der Spaltung benötigt (Abb. 4).[33] Kayushin et al. zeigten mit der Verwendung des Oxalyllinkers, dass eine Trinukleotidsynthese an der *Festphase* mit Hilfe der Phosphotriesterchemie mit guten Ausbeuten möglich ist, jedoch im Vergleich zur klassischen Oligonukleotidsynthese eine deutliche Verlängerung der Kopplungszeiten (~40 min) und Entschützungszeiten (15-30 min) benötigt.

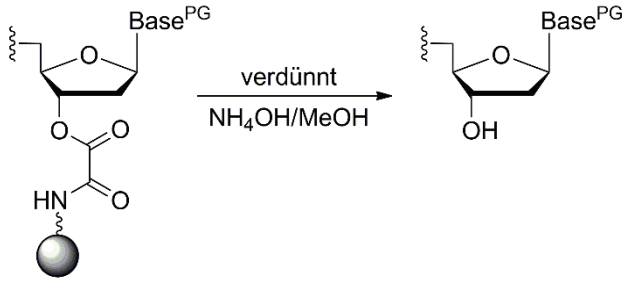

Abbildung 4: Immobilisierung- und Spaltungsstrategie der Synthese von Trinukleotiden an eine *Festphase* mittels Oxalyllinker nach Kayushin et al. (adaptiert nach Publikation 2.2).

Andere Untersuchungen befassten sich mit der Verwendung einer inversen Festphasenstrategie, bei der die Reagenzien an die *Festphase* gebunden werden.[47,48] Dies sollte die Vorteile des größeren Maßstabs der Lösungschemie mit der vereinfachten Reinigung der Festphasenchemie vereinen. Jedoch wurden im Vergleich zur Trinukleotidsynthese *in Lösung* niedrigere Kopplungsausbeuten und die Bildung von Nebenprodukte festgestellt.[47,48]

Eine weitere und neuere Möglichkeit bei der Trinukleotidsynthese, welche die Vorteile des größeren Maßstabs und des geringen Kostenaufwandes der Lösungschemie mit der vereinfachten Reinigung der Festphasenchemie kombinieren soll, ist die Verwendung von *löslichen Trägern*. Hierbei findet die Synthese in Lösung am *löslichen Träger* statt und die Reinigung erfolgt durch Ausfällen im geeigneten Lösungsmittel.[49] Für diese Art der Synthesen von Oligonukleotiden gibt es eine Reihe von Beispielen.[49] Viele Strategien nutzen Polyethylenglycol (PEG) als Grundgerüst für *lösliche Träger*.[50-53] Für die Synthese von vollständig geschützten Trinukleotiden an *löslichen Trägern* gibt es weniger Beispiele und diese verwenden statt PEG-basierter tetrapodale Trägermoleküle mit Pentaerythritoleinheit und Phosphotriesterchemie (Abb. 5).[34,35]

Wie bei der Festphasensynthese ist hier eine orthogonale Spaltung der Trinukleotide vom *löslichen Träger* zur Schutzgruppenchemie essenziell.

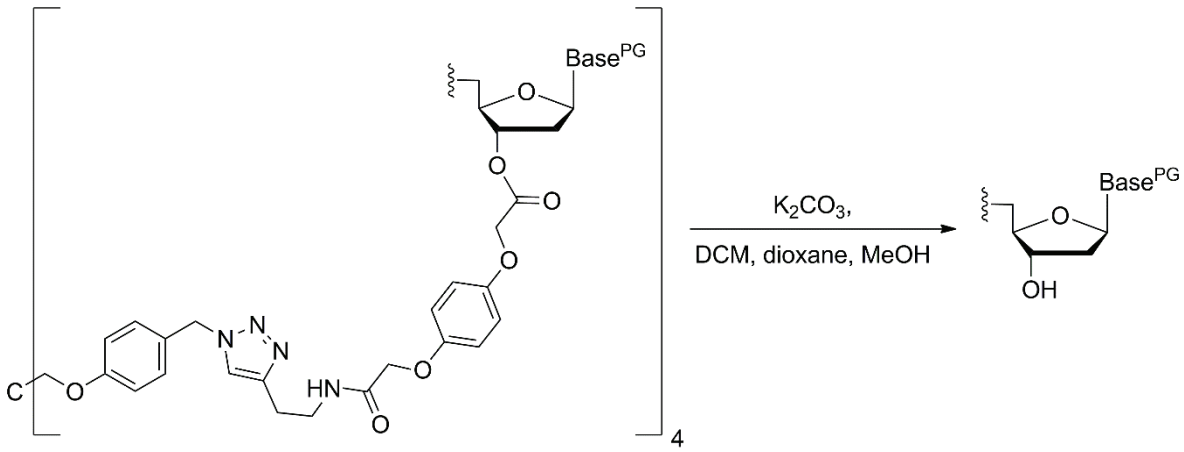

Abbildung 5: Immobilisierung- und Spaltungsstrategie der Synthese von Trinukleotiden an einem *löslichen Träger* nach Kungurtsev et al. (modifiziert nach Publikation 2.2).

Die Trinukleotidsynthese ist ein bedeutendes, jedoch nicht triviales, Anliegen. Es existieren zwar Lösungsansätze für geltende Einschränkungen bezüglich Synthesestrategien, Aufwand, Kosten und weiterer Faktoren, aber bisher lieferte keiner dieser Ansätze ein umfassend befriedigendes Resultat, sodass neue Protokolle benötigt werden. Diese Herausforderung wurde in dieser Arbeit adressiert.

1.2 Zielsetzung und experimentelle Strategie

Ziel dieser Arbeit war die Synthese von vollständig geschützten Trinukleotiden an *fester Phase* sowie an *löslichem Träger*. Im Vergleich zur Synthese in Lösung bieten beide Vorgehensweisen deutlich leichtere Reinigungs- bzw. Isolierungsmöglichkeiten. Die Synthese von Trinukleotiden an der *Festphase* findet nach der Immobilisierung automatisiert am Oligonukleotid-Synthesizer statt. Grundsätzlich stehen mit *controlled pore glass* (CPG) und diversen Polystyrolpolymeren verschiedene *Festphasen* zur Oligonukleotidsynthese zur Verfügung. Die möglichen Trägermaterialien sollten auf ihre Eignung für die Trinukleotidsynthese untersucht werden. Die Synthese von Trinukleotiden am *löslichen Träger* findet in Lösung statt, wobei anstelle aufwendiger Reinigungsschritte die Reaktionsprodukte einfach ausgefällt werden. Sowohl bei der Verwendung einer *Festphase* als auch beim Nutzen eines *löslichen Trägers* wird eine zusätzliche Verbindung zwischen Trinukleotid und gewählter Phase benötigt.

Es wurde die Verknüpfung von Trinukleotiden mit verschiedenen *Festphasen* bzw. einem *löslichen Träger* über eine Disulfidbrücke als Anbindungsstrategie gewählt. Diese ist, anders als die kommerziell verwendeten Succinatlinker, reduktiv und damit vollständig orthogonal zu der säurelabilen 5'-Dimethoxytrityl-Schutzgruppe (DMT) und den nukleophil angreifbaren Phosphat- oder Aminoschutzgruppen spaltbar (Abb. 6).[54-56] Diese Schutzgruppen weisen alle den Vorteil auf, dass sie unter Phosphitylierungsbedingungen stabil und kompatibel mit der Phosphoramiditmethode am Oligonukleotid-Synthesizer sind. Damit die Trinukleotide als Synthons am Oligonukleotid-Synthesizer beispielsweise zur Erstellung einer Oligonukleotidbibliothek eingesetzt werden können, muss vorher die Phosphitylierung zum Phosphoramidit erfolgen (Abb. 7). Hierfür ist die Disulfidbrücke ideal, da die reduktive Spaltung im Kontext des gewählten Linkers dazu führt, dass ein zunächst gebildetes *S,O*-Halbacetal spontan zur freien 3'-OH-Gruppe und Thioformaldehyd zerfällt, während alle anderen Schutzgruppen erhalten bleiben. Die 3'-OH-Gruppe steht dann direkt für die Phosphitylierung zur Verfügung. Die optimalen Bedingungen für die reduktive Spaltung der Disulfidbrücke sollten erarbeitet werden.

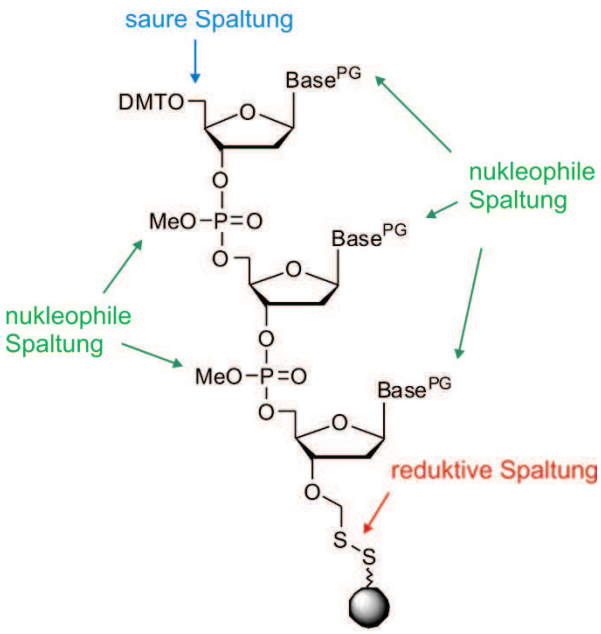

Abbildung 6: Spaltungsbedingungen der verwendeten Schutzgruppen und Immobilisierungsstrategie.

Die grundsätzliche Strategie der Synthese von Trinukleotiden in dieser Arbeit umfasst – nach Immobilisierung des Startnukleosids über eine Disulfidbrücke an eine geeignete Phase – den Aufbau des Trinukleotids mit Hilfe der Phosphoramiditmethode. Es folgt die reduktive Spaltung der Disulfidbrücke unter Erhalt des vollgeschützten Trinukleotids, welches anschließend in ein Trinukleotidphosphoramidit überführt werden kann und dann zum Einsatz am Oligonukleotid-Synthesizer zur Verfügung steht (Abb. 7).

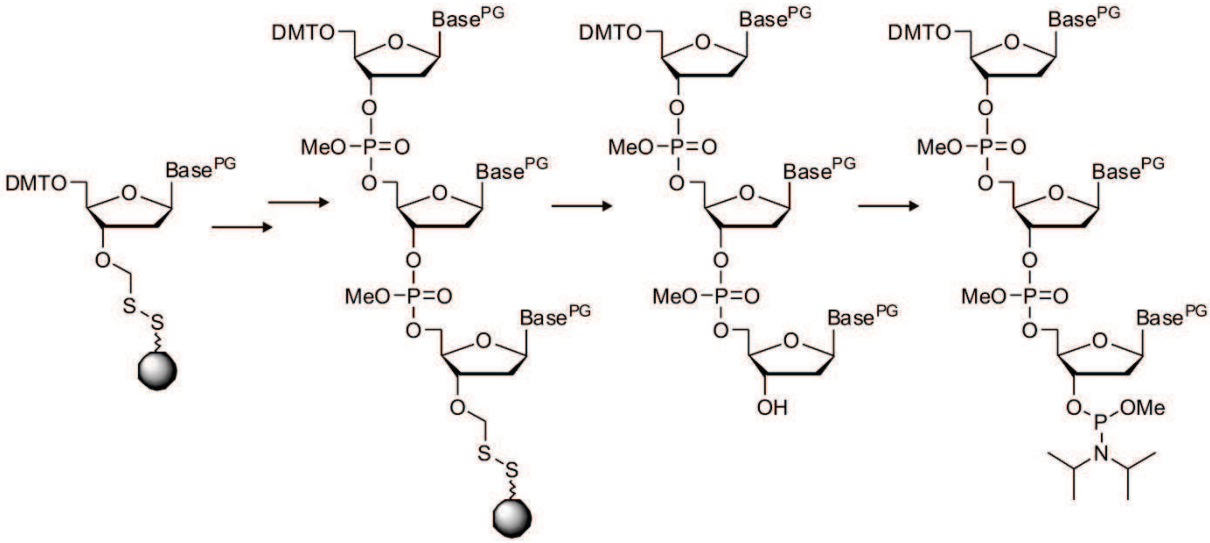

Abbildung 7: Schema der Synthese von Trinukleotidsynthons mittels Immobilisierung des Startnukleosids an eine *Festphase* bzw. an einen *löslichen Träger* über eine Disulfidbrücke.

Sowohl die Synthese von Trinukleotiden an der *Festphase* als auch am *löslichen Träger* sollte entsprechend mittels Immobilisierung des Startnukleosids über eine Disulfidbrücke stattfinden, jedoch unterschied sich die Art der Disulfidverknüpfung. Für die *Festphasensynthese* wurde zum einen thiofunktionalisiertes Polystyrol gewählt, um eine direkte Disulfidbrückenbindung zu ermöglichen, zum anderen sollte aminofunktionalisiertes Polystyrol zum Einsatz kommen, um durch den Einbau eines Linkermoleküls die Länge des Linkers variieren zu können und den Einfluss eines sekundären bzw. primären Kohlenstoffs in Nachbarschaft zur Disulfidbrücke zu untersuchen. Weiterhin wurden aminofunktionalisiertes CPG und hochvernetztes Polystyrol als Trägermaterialien für die Trinukleotidsynthese ausgewählt, welche beide bereits in der Oligonukleotidsynthese erfolgreich verwendet wurden. Als Linkerkomponente für die aminomodifizierten Träger wurden Mercaptosäuren gewählt, da die Thioleinheit in der Lage ist eine Disulfidbrücke aufzubauen und die Säureeinheit Amidbindungen mit den Aminofunktionen der entsprechenden Festphase eingehen kann.

Als Grundlage für die Synthese von Trinukleotiden an einem *löslichen Polymer* wurde das in der Literatur vielversprechend eingesetzte Tetrakis-*O*-[4-(azidomethyl)phenyl]pentaerythritol bestimmt.[35] An dieses kann über Click-Chemie eine Thiolfunktionalität geknüpft werden, um eine direkte Disulfidbrücke zwischen Trinukleotid und *löslichem Träger* zu gewährleisten.

Die verwendeten Schutzgruppen für die Trinukleotide orientieren sich größtenteils an der klassischen Phosphoramiditmethode, um die Kompatibilität am Oligonukleotid-Synthesizer zu gewährleisten. Statt der üblichen β-Cyanoethylschutzgruppe wurde jedoch die Methylschutzgruppe für das Phosphat gewählt, da die Methylschutzgruppe unter den Phosphitylierungsbedingungen äußerst stabil ist. Weiterhin wurde statt der häufig verwendeten Isobutyryl-Schutzgruppe auf Formamidin- und Benzoyl-Schutzgruppen zurückgegriffen, da Vorarbeiten ergaben, dass bei der Verwendung einer Isobutyryl-Schutzgruppe unter den gegebenen Synthesebedingungen die Gefahr von Schutzgruppenwanderung besteht.

1.3 Ergebnisse und Diskussion

1.3.1 Synthese von Trinukleotiden an der *Festphase* sowie anschließende Phosphitylierung und Kopplung (Publikation 2.3 und 2.4)

Die Synthese von Trinukleotiden an einer *Festphase* erleichtert die Herstellung sowie die Reinigung der Produkte deutlich. Ein Großteil der Synthese der Trinukleotide findet automatisiert am Oligonukleotid-Synthesizer statt und durch die Natur der *Festphase* erfolgt die Reinigung durch einfaches Waschen. Die verschiedenen Schritte zur Immobilisierung des Startnukleosids sind in Abbildung 8 und Tabelle 1 zusammengefasst.

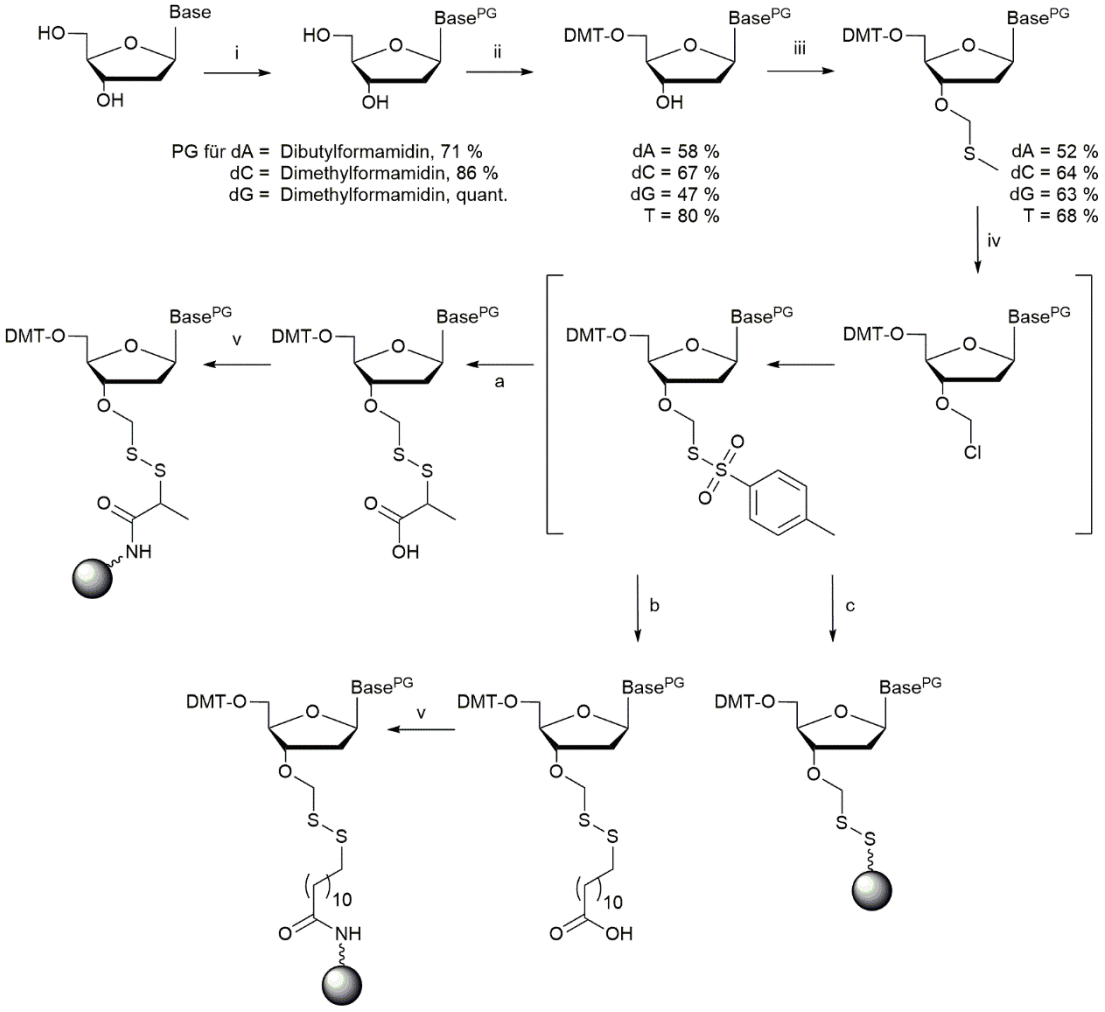

Abbildung 8: Syntheseschema zur Immobilisierung der Startnukleoside an die *Festphasen*. Die Anbindung der Nukleoside erfolgte über eine Disulfidbrücke via a) kurzem Linker (sekundärer Kohlenstoff in Nachbarschaft zur Disulfidbrücke), b) langem Linker (primärer Kohlenstoff in Nachbarschaft zur Disulfidbrücke), c) einer direkten Verbindung; i) dC/dG: 5 Äq. Dimethylformamid-Dimethylacetal, 1 h, 50 °C, in DMF; dA: 1,4 Äq. Dibutylformamid-Dimethylacetal, RT, ü.N., 1 h, 80 °C, in DMF; ii) 1,3 Äq. 4,4′-Dimethoxytrityl-Chlorid, 3 h bis zu ü.N. in Pyridin; iii) 70 Äq. DMSO, 53 Äq. Eisessig, 53 Äq. Acetanhydrid, RT, 20 h; iv) 3 Äq. TEA, 1 Äq. Sulfurylchlorid, 1,5 Äq. Kaliumthiotosylat in DCM; a) 2 Äq. 2-Mercaptopropionsäure, RT, 2 h; b) 2 Äq. 12-Mercaptododecansäure, RT, 2 h; c) *Festphase* B, RT, 1 h; v) 2 Äq. TSTU, 5 Äq. TEA, *Festphase* (A/C/D), RT, ü.N. in DMF/DCM/H_2O (Abbildung entnommen und angepasst aus Publikation 2.4).

Tabelle 1: Verwendete Linker und Trägermaterialien.

Bezeichnung des Trägers	Trägermaterial	funktionelle Gruppe	Linker
A	Amino CPG, Chem Genes	NH_2	2-Mercapto-propionsäure
A	Amino CPG, Chem Genes	NH_2	12-Mercapto-dodecansäure
B	Polystyrol A SH, Rapp Polymere	SH	-
C	Polystyrol AM NH_2, Rapp Polymere	NH_2	2-Mercapto-propionsäure
C	Polystyrol AM NH_2, Rapp Polymere	NH_2	12-Mercapto-dodecansäure
D	Custom Primer Support Amino, GE Healthcare (hochvernetzt)	NH_2	2-Mercapto-propionsäure
D	Custom Primer Support Amino, GE Healthcare (hochvernetzt)	NH_2	12-Mercapto-dodecansäure

Um über die 3'-OH-Gruppe eine Thiolfunktion in das Startnukleosid einzuführen, mussten zuerst die exozyklische Aminogruppe und die 5'-OH-Gruppe geschützt werden. Die exozyklischen Aminogruppen der Nukleobasen wurden mit Dimethylformamidin- (dG und dC) bzw. Dibutylformamidinschutzgruppen (dA) und die 5'-OH-Gruppe mit der für die Oligonukleotid-Festphasensynthese üblichen DMT-Schutzgruppe versehen (Abb. 8).

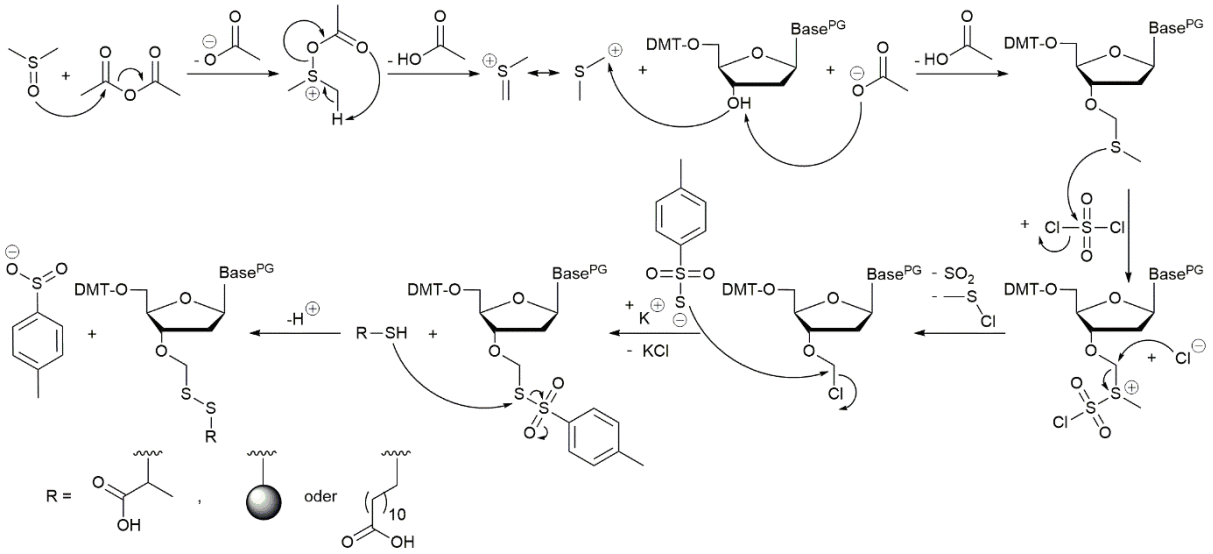

Abbildung 9: Mechanismus zur Disulfidbrückenbildung zwischen Startnukleosid und *Festphase* bzw. Linker

Anschließend wurde eine Methylthiomethylgruppe (MTM) am 3'-OH eingeführt.[57] Hierbei fand eine Pummerer-Umlagerung durch Dimethylsulfoxid (DMSO) und Essigsäureanhydrid statt (Abb. 9),[58,59] wobei trockenes Arbeiten essenziell ist, um die 5'-DMT-Schutzgruppe zu erhalten. Das entstandene MTM-funktionalisierte Nukleosid wurde daraufhin mit frisch destilliertem Sulfurylchlorid unter SO_2-Entwicklung zum Nukleosid mit

Chlormethyletherfunktion umgesetzt, sodass anschließend die Aktivierung mittels Kaliumthiotosylat stattfand (Abb. 9). Hier konnte die freie Thiolfunktion des thiofunktionalisierten Polystyrols (Tabelle 1, B; Abb. 9) angreifen und eine Disulfidbrücke bilden.[60,61] Der verwendete thiofunktionalisierte Polystyrolträger (B) wurde hier direkt eingesetzt, um das Startnukleosid über eine Disulfidbrücke am Träger zu immobilisieren.

Zusätzlich wurden Disulfidbrücken zwischen Startnukleosiden mit 2-Mercaptopropionsäure und 12-Mercaptododecansäure, durch den Angriff der Thioleinheiten der Linker am mit Thiotosylat aktivierten Nukleosid, gebildet (Abb. 8 und 9). Die Säurefunktionalitäten der 2-Mercaptopropionsäure und 12-Mercaptododecansäure gingen über eine Aktivesterbildung mittels *N,N,N′,N′*-Tetramethyl-*O*-(*N*-succinimidyl)uroniumtetrafluorborat (TSTU) und Austritt des stabilen Tetramethylharnstoffs eine Amidbindung mit aminofunktionalisierten Trägermaterialien (A, C, D) ein (Abb. 8).[62] Der Zusatz von Triethylamin (TEA) ist notwendig, um eine vorzeitige Spaltung der DMT-Gruppe zu verhindern und eine entsprechend korrekte Bestimmung der Beladungsdichte zu ermöglichen. Entsprechend wurden Startnukleoside mittels 2-Mercaptopropionsäure- und 12-Mercaptododecansäure-Linker an aminofunktionalisiertes Polystyrol (C), hochvernetztes aminofunktionalisiertes Polystyrol (D) und aminofunktionalisiertes CPG (A) immobilisiert (Abb. 8, Tabellen 1 und 2).

Tabelle 2: Beladung und Spaltung der Festphasen-Trägermaterialien, hv = hochvernetzt (Tabelle entnommen und angepasst aus Publikation 2.4).

Festphase	Linker	Startnukleosid	Beladungsdichte [µmol/g]	Spaltung vom Träger
A Amino CPG	12-Mercapto-dodecansäure	Thymidin	7,9	quantitativ
A Amino CPG	2-Mercapto-propionsäure	Thymidin	10,5	quantitativ
B Thiol PS	-	Thymidin	238,4	< 10 %
C Amino PS	12-Mercapto-dodecansäure	Thymidin	245,0	< 10 %
C Amino PS	2-Mercapto-propionsäure	Thymidin	57,8	< 10 %
D Amino PS (hv)	12-Mercapto-dodecansäure	Thymidin	89,3	quantitativ
D Amino PS (hv)	2-Mercapto-propionsäure	Thymidin	58,9	quantitativ
		Desoxyguanosin	51,6	quantitativ
		Desoxycytidin	58,8	quantitativ
		Desoxyadenosin	113,0	quantitativ

Tabelle 2 zeigt die verschiedenen Beladungsdichten, welche mittels photometrischer Messung des sauer abgespaltenen DMT-Kations bei 498 nm bestimmt wurden, der verschiedenen Trägermaterialien mit Startnukleosid.[63] (sowie Publikation 2.3 und 2.4) Hierbei lässt sich erkennen, dass CPG (A) als Trägermaterial die geringste Beladungsdichte aufwies, hochvernetztes aminofunktionalisiertes Polystyrol (D) eine mittlere Beladungsdichte ergab und thiol- sowie aminofunktionalisiertes Polystyrol (B, C) die höchsten Beladungsdichten zeigten.

Die Spaltung der Nukleoside vom Trägermaterial wurde mit Dithiothreitol (DTT) als klassischem Reduktionsmittel und Tris(2-carboxyethyl)phosphin (TCEP) durchgeführt. TCEP erwies sich als das geeignete Reduktionsmittel. Die Spaltung mit TCEP lieferte die besseren Ergebnisse und als Spaltungsreagenz ist es grundsätzlich zu bevorzugen, wenn Methylschutzgruppen am Phosphat für das geschützte Trinukleotid vorgesehen sind, da DTT als Thionukleophil diese spalten würde.[64] (und Publikation 2.4) Methylschutzgruppen sind der β-Cyanoethylschutzgruppe im Falle der Synthese von Trinukleotidsythons vorzuziehen, da sie unter den Phosphitylierungsbedingungen stabil sind und die Phosphitylierung für den Einsatz als Trinukleotidynthons am Oligonukleotid-Synthesizer notwendig ist.[38] Die Spaltung der Disulfidbrücke fand reduktiv und bei neutralem bis leicht basischem pH in HEPES-Puffer/Acetonitril(ACN)-Gemisch (1/2) statt, um eine Spaltung der DMT-Gruppe zu verhindern. Bei der Spaltung der Disulfidbrücke wurde zunächst ein *S,O*-Halbacetal gebildet, welches dann spontan zum freien 3'-OH und Thioformaldehyd zerfiel (Abb. 10).

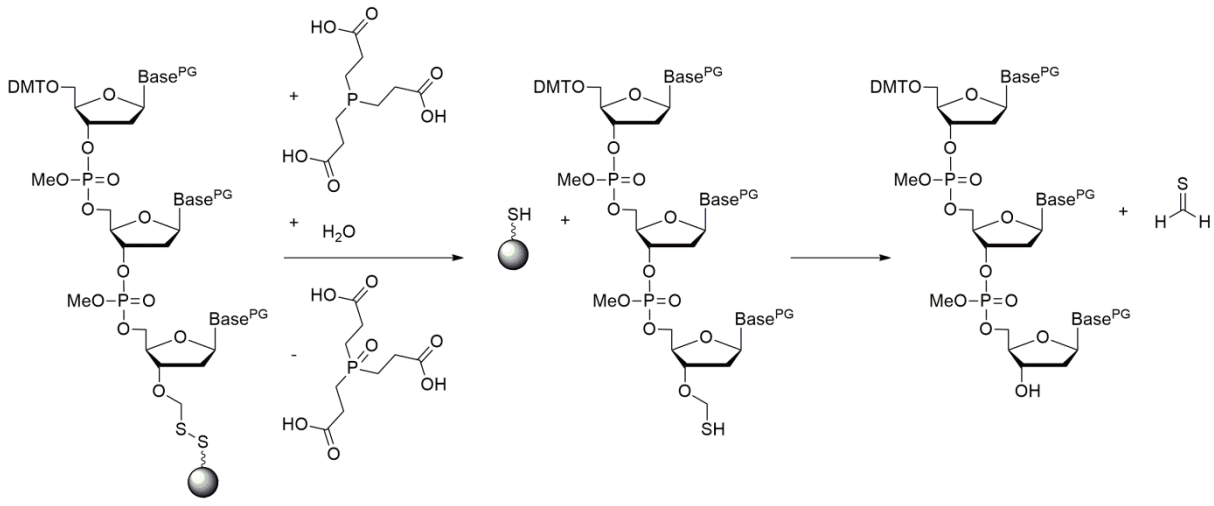

Abbildung 10: Schematische Darstellung der Spaltung der Disulfidbrücke mittels TCEP.

So wie sich die Beladungsdichten je nach Trägermaterial unterscheiden, ergaben sich auch deutliche Unterschiede in der Wiedergewinnung der Nukleoside vom Träger. Wie Tabelle 2 zu entnehmen ist, zeigen zwar thiol- und aminofunktionalisiertes Polystyrol (B, C) die besten Beladungsdichten, jedoch keine gute Rückgewinnung der Nukleoside vom Trägermaterial. Dies könnte in unspezifischen Wechselwirkungen zwischen Nukleosid und Trägermaterial begründet sein, welche ein vollständiges Abtrennen der Nukleoside durch Waschen vom Träger verhinderten. Von CPG (A) und hochvernetztem Polystyrol (D) hingegen ließen sich die Nukleoside quantitativ wiedergewinnen. Entsprechend erwies sich das hochvernetzte, aminofunktionalisierte Polystyrol (D) als am vielversprechendsten, da es bessere Beladungsdichten als CPG (A) und quantitative Spaltung der Nukleoside vom Träger ermöglichte. Die beiden verwendeten Linker 2-Mercaptopropionsäure und 12-Mercaptododecansäure zeigten im Spaltungsverhalten keine signifikanten Unterschiede, weshalb die Linkerlänge zumindest in diesem Bereich keine übergeordnete Rolle zu spielen scheint. Die Stabilität der Disulfidbrücke ist jedoch vom Einfluss der benachbarten Alkylgruppe und entsprechender Elektronendonorfähigkeit abhängig.[38,57] (und Publikation 2.4) Folglich wurde der 2-Mercaptopropionsäurelinker mit sekundärem Kohlenstoff in Nachbarschaft zur Disulfidbrücke im Vergleich zum 12-Mercaptododecansäurelinker mit primärem Kohlenstoff bevorzugt, um die Stabilität der Disulfidbrücke unter den Synthesebedingungen am Oligonukleotid-Synthesizer zu gewährleisten. Daher wurden geschütztes Thymidin, 2'-Desoxyadenosin, 2'-Desoxyguanosin und 2'-Desoxycytidin mittels 2-Mercaptopropionsäurelinker an hochvernetztes Polystyrol (D) gebunden.

Nach der Beladung der Träger mit Startnukleosid wurden eventuell noch vorhandene freie Aminogruppen des hochvernetzten, aminofunktionalisierten Polystyrolträgers (D) acetyliert und die Beladungsdichten jedes Mal erneut bestimmt (Tabelle 2). Die Startnukleoside am Träger wurden nun am Oligonukleotid-Synthesizer automatisiert zu Trinukleotiden verlängert. Nachdem das Trägermaterial in die für den Oligonukleotid-Synthesizer spezifischen Synthesesäulchen überführt wurde, begann der Standard-Synthesezyklus. Nachdem die 5'-DMT-Gruppe sauer entschützt wurde, folgte nach Aktivierung durch Benzylmercaptotetrazol die Kopplung mit geschütztem Phosphoramidit.[65] Anschließend wurde die Phosphitgruppe mittels Iodlösung zum Phosphat oxidiert. Bei der Oxidation wurde beachtet, dass die I_2-Konzentration nicht über 0,2 M lag, um Schäden an der Disulfidbrücke zu verhindern.[66]

Zusätzlich wurden eventuell ungekoppelte freie 5'-OH-Gruppen acetyliert und der Synthesezyklus wiederholt, sodass der Träger am Ende mit geschütztem Trinukleotid beladen war (DMT-on, Abb. 11).

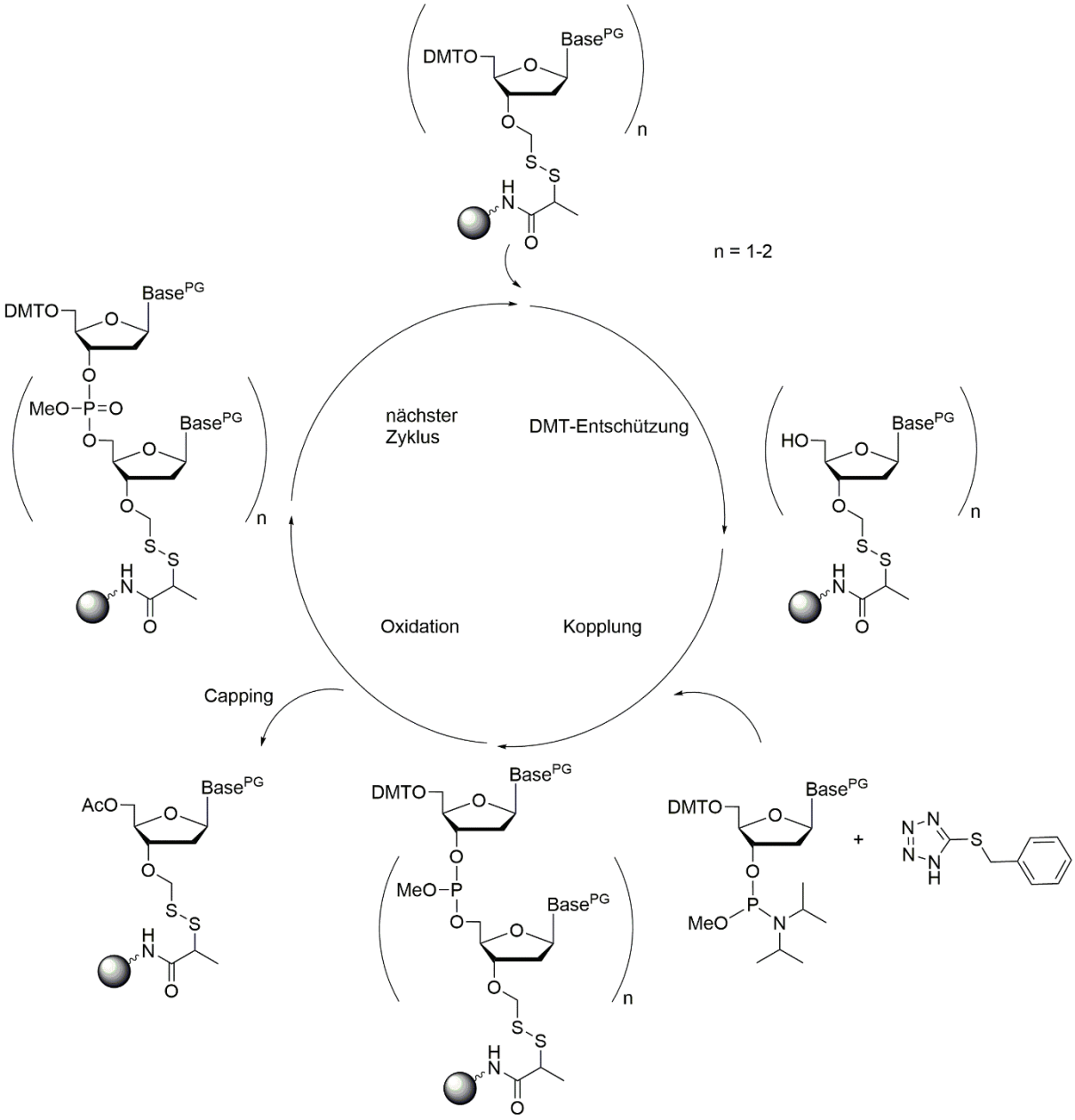

Abbildung 11: Schema der Trinukleotidsynthese am Oligonukleotid-Synthesizer.

Mit Hilfe der beschriebenen Methode wurden die geschützten Trinukleotide ATT, AAA, CAG, GCG, GCC, CAC, GTG, AAT und CTG erfolgreich im 1 µmol-Maßstab synthetisiert und mittels TCEP reduktiv vom hochvernetzten, aminofunktionalisierten Polystyrolträger gespalten (D) und mittels Massenspektrometrie (MS) nachgewiesen (Publikation 2.3 und 2.4).

Im folgenden Experiment sollte das Kopplungsverhalten einer Mischung von Trinukleotiden untersucht werden, um eine statistische oder anderweitig definierte Verteilung der verwendeten Trinukleotide, welche bei dem Einsatz in Genbibliotheken später den Aminosäuren an einer Position entsprechen würden, zu sichern. Insbesondere in der Anwendung von Trinukleotidsynthons bei der Erstellung von vollständig randomisierten Genbibliotheken ist die Einstellung der Codonhäufigkeiten im Gen wesentlich. Da verschiedene Trinukleotidsynthons unterschiedliche Kopplungsaktivitäten aufweisen können, ist eine Korrektur des Mischungsverhältnisses nach Maßgabe der zuvor experimentell zu bestimmenden, relativen Kopplungseffizienzen nötig.[67] Entsprechend war das Ziel, beispielhaft eine Oligonukleotidsequenz zu synthetisieren, welche an einer definierten Stelle aus einer Mischung von verschiedenen Trinukleotidphosphoramiditen zusammengesetzt ist. Diese Sequenz sollte kloniert und in ausreichender Menge für die statistische Auswertung, insbesondere hinsichtlich der Überprüfung von Fehlsequenzen und relativen Einbauraten, sequenziert werden.

Hierfür wurden die am Oligonukleotid-Synthesizer synthetisierten Trinukleotide (ATT, AAA, CAG, GCG, GCC, CAC, GTG, AAT, CTG) posphityliert (Abb. 12).[71] Der Reaktionsverlauf wurde mittels Dünnschichtchromatographie (DC) verfolgt, wobei sich die entstehende Phosphoramiditgruppe am Trinukleotid mittels Ninhydrins anfärben ließ. Nach vollständiger Umsetzung der Edukte wurden die Trinukleotidphosphoramidite wässrig aufgearbeitet, getrocknet und unverzüglich für die Kopplung am Oligonukleotid-Synthesizer eingesetzt.

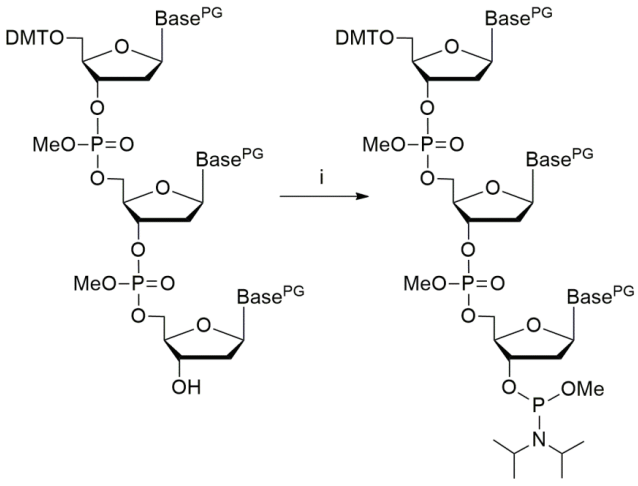

Abbildung 12: Phosphitylierung der Trinukleotide, i = 1,9 Äq. Methyl-*N,N*-Diisopropylchloro-phosphoramidit, TEA, 3,5 h, RT, in DCM.

Dabei stellte zunächst die begrenzte Löslichkeit der Trinukleotidphosphoramiditmischung ein Problem dar. Anstelle des Standardlösungsmittels ACN, in dem die Trinukleotid-phosphoramidite nicht löslich waren, wurde daher Dichlormethan (DCM, ca. 0,1 M) gewählt, in welchem eine bessere, jedoch noch immer unvollständige Löslichkeit vorlag.

Die Trinukleotidphosphoramidite wurden als Mischung zur Synthese des folgenden 26mers eingesetzt: 5'-ACTCAGATCTCGAGC-Y-TCAAGCTT-3', Y = ATT, AAA, CAG, GCG, GCC, CAC, GTG, AAT, CTG. Die Synthese des Oligonukleotids fand an CPG statt und wurde, abgesehen von Position Y, aus DNA-Monomerphosphoramiditen aufgebaut. Die Kopplungsfähigkeit der Trinukleotidmischung ermöglichte die Synthese des gewünschten 26mers. Dieses wurde nach Aufreinigung mittels PCR Amplifiziert (Abb. 13).

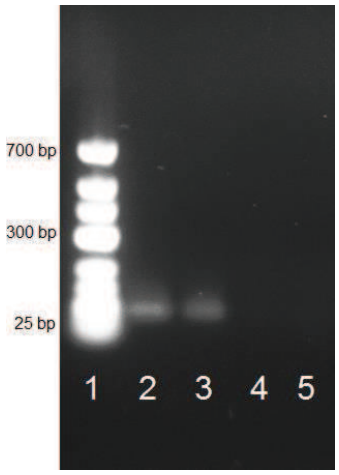

Abbildung 13: 1% Agarose-Gel der PCR des synthetisierten 26mers mit Ethidiumbromidfärbung; 1 = DNA-Leiter (GeneRuler, low range), 2 und 3 = PCR Produkt, 4 = neg. Kontrolle ohne Polymerase, 5 = neg. Kontrolle ohne Template.

Es folgte die Klonierung mit Hilfe der FastCloning Methode nach Li et al. unter Verwendung des pGEM-3Z Vektors (Promega) in NEB 5-alpha kompetente *E. coli* Zellen.[68] Wie in Abbildung 14 zu sehen ist, zeigt das Blau-Weiß-Screening den Klonierungserfolg an: Zum einen konnten durch die Selektion, der mit Ampicillin versetzten Agarplatten nur Kolonien wachsen, welche bei der Transformation die Vektor-DNA mit Resistenzgen aufgenommen haben, und zum anderen zeigen die weißen Kolonien (A) die Insertion eines DNA-Abschnitts in die *multiple cloning site* an, da in diesem Fall das β-Galactosidase-Gen unterbrochen wurde und somit das zugesetzte farblose X-Gal nicht in das blaue Derivat gespalten wurde.

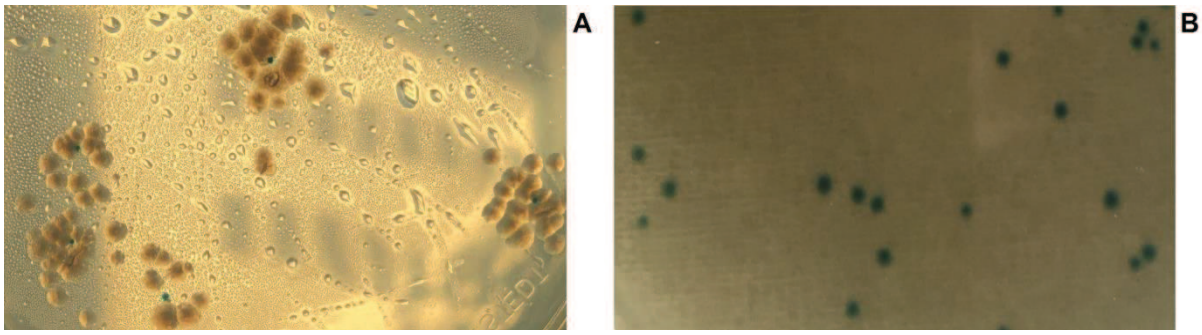

Abbildung 14: *E. coli* Kolonien nach Klonierung. A: mit Insertion des synthetisierten Oligonukleotids (rekombinierter Vektor), B: ohne Insertion des synthetisierten Oligonukleotids (nicht-rekombinierter Vektor, neg. Kontrolle).

Aus 20 Kolonien wurden Übernachtkulturen angelegt (Transfer einer Kolonie in 5 mL LB-Medium, 37 °C, 180 rpm) und anschließend Plasmidpräparationen (NEB Monarch Plasmid Miniprep Kit) durchgeführt. Diese 20 Proben wurden zum Sequenzieren verschickt. Jedoch führte dies nicht zur erhoffen Aussage über die Kopplungshäufigkeit der einzelnen Trinukleotidphosphoramidite in der verwendeten Mischung. Aus den Sequenzierdaten war keine eindeutige Sequenzabfolge lesbar. Weder zwischen den Sequenzdaten der verschiedenen Kolonien noch im Vergleich mit dem verwendeten Plasmid (mit oder ohne Insertion) konnten übereinstimmende DNA-Abschnitte gefunden werden. Eine Wiederholung der Sequenzierung mit Übernachtkulturen von acht weiteren Kolonien und anschließender Plasmidpräperation zeigte ähnliche Ergebnisse.

Ungeachtet dessen wurden mit Hilfe der beschriebenen Methode die vollständig geschützten Trinukleotide AAA, TTT, ATT, CAG, GCG, GCC, CAC, GTG, CTG und AAT erfolgreich im 1 µmol-Maßstab synthetisiert und mittels TCEP reduktiv vom hochvernetzten, aminofunktionalisierten Polystyrolträger gespalten (D). Die erfolgreiche quantitative Spaltung vom Träger wurde durch Säurebehandlung überprüft, da eventuell am Träger verbliebenes Trinukleotid durch die orangene Färbung des DMT-Kations detektierbar ist. Die Trinukleotide wurden vom Träger gewaschen, in Wasser ausgefällt und via MS nachgewiesen. Die Trinukleotide wurden phosphityliert und deren Kopplungsfähigkeit durch die Synthese eines 26mers nachgewiesen. Damit ist erfolfreich gezeigt, dass mit hochvernetztem, aminofunktionalisiertem Polystyrol über die Anbindung einer Disulfidbrücke geschützte Trinukleotide an fester Phase synthetisiert und nach Überführung in ein Phosphoramidit im Standard-DNA-Synthesezyklus an einem Oligonukleotid-Synthesizer gekoppelt werden können.

1.3.2 Synthese von Trinukleotiden an einen *löslichen Träger* sowie anschließende Phosphitylierung und Kopplung (Publikation 2.5)

Da die Synthese von Trinukleotiden an der *Festphase* in größeren Maßstäben recht kostenintensiv ist und entsprechend große Oligonukleotid-Synthesizer (z.B. OligoPilot 400) benötigt, ist die Etablierung einer Methode, welche die Synthese in größeren Maßstäben kostengünstig und ohne besondere Hilfsmittel erlaubt, eine attraktive Alternative. Hierfür ist besonders die Verwendung *löslicher Träger* geeignet, da sie nicht auf kleinere Maßstäbe begrenzt ist, keine besondere Laborausstattung benötig und zusätzlich durch das Ausfällen der Produkte im geeigneten Lösungsmittel keine aufwendigen Reinigungsschritte efordert. Für die Synthese von Trinukleotiden wurde in dieser Arbeit der *lösliche Träger* Tetrakis-*O*-[4-(azidomethyl)phenyl]pentaerythritol, welches freundlicherweise von der Arbeitsgruppe von Pasi Virta (Univ. Turku) zur Verfügung gestellt wurde, verwendet.

Zur Einführung der Thiolgruppe wurde zwischen dem *löslichen Träger*, welches je vier Azidgruppen trägt, und Propargylthioacetat eine kupferkatalysierte Azid-Alkin-Cycloadditionsreaktion (Click-Chemie) ausgeführt (Abb. 15).[69,70] Im Vergleich zur Originalpublikation erwies sich die Verwendung der Hälfte des teuren Propargylthioacetats (5 Äquivalente vs. 10 Äquivalente (Äq.)) als ebenso effektiv, da in beiden Fällen Ausbeuten von 70 % erreicht wurden.[34] (und Publikation 2.5) Die auf diese Weise eingeführte Thiolgruppe bildete nach Deacetylierung in einer 5 M Butylamin-Lösung eine Disulfidbrücke mit einem geschützten 3'-*O*-MTM-funktionalisierten Nukleosid aus. Wie bereits unter 1.3.1 genauer beschrieben, wurde das MTM-funktionalisierte Nukleosid mit Sulfurylchlorid und Kaliumthiotosylat aktiviert, um dann *in situ* mit einer freien Thiolgruppe eine Disulfidbrücke auszubilden. Auf diese Weise wurden Startnukleoside an den *löslichen Träger* gebunden und durch Fällung in Methanol gereinigt.

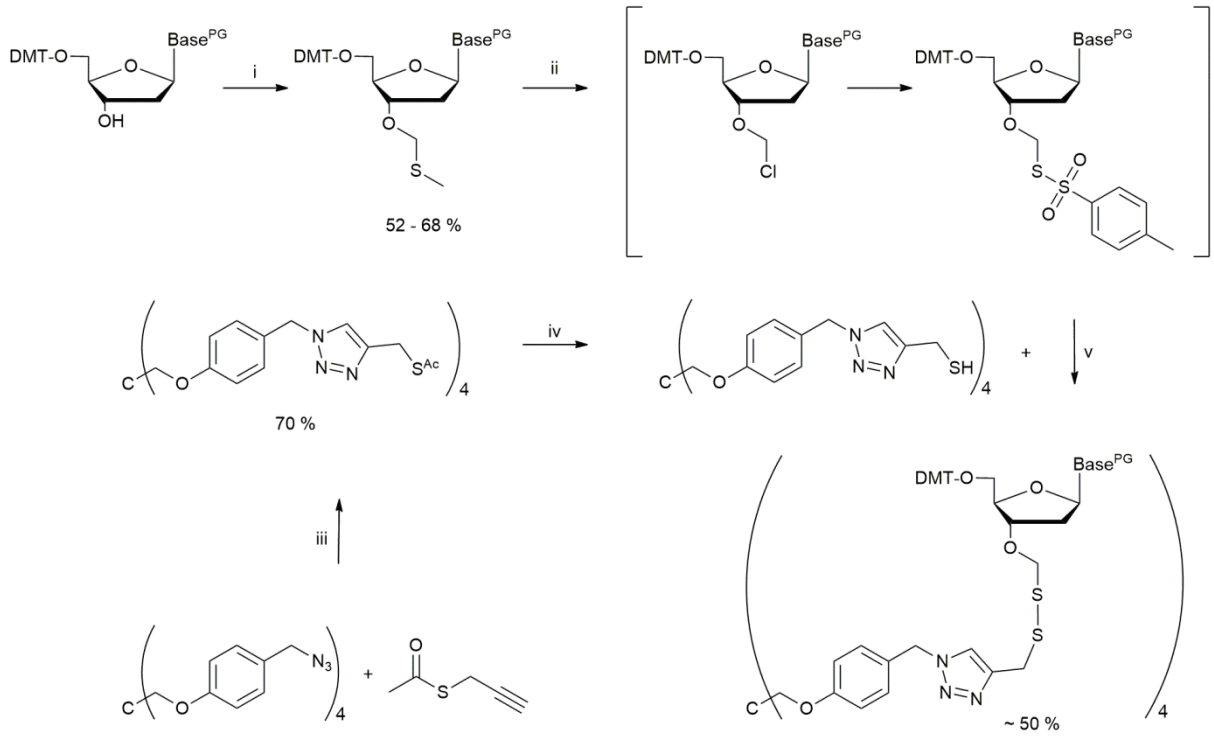

Abbildung 15: Syntheseschema zur Immobilisierung der Startnukleoside an den *löslichen Träger* mittels Disulfidbrücke, a: Base = dA, PG = Bz; b: Base = T; i) 70 Äq. DMSO, 53 Äq. Eisessig, 53 Äq. Acetanhydrid, RT, 20 h; ii) 3 Äq. TEA, 1 Äq. Sulfurylchlorid, 1,5 Äq. Kaliumthiotosylat in DCM mit v) 0,125 Äq. deacetylierter *löslicher Träger* in ACN/DCM, RT, 3,5 h; iii) 5 Äq. Propargylthioacetat, 0,1 Äq. Natriumascorbat, 0,4 Äq. CuI in DMAc, 50 °C, ü.N.; iv) 5 M Butylamin in MeOH, RT, ü.N. (modifiziert nach Publikation 2.5).

Anschließend erfolgte die saure Entschützung der 5'-*O*-DMT-Schutzgruppe mit Dichloressigsäure in Dichlorethan (DCE) (Abb. 16). Gerade die Verwendung der DMT-Schutzgruppe am 5'-OH ermöglicht durch die deutliche orange Färbung des DMT-Kations bei Säurebehandlung die leichte visuelle Überprüfung der Beladung des Trägers mit Startnukleosid, bzw. im späteren Verlauf der Kopplung zum Dinukleotid. Anschließend fand eine Reinigung des Produktes durch Ausfällung in Methanol statt. Das an den *löslichen Träger* gebundene Startnukleosid mit freier 5'-OH-Gruppe wurde daraufhin mit einem geschützten Phosphoramidit, welches mit Benzylmercaptotetrazol aktiviert wurde, gekoppelt.[65] Zur anschließenden Oxidation der entstandenen Phosphitgruppe zum Phosphat wurde Iodlösung hinzugegeben, bis deren gelbe Färbung bestehen blieb. Daraufhin wurde die Reaktion mittels Trimethylphosphit gequencht. Die Reinigung fand erneut über Ausfällen in Methanol statt. Um die gewünschten Trinukleotide zu erhalten, wurden Entschützung, Kopplung und Oxidation wiederholt (Abb. 16).

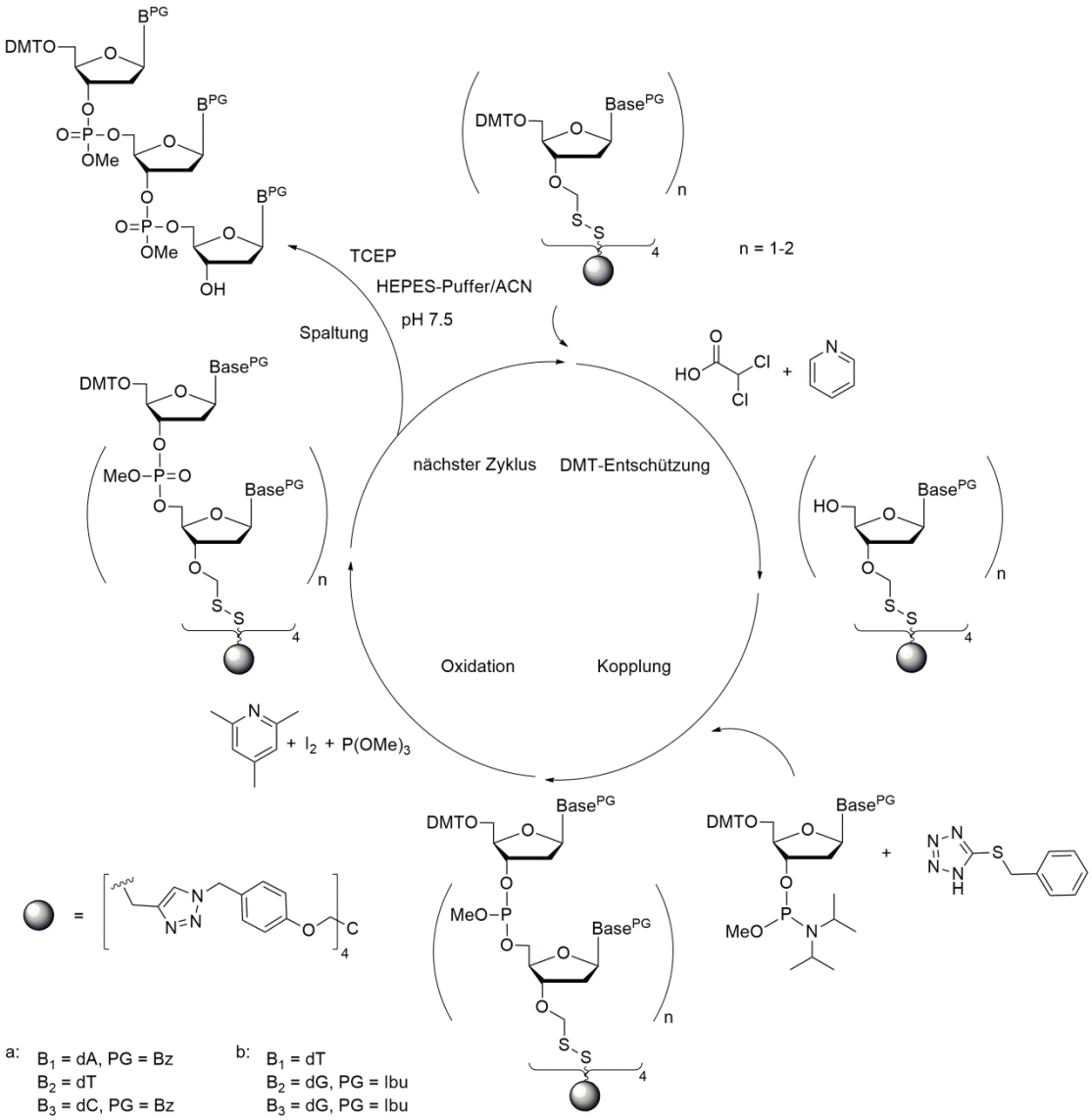

Abbildung 16: Syntheseschema zur Trinukleotidsynthese am *löslichen Träger* sowie Spaltung vom Träger (Abbildung adaptiert nach Publikation 2.5).

Die Spaltung des Trinukleotids vom *löslichen Träger* fand wie bereits unter 1.3.1 genauer erläutert mit Hilfe des Reduktionsmittels TCEP statt. TCEP wurde in einem HEPES-Puffer bei pH 7,5 gelöst, da niedrigere pH-Werte den Verlust der DMT-Gruppe zur Folge hatten. Zusätzlich wurde ACN hinzugefügt, um eine bessere Löslichkeit zu gewährleisten. Anschließend wurden die Trinukleotide vom Träger gewaschen. Eine NMR-Analyse zeigte jedoch noch deutliche TCEP-Rückstände, sodass eine Reinigung mittels präparativer DC erfolgte. Hierbei war die Zugabe von TEA jeweils zum Lösen, zum Laufmittel und zum Eluieren essenziell, um die Spaltung der DMT-Gruppe zu verhindern. Auf diese Weise wurden 27 µmol CTA und 48 µmol GGT isoliert und mittels NMR analysiert (^{1}H, ^{13}C, DEPT, DQF-COSY, HSQC, Publikation 2.5).

Die synthetisierten Trinukleotide CTA und GGT wurden, wie in Abschnitt 1.3.1 dargestellt, in Trinukleotidphosphoramidite überführt.[71] Wie auch für die an der Festphase synthetisierten Trinukleotide erwies sich ACN für das Trinukleotidphosphoramidit CTA als ungeeignetes Lösungsmittel. Entsprechend wurde erneut DCM als Ersatzlösungsmittel gewählt, in welchem eine bessere, jedoch auch in diesem Fall unvollständige Löslichkeit vorlag. Das Trinukleotidphosphoramidit CTA wurde weitestgehend in DCM gelöst, sodass eine ca. 0,1 M Lösung entstand. Diese Lösung wurde verwendet, um die aus Monomerbausteinen aufgebaute Sequenz CTT zum 6mer 5'-CTACTT-3' unter Erhalt der letzten DMT-Gruppe am Oligonukleotid-Synthesizer zu synthetisieren (DMT-on). Abbildung 17 zeigt das Chromatogramm der Reinigung des 6mers via RP-HPLC. Hierbei lässt sich erkennen, dass die eingeschränkte Löslichkeit des Trinukleotidphosphoramidits zu einer unvollständigen Kopplung und einem entsprechend großen Vorkommen an Abbruchfragmenten führte. Die Kopplungsausbeute wurde mit Hilfe der HPLC-Peakflächen und Extinktionskoeffizienten abgeschätzt und betrug für das Trinukleotidphosphoramidit CTA 17 %.

Um die Löslichkeit zu optimieren, wurde das Trinukleotidphosphoramidit GGT in DCM:ACN (3:1) gelöst. Diese 0,1 M Lösung des Trinukleotidphosphoramidits wurde am Oligonukleotid-Synthesizer eingesetzt, um die aus Monomerbausteinen aufgebaute Sequenz CTT zum 6mer 5'-GGTCTT-3' im Modus DMT-on zu synthetisieren. In dessen Chromatogramm der HPLC-Aufreinigung (Abb. 17) lässt sich erkennen, dass die verbesserte Löslichkeit des Trinukleotids sowie der Zusatz von ACN als geeignetes Lösungsmittel für die Kopplungsreaktion eine bessere Kopplung mit einem deutlich besseren Verhältnis zwischen Produkt und Abbruchfragmenten zur Folge hatte. Die geschätzte Kopplungsausbeute für das Trinukleotidphosphoramidit GGT betrug 72 %. Die 6mere 5'-CTACTT-3' und 5'-GGTCTT-3' wurden mittels MS bestätigt (Publikation 2.5, SI).

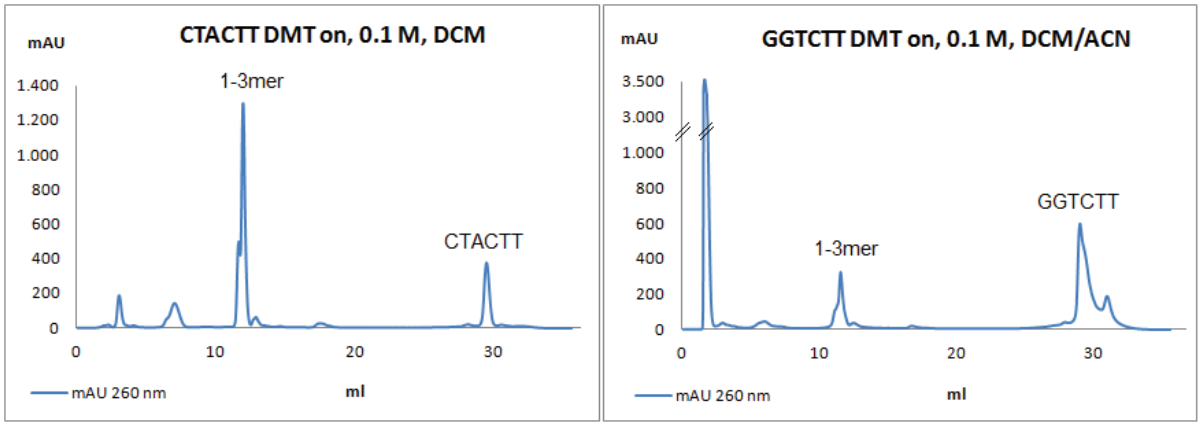

Abbildung 17: HPLC-Chromatogramm der 6mere 5'-CTACTT-3' und 5'-GGTCTT-3', beide DMT-on, AU = *absorption unit*, Nucleodur 125/4, *column volume* (CV) = 1,571 ml, 1 ml/min, Puffer A: 5% ACN, 0,1 M TEAAc; Puffer B: 30% ACN, 0,1 M TEAAc, Gradient: Start mit 0% Puffer B für 4 CV, bis 40% Puffer B über 3 CV, bis 60% Puffer B über 7 CV, bis 100% Puffer B über 2 CV, 100% Puffer B für weitere 2 CV, bis 0% Puffer B über 3 CV (adaptiert nach Publikation 2.5).

Der *lösliche Träger* Tetrakis-*O*-[4-(azidomethyl)phenyl]pentaerythritol erwies sich als geeignete Basis für die erfolgreiche Synthese in größerem Maßstab (27 und 48 µmol im Vergleich zum 1 µmol Synthesemaßstab an der *Festphase*) von geschützten Trinukleotiden über die Immobilisierung der Trinukleotide mittels einer Disulfidbrücke an den *löslichen Träger* und die Verwendung der Phosphoramiditchemie. Weiterhin ist es gelungen, die synthetisierten Trinukleotide in Trinukleotidphosphoramidite zu überführen und deren Kopplungsfähigkeit am Oligonukleotid-Synthesizer nachzuweisen.

1.4 Zusammenfassung

Die Verwendung von Genbibliotheken ist ein Schlüsselelement der gerichteten Evolution und folglich Teil der Erzeugung maßgeschneiderter Proteine. Die Herstellung einer oligonukleotidbasierten Genbibliothek mit Hilfe von Trinukleotiden, welche den 20 kanonischen Aminosäuren oder einer Auswahl entsprechen, hat mehrere Vorteile. Zum einen wird die Qualität der Bibliothek verbessert, da weder Stoppcodons entstehen noch die degenerierte Natur des genetischen Codes eine unausgewogene Codonhäufigkeit innerhalb der Bibliothek hervorruft. Zum anderen erhält die Genbibliothek durch vollständige Kontrolle der Randomisierung viele aussichtsreiche Kandidaten bei handhabbarer Größe, was das anschließende Screening erleichtert. Eine weitere Verwendung der Trinukleotide liegt in der Anwendung als Blockmere, um gegebenenfalls die Synthese von in größeren Mengen benötigten Oligonukleotiden effizienter zu gestalten.

In dieser Arbeit wurde die erfolgreiche Synthese von Trinukleotiden sowohl an der *Festphase* als auch am *löslichen Träger* mittels Phosphoramiditmethode gezeigt. Die Synthese an der *Festphase* hat den Vorteil der erleichterten Reinigung durch die Natur der Festphase sowie den Vorteil des automatisierten Prozesses durch die Verwendung des Oligonukleotid-Synthesizers. Für die Trinukleotidsynthese in größerem Maßstab unter Beachtung der Kosteneffizienz hingegen ist die Synthese am *löslichen Träger* von Vorteil, da sie ähnlich wie die Synthese *in Lösung* ohne besondere Laborausstattung besser zum *upscaling* geeignet ist und zusätzlich durch Ausfällen eine erleichterte Reinigung ermöglicht.

- Die grundlegende Strategie der Synthese von Trinukleotiden in der vorliegenden Arbeit ist die Immobilisierung der Startnukleoside an die *Festphase* bzw. den *löslichen Träger* über eine Disulfidbrücke, sodass die Synthese der Trinukleotide an der gewählten Phase stattfinden kann. Damit die Trinukleotidphosphoramidite am Oligonukleotid-Synthesizer zur Erstellung einer Oligonukleotidbibliothek genutzt werden können, müssen alle Funktionalitäten des Trinukleotids vollständig mit Schutzgruppen versehen sein. Folglich muss die Spaltungschemie von der *Festphase* bzw. dem *löslichen Träger* orthogonal zur Schutzgruppenchemie erfolgen. Dies leistet die reduktiv spaltbare Disulfidbrücke. Die Thiofunktionalität wurde an das geschützte Startnukleosid mit Hilfe einer Methylthiomethylfunktionalisierung sowie deren

Umsetzung mittels Sulfurylchlorid und Kaliumthiotosylat an die 3'-OH-Gruppe eingeführt.

- Für die Synthese an der *Festphase* wurden Disulfidbrücken mit einem thiofunktionalisierten Polystyrolträger, einem 2-Mercaptopropionsäurelinker und einem 12-Mercaptododecansäurelinker aufgebaut. Im Falle der Mercaptosäurelinker fand die Immobilisierung über eine Amidbindung an aminofunktionalisiertes Polystyrol, an hochvernetztes, aminofunktionalisiertes Polystyrol und an einen aminofunktionalisierten CPG-Träger statt. Hierbei zeigte sich, dass die Beladungsdichten mit Startnukleosid für das thiofunktionalisierte Polystyrol und das aminofunktionalisierte Polystyrol am höchsten waren. Jedoch war von diesen Trägern keine gute Rückgewinnung der Nukleoside möglich. Die Nutzung des hochvernetzten Polystyrolträgers erwies sich als erfolgreichste Variante, da gute Beladungsdichten erreicht wurden und zusätzlich eine quantitative Rückgewinnung der Nukleoside nach der reduktiven Spaltung möglich war. Der Einbau des 2-Mercaptopropionsäurelinkers wurde bevorzugt, da der sekundäre Kohlenstoff in Nachbarschaft zur Disulfidbrücke eine höhere Stabilität gewährleistet. Entsprechend wurden mit Schutzgruppen versehenes Thymidin, 2'-Desoxyadenosin, 2'-Desoxyguanosin und 2'-Desoxycytidin mittels 2-Mercaptopropionsäurelinker an hochvernetztes Polystyrol gebunden. Mit Hilfe des Oligonukleotid-Synthesizers wurden die Trinukleotide AAA, TTT, ATT, CAG, GCG, GCC, CAC, GTG, CTG und AAT synthetisiert sowie, nach Spaltung mittels TCEP und Nachweis durch MS, zu einem 26mer gekoppelt.

- Bei der Synthese von Trinukleotiden an einem *löslichen Träger* wurde Tetrakis-*O*-[4-(azidomethyl)phenyl]pentaerythritol mit Propargylthioacetat via Click-Chemie thiofunktionalisiert. Nach Deacetylierung der Thiolgruppe wurde mit geschütztem 3'-*O*-MTM-funktionalisiertem Nukleosid eine Disulfidbrücke aufgebaut. Vergleichbar mit der Standardoligonukleotidsynthese folgten DMT-Entschützung, Kopplung mit einem DNA-Phosphoramidit und Oxidation in Lösung. Hierbei wurden die Produkte nach dem jeweiligen Syntheseschritt in Methanol ausgefällt, sodass aufwendige Reinigungsschritte entfielen. Der Synthesezyklus wurde je zweimal durchgeführt, um entsprechend das Trinukleotid zu erhalten. Auf diese Weise wurden die Trinukleotide CTA und GGT am *löslichen Träger* synthetisiert, reduktiv vom Träger gespalten und mittels NMR nachgewiesen. Anschließend wurden die Trinukleotide in

Trinukleotidphosphoramidite überführt und am Oligonukleotid-Synthesizer zum 6mer gekoppelt.

Im Rahmen dieser Arbeit wurden erfolgreich Trinukleotide sowohl automatisiert über eine *Festphase* als auch an einem *löslichen Träger* synthetisiert. Entsprechend wurde die Verwendung einer Disulfidbrücke sowohl an der *Festphase* als auch am *löslichen Träger* als gelungene Strategie zur Synthese von Trinukleotidsynthons etabliert. Weiterhin ist es gelungen, die Kopplungsfähigkeit der synthetisierten Trinukleotidphosphoramidite nachzuweisen. Folglich können die Synthesestrategien zukünftig dazu genutzt werden, optimale Genbibliotheken zu erstellen oder die Oligonukleotidsynthese ökonomischer zu gestalten.

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Preparation of trinucleotide synthons for the synthesis of gene libraries

KEYWORDS: Codon, gene library, randomization, synthesis, trinucleotide.

Abstract Over the past decades, the interest in efficient methods for site-directed mutagenesis in protein engineering has strongly grown. Oligonucleotide-directed mutagenesis is the favoured method for preparation of large peptide or protein libraries. Among a host of methods, the use of mixtures of pre-formed trinucleotide blocks representing codons for the 20 canonical amino acids stands out as allowing fully controlled partial (or total) randomization at any arbitrarily chosen codon positions of a given gene. Such trinucleotide synthons, however, are not easy to come by. Several strategies have been suggested making use of various 3'-O- and phosphate protecting groups. However, most of these methods suffer from one or more limitations, such as di- and mononucleotide side products, loss of protecting groups, bad coupling yields or false linked nucleotides. More recent work has successfully overcome these limitations. Herein we review the strategies for preparation of fully protected trinucleotide synthons.

INTRODUCTION

The generation of novel proteins (enzymes, pharmaceutic drugs etc.) with experimentally pre-defined properties has been a long-standing goal in the field of molecular biotechnology. Over the past years, this has been achieved in a number of cases, although rather rudimentary, due to methodological progress in combinatorial and evolutionary protein engineering, which is based on the generation of highly diverse libraries of partially randomized genes (1). Gene libraries are expressed into protein libraries, which subsequently can be screened for a desired property. Some of such protein libraries have reached commercial level, whereas methodological optimization and extension of the strategy to larger proteins and more complex scenarios are still a major aim of research (2). Gene libraries are assembled from partially randomized oligonucleotides, for the synthesis of which various options exist. Most attractive is the use of trinucleotide synthons, each representing the codon of a specific amino acid (3). For each codon position to be randomized, a defined mixture of up to 20 different trinucleotides (dependent on the sort and number of amino acids that shall be incorporated) in arbitrary molar ratios is prepared and used for statistical coupling in oligonucleotide synthesis. This strategy allows full control over the nature of partial randomization and in consequence, preparation of a gene library with the largest amount possible of promising candidates.

Extrapolated sentences:
- Gene libraries are most efficiently synthesized by the use of trinucleotide phosphoramidites, each representing the codon of a specific amino acid.
- The use of trinucleotide synthons allows full control over the nature of randomization and in consequence, preparation of a gene library with the largest amount possible of promising candidates.
- Key to trinucleotide synthesis is the definition of a suitable set of orthogonal protecting groups.

Fully protected trinucleotides can be prepared in solution and, potentially also on the solid phase. The key is the definition of a suitable composition of orthogonal protecting groups that on one side allow selective deblocking if required, and on the other are sufficiently stable under the conditions of phosphoramidite preparation and oligonucleotide synthesis (Figure 1). Both, phosphorous (V) and phosphorous (III) chemistries have been used for preparation of trinucleotides, so far mainly in solution (3). Actually, the efforts started already back in 1994 with trinucleotide synthesis by phopsphite triester chemistry, which however at that time was limited to only two trinucleotides dGCT and dGGT, both obtained with low yield and quality (4). Due to the problems associated with this early synthesis, other strategies for preparation of trinucleotides first based on phosphite triester chemistry, later more on phosphotriester chemistry were

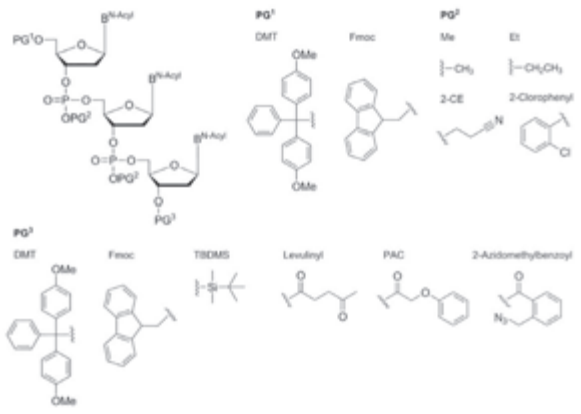

Figure 1. Structure of a trinucleotide and various protecting groups for 5'-OH, 3'-OH and phosphate moiety.

developed (vide infra). The individual strategies differ mainly in the choice of the protecting schemes, and connected with this, in the quality of the products and synthesis steps involved. In recent years, we have reinvestigated trinucleotide synthesis by phosphite triester chemistry, and we demonstrated that, in conjunction with the clever choice of protecting groups, it is a superior method. Both, trinucleotide preparation by phosphotriester and phosphite triester chemistry are discussed in more detail below.

TRINUCLEOTIDE SYNTHESIS BY PHOSPHOTRIESTER CHEMISTRY

One of the first preparations of trinucleotides by phosphotriester chemistry in 5' to 3' direction was described by Ono *et al.*, based on a previously developed procedure for the synthesis of short oligonucleotide blocks (5, 6). The 3'-hydroxyl group of initially synthesized dimers was converted into a 2-chlorophenylphosphoric ester (according to the scheme shown in Figure 2) and reacted with an *N*-acyl-nucleoside in the presence of MSNT (1-mesitylenesulphonyl-3-nitro-1,2,4-

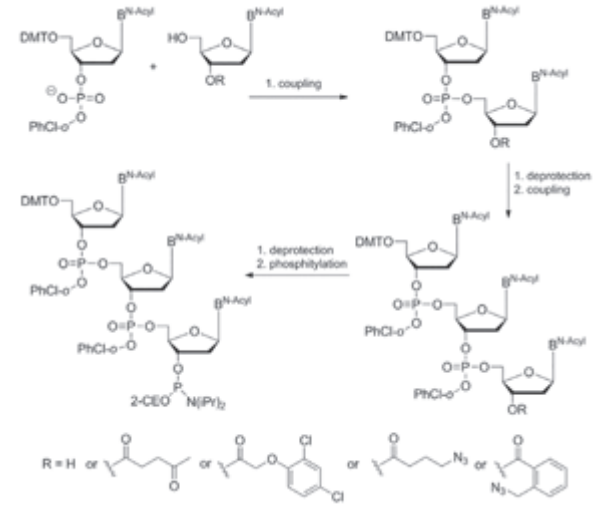

Figure 2. Synthesis of trinucleotide synthons by phosphotriester chemistry.

triazole) as coupling reagent. The 3'-OH group of the *N*-acyl-nucleoside was left free, based on the predicted higher selectivity of the reaction for the primary 5'-OH group. The purified trinucleotides were phosphitylated, and the resulting phosphoramidites were applied in oligonucleotide synthesis as a mixture of seven different triplet-amidites. Coupling yields of up to 90% were achieved. A similar route for trinucleotide synthesis in 5' to 3'-direction, was reported by Kayushin *et al.*, also trusting on the higher reactivity of the 5'-OH in comparison to the 3'-OH function, and consequently no need for protection of the latter (7). With both strategies, not surprisingly a number of side products, mainly resulting from reaction of the unprotected 3'-OH group (3'-3' linked dimers and trimers) were observed. Nevertheless, trinucleotides could be isolated from the product mixture with sufficient purity for following phosphitylation and use in oligonucleotide synthesis with coupling yields of 94 up to 98%.

In a report from 1996 (8) the problem of appearing by-products in conjunction with the unprotected 3'-hydroxyl group was tried to circumvent by the use of the levulinoyl group for 3'-*O*-protection (Figure 1, 2). As in the methods described above, the 2-chlorophenyl group was used for protection of the phosphate moiety. As an elegant element, the reported strategy involves the synthesis of a set of trinucleotides by extension of previously synthesized dimers in either 5'- or 3'-direction. This reduces the number of dimers necessary to synthesize a given set of trinucleotides. Cleavage of the levunlinoyl group at the 3'-position to allow conversion of the dinucleotide into the trinucleotide or final phosphitylation, respectively, was conducted with a 0.5 M solution of hydrazine in pyridine/acetic acid (3:5, v/v). Subsequently, trinucleotides were phosphitylated with 2-cyanoethyl-*N,N*-diisopropylchlorophosphoramidit and coupled under standard conditions for oligonucleotide synthesis using a 0.1 M concentration of trinucleotide synthons in acetonitrile and a coupling time of 15 minutes. Coupling yields in the range of 66 to 99% were reached with the lowest yield of 66% observed for the GGG-triplet.

In the following years, a number of other protecting groups for the 3'-hydroxyl position were investigated. In continuation of their previous work (7), Yagodkin *et al.* used 2,4-dichlorophenoxyacetyl, 4-azidobutanoyl and 2-azidomethylbenzoyl for 3'-OH protection (9) (Figure 2). Among these variants, the 2-azidomethylbenzoyl group has proven most suitable due to its convenient removal (Figure 3a). As in their previous studies (7) the authors used the 2-chlorophenyl group for protection of the phosphate. After condensation of *N*-acyl-3'-*O*-(2-chlorophenylphosphate)-5'-*O*-DMT-nucleosides to 3'-*O*-(2-azidomethylbenzoyl)-nucleosides in the presence of MSNT, the DMT-group of the resulting dimer was cleaved off under mild acidic conditions. Subsequently, dinucleotides were transformed, by coupling of another *N*-acyl-3'-*O*-(2-chlorophenylphosphate)-nucleoside, into fully protected trinucleotides. The 3'-*O*-(2-azidomethylbenzoyl) group was selectively removed with triphenylphosphine in the presence of water. Under these conditions, the azide is reduced to a primary amine, and upon spontaneous intramolecular cyclization, isoindoline-1-one and the trinucleotide with free 3'-hydroxyl function are generated (Figure 3a). Subsequent 3'-phosphitylation afforded the fully protected trinucleotide phosphoramidite. A set of 20 trinucleotide phosphoramidites on a 5-10 g scale each was produced. The amidites were coupled in oligonucleotide synthesis as 0.1-0.15M solutions in acetonitrile/

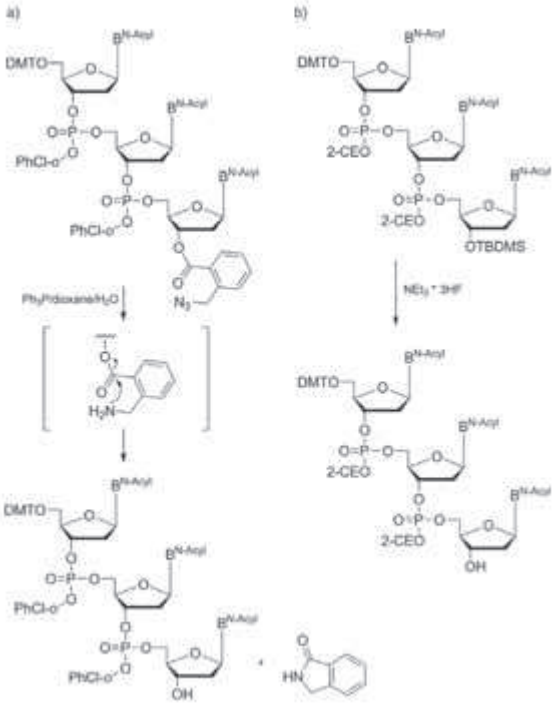

Figure 3. Final removal of 3'-OH protecting groups after trinucleotide preparation following the phosphotriester approach (a) or phosphite triester chemistry (b).

dichloromethane (1:3, v/v) with double and triple couplings and a twofold extended coupling time. Coupling yields of 71 to 98% were obtained. Due to the superior properties of the 2-azidomethylbenzoyl group for 3'-*O*-protection/deprotection, the protocol by Yagodkin *et al.* (9) is the most favourable among the reported strategies for trinucleotide synthesis by phosphotriester chemistry.

Also worth mentioning is a more recent approach for preparation of a trinucleotide synthon to be used in stop-codon scanning mutagenesis (10). A 5'-Fmoc protected TAG trinucleotide was synthesized in 5' to 3' direction, basically following the reaction sequence illustrated in Figure 2, although with Fmoc instead of DMT as 5'-blocking group. The important novel step is the *in situ* production of an intermediate phosphorylation reagent, 2-chlorophenylphosphoroditriazolide, by titrating 2-chlorophenyl dichlorophosphate into triazole and trimethylamine. By reaction with 5'-Fmoc thymidine, the first triazolide on the phosphate is replaced by the nucleoside, and reactivity of the second triazolide is sufficiently reduced to couple *N*-benzoyladenosine without further sugar protection. The in situ activation step was repeated for coupling of the dinucleotide with *N*-isobutyrylguanosine. Not surprisingly, as mentioned already above, 3'-3'-linked dimers and trimers were identified as side products, in spite of the higher selectivity of the reaction for the 5'-OH group over the 3'-OH group. However, as reported (10) 3'-3'-coupling products could be easily removed by chromatography. Subsequent phosphitylation delivered the TAG trinucleotide phosphoramidite, which was used in DNA library synthesis in parallel with DMT-protected phosphoramidite monomers producing stop-codons at specific positions. Removal of the *5'-O-Fmoc* group was conducted with 0.1 M DBU solution directly at the synthesizer. Unfortunately, the reactivity of Fmoc-TAG reached only ~20% of that of the DMT-mononucleotides.

TRINUCLEOTIDE SYNTHESIS BY PHOSPHITE TRIESTER CHEMISTRY

As mentioned in the introduction, preparation of trinucleotide synthons for DNA library synthesis actually started with phosphite triester chemistry (4). The intermittent preference for phosphotriester chemistry was certainly owing to the higher stability of phosphotriesters over phosphite triesters. Nevertheless, we decided to reinvestigate trinucleotide synthesis by phosphite triester chemistry, and focused our attention on defining a suitable set of orthogonal protecting groups for the 5'- and 3'-OH functionalities and for the phosphite/phosphate (Figure 3b, 4). A suitable set was found consisting of DMT for protection of 5'-OH, TBDMS (*tert*-butyldimethylsilyl) for protection of 3'-OH and β-cyanoethyl for protection of the phosphite/phosphate (11). An *N*-acyl-5'-*O*-DMT protected nucleoside-β-cyanoethyl phosphoramidite was coupled by activation with tetrazole with a 3'-*O*-TBDMS protected *N*-acylnucleoside followed by oxidation to the phosphate and deprotection of the 5'-OH group. The dinucleotide was extended in 5'-direction by reaction with another *N*-acyl-5'-*O*-dimethoxytrityl nucleoside phosphoramidite. Subsequently, the TBDMS group was selectively removed by treatment with the mild agent 3 HF/triethylamine for further conversion into the trinucleotide phosphoramidite (Figure 3b). According to this protocol we have synthesized 20 fully protected trinucleotide synthons that were successfully coupled to short test-sequences (11). Our strategy has overcome the problems of previous trinucleotide synthesis associated with the used protection schemes. In their initial work back in 1994, Virnekäs and co-workers (4) used phenoxyacetyl (PAC) for 3'-OH protection (Figure 1, 4). This group was removed with methanolic ammonia, conditions that are known to also cause cleavage of phosphrtriesters, and thus to damage the synthesized trinucleotide. Accordingly, the overall yields of the synthesized trinucleotides were rather low, and a significant amount of by-products was found (4).

There has been also a report in the past on trinucleotide synthesis with TBDMS as 3'-*O*-protecting group (12). At that time, TBAF (tetrabutymammonium fluoride) was the typical reagent for removal of the TBDMS group. However, it also cleaves phosphotriesters, and therefore cannot be used for deprotection of the 3'-OH group. Taking this into account, Lyttle and co-workers (12) used 6 N HCl for simultaneous cleavage of 5'-DMT and 3'-TBDMS, and trusted on the higher selectivity of the following coupling reaction with an activated nucleotide for the primary 5'-OH group. Of course, as already discussed in the cases above, side products, in particular 3'-3'-coupling products were observed. Moreover, the strong acidic conditions (6N HCl!) can cause a number of other side reactions such as for example depurinations, and accordingly, obtained yields were rather low.

Another protocol is based on Fmoc for 5'-*O*-protection, and DMT for 3'-*O*-protection (13) (Figure 4). This turned out being a suitable strategy in terms of selective deprotection, however, due to the 5'-Fmoc group at the final trinucleotide synthons, their use in automated oligonucleotide synthesis required substantial modification and implementation of additional steps in the program. Therefore, later, the protection scheme was reversed to 5'-*O*-DMT and 3'-*O*-Fmoc, although coupling chemistry was switched to P(V) using the phosphotriester approach (Figure 2) (14).

CONCLUSION

Trinucleotide synthons are superior building blocks for the codon based synthesis of genes, and moreover, owing to their easy use in mixtures of defined stoichiometry, for the preparation of fully

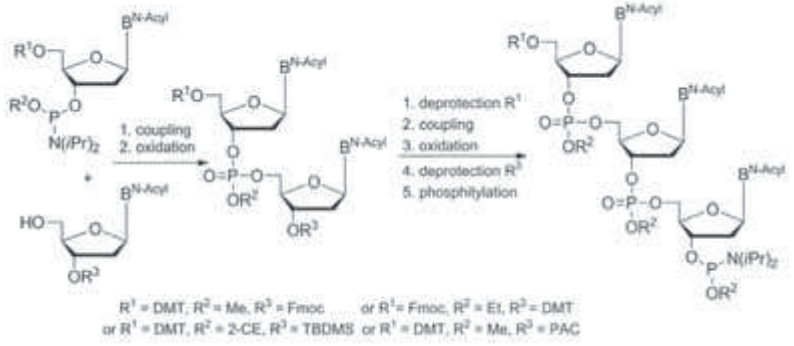

Figure 4. Synthesis of trinucleotide synthons by phosphite triester chemistry.

controlled total or partial randomized gene libraries. There are several ways of producing trinucleotide synthons using P(III) or P(V) chemistry. It is difficult, if not impossible, to compare the individual strategies with regard to special parameters such as yields, estimated costs or full-scale production abilities. The reported syntheses were conducted under rather different conditions, and yields, if given at all, are hardly comparable. In general, the higher stability of phosphotriester over phosphite triesters, in particular when 2-chlorophenyl is used for phosphate protection makes preparation of trinucleotides by phosphrtriester chemistry advantageous. However, reactivity of phosphotriester is lower compared with phosphite triesters, and an additional step for removal of 2-chlorophenyl groups has to be implemented in the program, when such trinucleotide synthons are used in the synthesis of oligonucleotides. Preparation of trinucleotide synthons by coupling of β-cyanoethyl phosphoramidites overcomes this limitation, because the ß-cyanoethyl group is routinely used in modern oligonucleotide synthesis and its removal is a standard step in the synthesis program. Among the host of related methods, the clever choice of orthogonal protecting groups decides on the quality of the trinucleotide products, with 5'-*O*-DMT/3'-*O*-(2-azidomethylbenzoyl) (9) and 5'-*O*-DMT/3'-*O*-TBDMS (11) being most convincing combinations. Beyond protocols for trinucleotide synthesis in solution, the further trend might go to solid phase strategies, which however, requires another layer of orthogonality: cleavage of the trinucleotide from the support must proceed under conditions that leave all other protecting groups intact (15).
Trinucleotide synthons can be used in standard phosphoramidite oligonucleotide synthesis requiring only small adaptations of the coupling protocol, such as slightly longer coupling times and/or repeated coupling cycles. With such optimized protocols, coupling yields of ~ 98% are reached (3). Thus, the preparation and use of trinucleotide synthons for the generation of gene libraries with partial or full randomization at any given position has the potential of greatly enhancing the prospects for protein design in the broad area of biotechnology.

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Preparation of trinucleotide phosphoramidites as synthons for the synthesis of gene libraries

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Review

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Abstract

The preparation of protein libraries is a key issue in protein engineering and biotechnology. Such libraries can be prepared by a variety of methods, starting from the respective gene library. The challenge in gene library preparation is to achieve controlled total or partial randomization at any predefined number and position of codons of a given gene, in order to obtain a library with a maximum number of potentially successful candidates. This purpose is best achieved by the usage of trinucleotide synthons for codon-based gene synthesis. We here review the strategies for the preparation of fully protected trinucleotides, emphasizing more recent developments for their synthesis on solid phase and on soluble polymers, and their use as synthons in standard DNA synthesis.

Introduction

Protein engineering is a highly actual research area with a number of potential applications [1-4]. The construction, adaptation and optimization of proteins can proceed by two major strategies: (i) rational design or (ii) directed evolution. The rational design is based on the introduction of point mutations, insertions or deletions at a defined position of the protein sequence, and requires detailed knowledge of the protein structure and the mechanism of action. On the opposite, directed evolution relies on the selection of a mutant with predefined properties from a random protein library. This strategy is advantageous over the rational design; whenever molecular properties of proteins are investigated that are not yet sufficiently understood, if properties like solvent or temperature stability need to be optimized, or regio-, chemo- or enantioselectivity and substrate specificity shall be changed. Thus, the optimization and variation of proteins, in particular of enzymes, by random mutagenesis and subsequent selection and identification of mutants with improved properties is a favoured method in the field of white biotechnology and biocatalysis, to improve the fitness of enzymes for industrial application [5].

In general, directed evolution may be summarized as an iterative two-step process which involves the generation of protein mutant libraries and high throughput screening processes to select for variants with improved traits. Protein mutant libraries are produced from gene libraries, which are generated by random mutagenesis at DNA level. Often polymerase chain raction (PCR)-based methods like error-prone PCR are used for this purpose as well as recombinant methods like DNA shuffling and related strategies [6,7]. One of the major challenges in gene library production is to generate libraries with a high number of promising candidates to enhance the chance of selecting functional protein variants. The methods mentioned above allow the degree and localization of randomization to be adjusted to a certain degree, however, full control over mutagenesis is still rather limited. Oligonucleotide-based methods with a number of sophisticated techniques [8] are advantageous here, as they offer a better possibility to control randomization. The basic principle consists of using chemically synthesized primers of mixed composition for introducing subsets of the 20 canonical amino acids at a defined position of the protein [9]. In the simplest way, a mixture of the four standard nucleotides is used for coupling at each randomized position of the primer in DNA synthesis. For a primer with 9 randomized positions (corresponding to three randomized amino acids in the resulting protein) this would lead to $4^9 = 262144$ sequence variants including stop codons and codons of undesired amino acids, and a bias towards amino acids encoded by multiple codons. Moreover, it is impossible to restrict randomization to a defined subset of amino acids at a desired position. Thus, the result is a rather large library, however, with only a small number of potentially successful candidates. There are strategies to at least partially circumvent this problem, like using NNS instead of NNN codons (with N = A, C, G, T; S = C, G) taking advantage of redundancy of the third nucleotide positions in the majority of codons [10], or using spiked oligonucleotides [11], which are synthesized from solutions of the four nucleotide building blocks, each of those contaminated with a "spiking mix" consisting of equal aliquots of each of the four building blocks [9,12]. The required volume of the spiking mix to achieve a desired amount of nucleotide replacements at a defined position of the oligonucleotide can be calculated, such that library size and degree of randomization can be restricted [13,14]. Nevertheless, although those methods and sophisticated variations of them [14-17] have improved library design and synthesis, full control over randomization is not possible. This can be achieved only by the usage of trinucleotide synthons for codon-based synthesis of a desired primer [18]. Taking the example from above, for a DNA fragment encoding three randomized amino acids, instead of nine nucleotide positions to be randomized, variation of trinucleotides (codons for the 20 amino acids) at only three positions is required. Therefore, the number of possible sequence variants in the gene library decreases from $4^9 = 262144$ to $20^3 = 8000$, if the full set of the 20 amino acids is desired at each of the three randomized positions. The library size can be even further decreased by using subsets of amino acids (e.g., only basic or only acidic amino acids) at the individual positions. Furthermore, stop codons as well as bias to amino acids with codon redundancy are completely prevented. Not at last, the coupling efficiency of individual trinucleotide synthons in chemical DNA synthesis can be considered when preparing the trinucleotide mixture, to ensure that each of the trinucleotides is coupled with identical statistical probability, or alternatively, to adjust the trinucleotide mixture to a desired amino acid distribution at the respective position. Thus, the application of trinucleotide building blocks for the synthesis of gene libraries stands out as facilitating fully controlled total or partial randomization at any predefined number and position of codons of a given gene. Trinucleotide synthons need to be chemically synthesized. Here, the challenge has been to find a suitable set of orthogonal protecting groups that allows the preparation of the trinucleotide, its conversion into a coupling competent building block, and its subsequent use in chemical DNA synthesis. Trinucleotides have been prepared in solution [19], on solid phase [20], and more recently on soluble polymers [21-23] (Figure 1), followed by phosphitylation to be used in standard DNA synthesis.

The preparation of mixed oligonucleotides for random mutagenesis including the strategy of using trinucleotide synthons has been reviewed recently [19,24]. Therefore, herein we will concentrate on more recent developments in trinucleotide synthesis.

Review

1. Preparation of trinucleotides in solution

Over the years, a number of methodologies has been published, varying in the protecting group for the phosphate moiety being methyl [25], ethyl [26], cyanoethyl [27] or *ortho*-chlorophenyl [28,29], and for the 3'-OH-group being phenoxyacetyl [25], dimethoxytrityl (DMTr) [26], *tert*-butyldimethylsilyl (TBDMS) [27,30], levulinoyl [26], or 2-azidomethylbenzoyl [27] (Figure 2), and applying either phosphotriester chemistry [28,29,31,32] or phosphite triester chemistry [25-27,30] in solution.

In general, trinucleotides can be assembled through the reaction of two suitably protected monomers to generate a dinucleotide, which then can be extended in either 5'- or 3'-direction (Figure 3).

Surprisingly, only one report has made use of this "economy", first coupling a 5'-*O*-DMTr-protected nucleoside-3'-*ortho*-

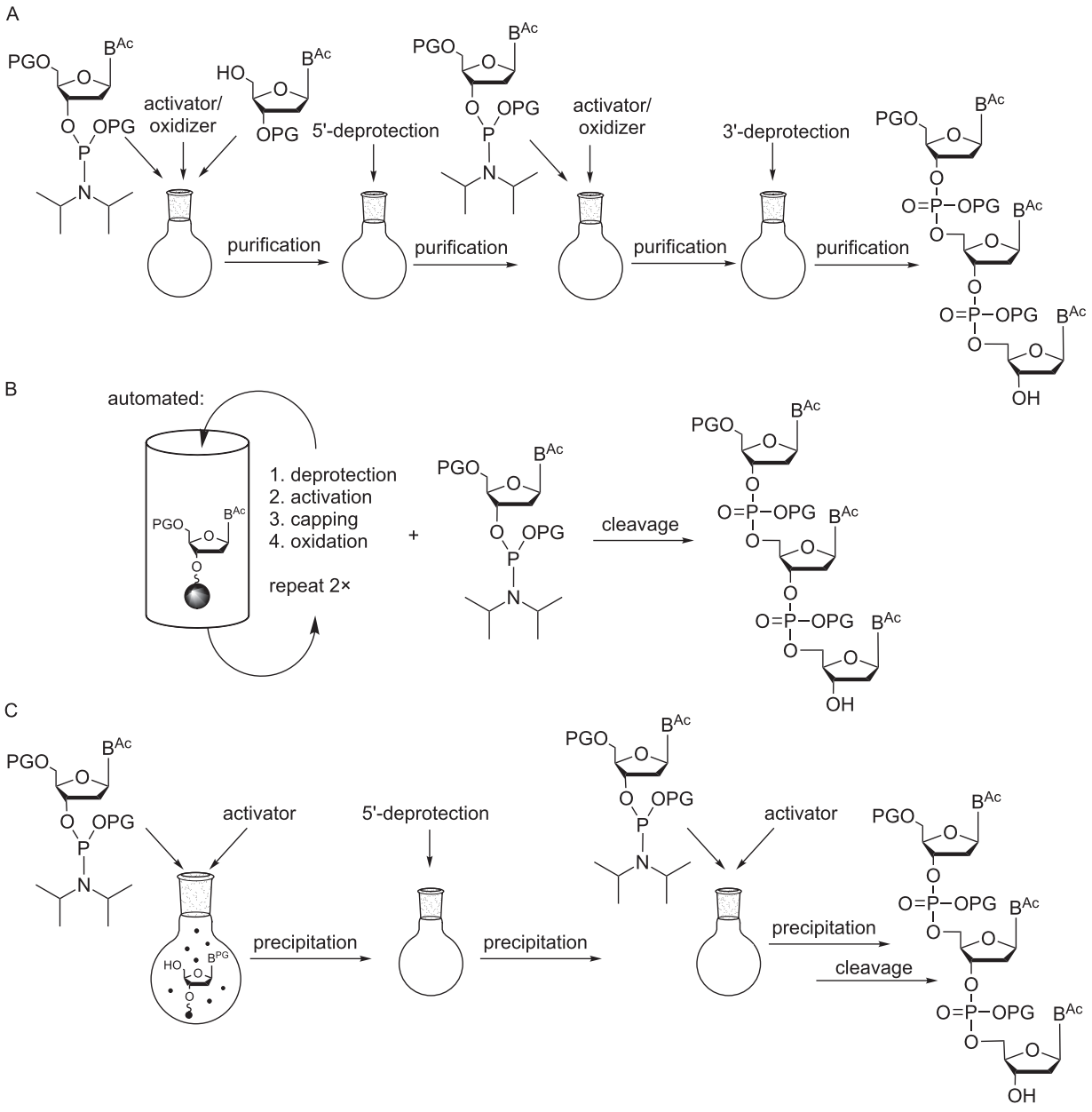


Figure 1: Preparation of fully protected trinucleotides in solution (A), on solid phase (B) and on soluble polymers (C).

chlorophenylphosphotriester to a 3'-*O*-levulinoyl-protected monomer. Upon selective removal of either the 5'-*O*-DMTr group or the 3'-*O*-levulinoyl group, the dimer was extended in 5' or 3' direction [26]. All other reports describe strategies, where the dimers are extended unidirectional, either in 5'-direction [25-27,29,30] or 3'-direction [31,32]. A key issue in all these methodologies is that the 5'- or the 3'-O-protecting group is selectively cleaved, whereas all other protecting groups (at the nucleobases, the phosphorous and the 5'- or alternatively 3'-OH group) remain intact.

Basically, this aim has been achieved, although in particular in earlier reports a number of problems associated with insufficient stability of protecting groups under synthesis conditions, as well as restricted orthogonality have been described, which was mirrored in the sometimes severely limited quality of the trinucleotide synthons and accordingly of the prepared oligonucleotide libraries [14,15,25,26,28,30,31,33]. Among the described procedures the use of *tert*-butyldimethylsilyl [25] and 2-azidomethylbenzoyl groups [29] for 3'-O-protection stands out as being the most successful in terms of high quality tri-

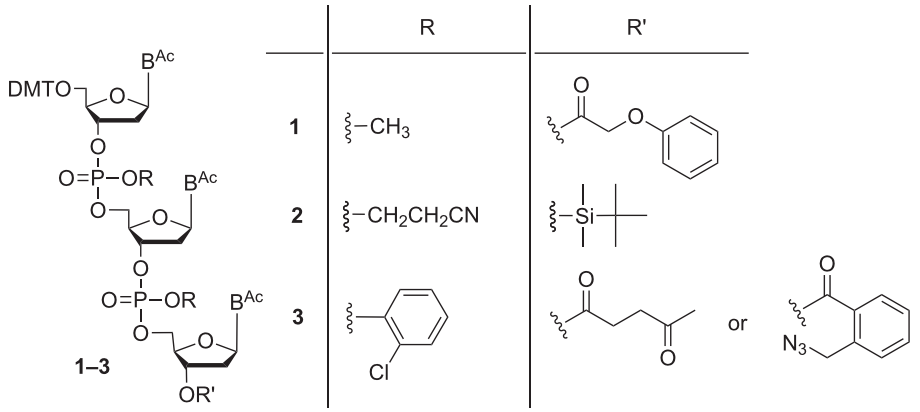


Figure 2: Strategies for trinucleotide synthesis using different pairs of orthogonal groups for protection of the phosphates and the 3'-OH-function.

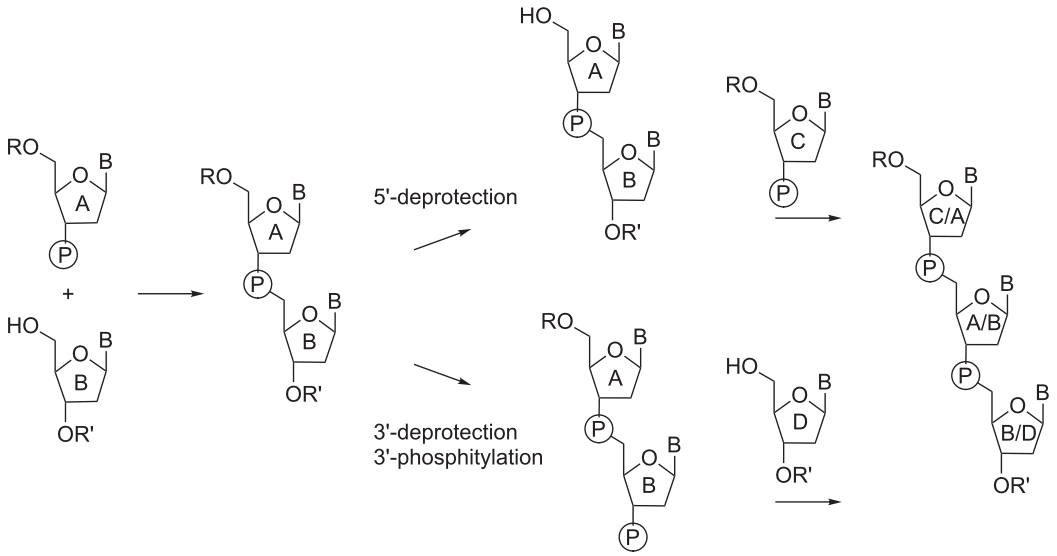


Figure 3: Strategy for the synthesis of nucleotide dimers and extension to the trimer in either 5'- or 3'-direction.

nucleotides. Both protecting groups, under the applied conditions, can be efficiently cleaved, at the same time leaving all other protecting groups intact. Thus, a full set of all 20 trimers was synthesized by phosphotriester chemistry starting with the condensation of *N*-acyl-3'-*O*-(*o*-chlorophenylphosphate)nucleosides to 3'-*O*-(2-azidomethylbenzoyl)-protected nucelosides, followed by removal of the 5'-*O*-DMTr group and extension of the dimer to the trimer by coupling of another *N*-acyl-3'-*O*-(*o*-chlorophenylphosphate)nucleoside. The final removal of the 2-azidomethylbenzoyl group occurred by reduction of the azide with triphenylphosphine in aqueous dioxane and subsequent spontaneous intramolecular cyclization leading to cleavage of the ester bond and release of the free 3'-OH group [29] (Figure 4A).

Also with 3'-*O*-TBDMS-protected monomers as mentioned above, a full set of trimers representing codons of all 20 amino acids was synthesized, although using phosphite triester chemistry [27]. In this case, the synthesis started with the coupling of an *N*-acyl-5'-*O*-DMTr-protected nucleoside-3'-*O*-phosphoramidite to an *N*-acyl-3'-*O*-TBDMS-protected nucleoside, followed by oxidation of the internucleotide phosphorous. Upon cleavage of the 5'-*O*-DMTr group, the dimer was reacted with another *N*-acyl-5'-*O*-DMTr-protected nucleoside-3'-*O*-phosphoramidite to afford the trimer. The 3'-*O*-TBDMS group was selectively removed under mild conditions with trimethylamine/3HF (Figure 4B) with strict control of pH to leave the β-cyanoethyl groups at the internucleotide phosphates intact [27]. With both procedures (3'-*O*-(2-azidomethylbenzoyl) and 3'-*O*-

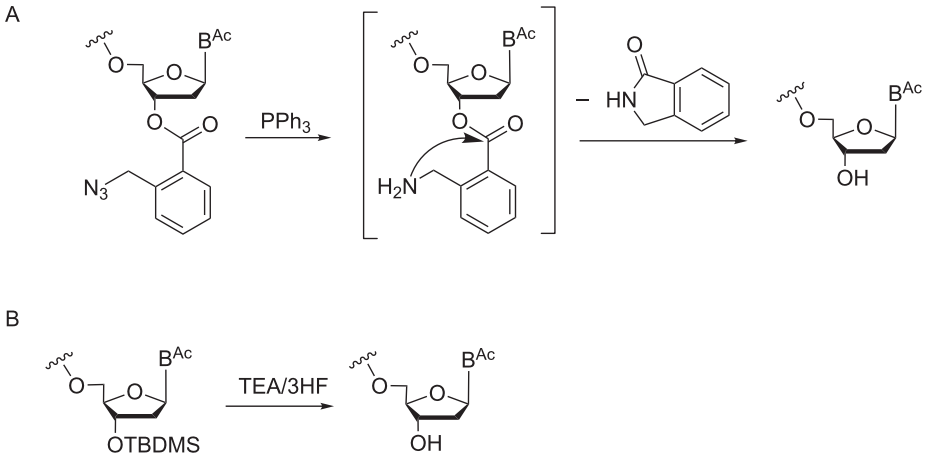


Figure 4: Removal of the 3'-O-protecting group under conditions that leave all other protecting groups at 5'-OH, nucleobases and internucleotide phosphates intact.

TBDMS protection), 20 trinucleotides of high purity were prepared and upon phosphitylation used as synthons in oligonucleotide synthesis [27,29].

In general, the reported syntheses of trinucleotides in solution proceed by either phosphite triester chemistry or phosphotriester chemistry with the latter being the more robust method. Also H-phosphonate chemistry has been used for assembling short oligomers in solution [34], although not with the aim of generating trinucleotide synthons for gene synthesis.

2. Preparation of trinucleotides on solid phase

Given the fact that trinucleotide synthesis in solution requires tedious purification and isolation of the products after each step of the synthesis, the assembly of trimers on a solid phase appears to be an attractive alternative. However, it has to be taken into account that the 3'-start nucleoside is required to be linked to the solid phase in a way that allows the cleavage of the trimer from the solid support, but leaves all other protecting groups intact. Therefore, the routinely used succinate linkage for immobilization of the start nucleotide cannot be used. Instead, linkers that allow a release of the trimers by a non-nucleophilic and/or non-basic treatment are required. In terms of trimer synthesis only one report in the literature describes such a strategy: The start nucleoside was loaded onto controlled pore glass (CPG) via an oxalyl anchor (Figure 5A), which after the synthesis was cleaved with a 5% solution of 25% aqueous ammonia in methanol, or with 20% pyridine in methanol [20].

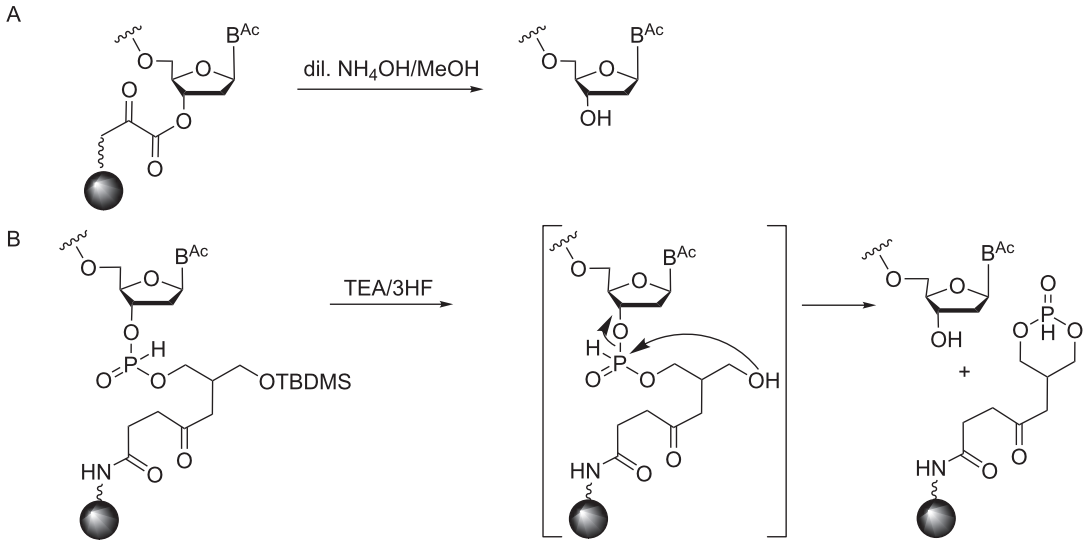


Figure 5: Release of trinucleotide blocks from the solid support by cleavage of an oxalyl anchor (A) and by a transesterification mechanism (B).

Combined with phosphotriester chemistry for trimer assembly, this treatment did not cause damage of the phosphotriester linkages and the nucleobase *N*-acyl groups. Using this strategy the large scale synthesis (5 g) of 3'-unprotected trinucleotides proceeded with a total 75–90% yield [20].

Other strategies with potential for the solid-phase synthesis of protected trinucleotides might rely on a universal solid support, from which oligomers with free 3'-OH function are released by a transesterification mechanism [35]. The 3'-start nucleoside is bound to one of the primary hydroxy groups of CPG-linked glycerol via an H-phosphonate linkage (Figure 5B). The removal of the TBDMS group from the remaining primary alcohol of glycerol induces the spontaneous cleavage of the H-phosphonate and the release of the oligomer with the free 3'-OH group leaving all other protecting groups intact. This strategy has been shown to be compatible with phosphoramidite chemistry and β-cyanoethyl protection of the internucleotide phosphates [33].

A more recent report describes the preparation of a polystyrene support decorated with a photolabile linker and its potential use for the synthesis of siRNA duplexes under mild and neutral conditions [36]. A similar strategy was used for the synthesis of partially 2'/3'-*O*-acetylated RNA oligonucleotides [37]. A photo-cleavable linker would also have potential for the synthesis of protected trinucleotides, as it would allow the cleavage of the trimer from the support by irradiation with UV light, without harming nucleobase and internucleotide phosphate protection. Nevertheless, photo-induced formation of byproducts may be an issue to be considered.

In our lab, we have been developing a strategy for solid-phase trinucleotide synthesis involving a disulfide linkage to the support (CPG or polystyrene), which can be cleaved under reductive conditions without harming nucleobase and phosphate protecting groups. The disulfide bridge is generated through the reaction of a 3'-*O*-methylthiomethyl-functionalized nucleoside with 2-mercaptopropionic acid and subsequent coupling to amino-functionalized CPG or polystyrene. After assembly of the trinucleotide on the support, the disulfide bridge is cleaved by treatment with dithiothreitol (DTT) [38] or tris-(2-carboxyethyl)phosphine (TCEP, Figure 6) leaving all other protecting groups intact.

The resulting hemi-(*S,O*)-acetal at the nucleotiode 3'-terminus is spontaneously degraded into the alcohol and thioformaldehyde, thus delivering the trimer with free 3'-OH group for subsequent phosphitylation. The detailed strategy and syntheses will be described elsewhere.

3. Preparation of trinucleotides by inverse solid-phase synthesis

Interestingly, also the use of polymer-supported reagents for H-phosphonate or phosphoramidite activation and phosphite oxidation has been described [34,39], thereby combining the advantages of solution chemistry and solid-phase methods. Thus, solid-supported acyl chloride or pyridinium tosylate as the activator of nucleoside-3'-*O*-H-phosphonates/phosphoramidites, and polystyrene-bound trimethylammonium periodate as oxidation reagent have been demonstrated to be superior for dimer and trimer synthesis, as complicated purification steps can be avoided, and excess reagents are easily removed by filtration. Compared with standard phosphotriester and phosphite triester chemistry, the limitations of this approach are lower coupling yields and side reactions hampering the yield and quality of the desired products [34,39].

4. Preparation of trinucleotides on soluble supports

Another strategy of combining the advantages of solution chemistry and solid-phase methods is the assembly of oligonucleotides on soluble supports. Among the supports used for this purpose, polyethylene glycol (PEG) has a prominent position, appearing as the routinely used polymer [40-44]. The isolation of intermediate and final products from the reaction mixture proceeds by precipitation from diethyl ether and filtration, thus significantly speeding up the process. In addition, the method is

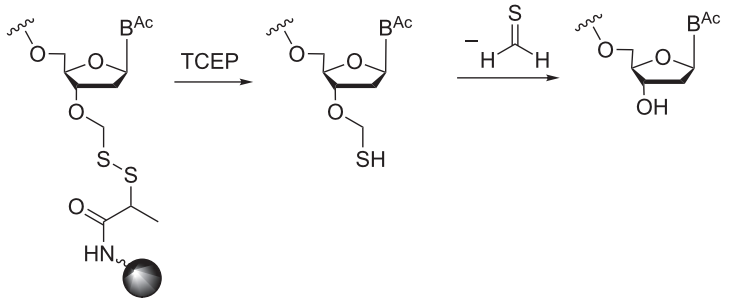


Figure 6: Release of the trinucleotide from the support under reductive conditions.

favorable in terms of producing oligonucleotides at a larger scale, since the reaction proceeds in homogeneous solution on a rather cheap polymer. The synthesis of oligonucleotides on soluble supports has been reviewed recently [45], showing that a variety of soluble polymers and precipitative supports are well suited to it. Also the solution-phase synthesis of protected trinucleotide building blocks has been described in the literature [21-23]. In an initial attempt, thymidine as a start nucleoside was tethered to a precipitative tetrapodal soluble support via a disulfide-linker [21] (Table 1, entry 1).

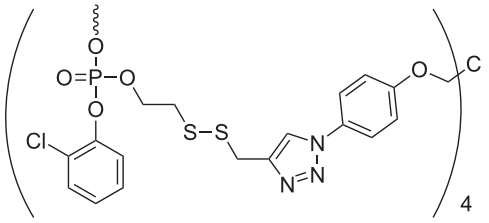
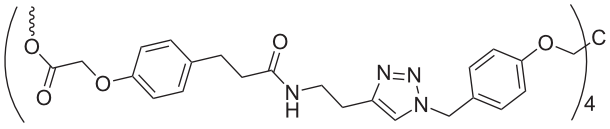
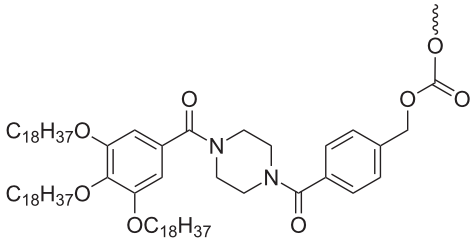
Upon detritylation, the support carrying the start nucleoside now having a free 5'-OH group was precipitated from methanol, followed by coupling with a 5'-*O*-DMTr-protected nucleoside-3'-*O*-(*o*-chlorophenyl)phosphate activated as benzotriazol and renewed precipitation with methanol. The resulting dimer was then extended to the trimer by another cycle of detritylation, precipitation, coupling and precipitation. During reductive cleavage of the disulfide bond to release the fully protected trimer from the support, unfortunately the loss of the 5'-DMTr group was observed. To overcome this hurdle, the disulfide tether was replaced in a following-up study with a Q-linker (hydroquinone-*O,O'*-diacetic acid), to be cleaved with dilute methanolic K_2CO_3 for the release of trimers in fully protected form. Five different trimers were assembled at 0.5 mmol scale and released form the support as described [22] (Table 1, entry 2). Thus, the fully protected trinucleotide building blocks were obtained with 65 to 70% yield from three coupling cycles, each containing two precipitations.

Yet another method for the synthesis of oligonucleotide blocks has been developed using a Cbz-type alkyl-chain-soluble support [23]. The support was attached via the benzyloxycarbonyl (Cbz) group to the 3'-OH of the starting nucleoside being adenosine, cytidine, guanosine or thymidine, and trimers were assembled by phosphoramidite chemistry (Table 1, entry 3). The support was found to disperse homogenously in the reaction solvents and to precipitate upon the addition of a polar solvent, typically methanol. After coupling of a standard phosphoramidite building block followed by oxidation with 2-butanone peroxide in dichloromethane, the resulting dimer on the support was again precipitated with methanol and filtered, before detritylation and coupling of the third monomer. The release of the trimer in fully protected form from the support was achieved by hydrogenation with Pd/C (10%) in tetrahydrofurane (THF) for 40 h at room temperature. Three fully protected trimers were prepared this way with isolated yields in the range of 44 to 49% [23].

5. Phosphitylation and coupling of trinucleotide synthons in solid phase DNA synthesis

To be used as building blocks in standard phosphoramidite synthesis, fully protected trimers need to be converted in phosphoramidites (Figure 7).

Table 1: Assembly of trimers on soluble supports.

entry	soluble support	5'-O-PG	chemistry	release conditions
1		H	phosphotriester	TCEP, NEt_3, MeOH, 3 h, 57%
2		DMTr	phosphotriester	K_2CO_3,DCM/MeOH/ dioxane, 30 min, 88–99%
3		DMTr	phosphoramidite	H_2/Pd, THF, 40 h, 44–49%

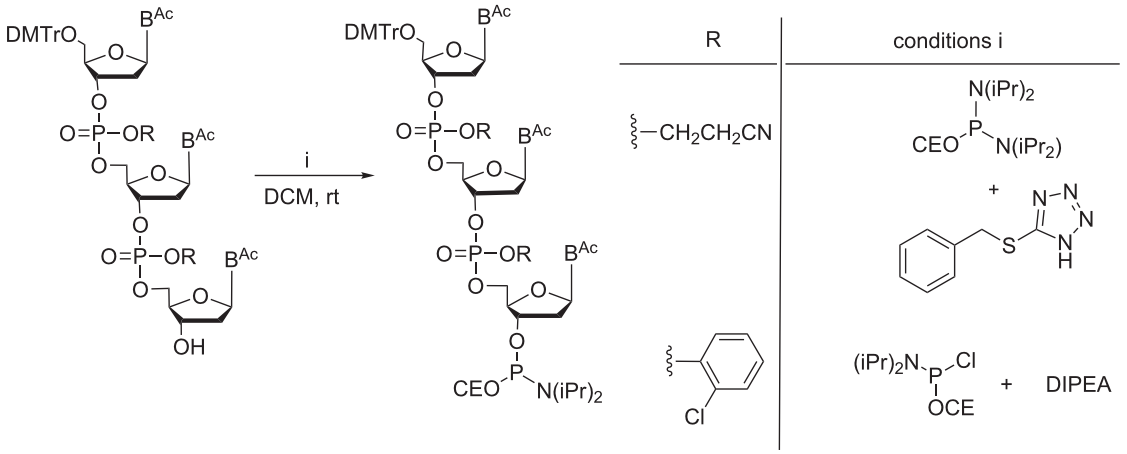


Figure 7: Phosphitylation of trimers. Reaction conditions, in particular the choice of the phosphitylation reagent, are dependent on the nature of the protecting group at the internucleotide phosphates.

This has been described in a number of reports [19,22,27,29], and is easily achieved with trimers having *o*-chlorophenyl groups for protection of the phosphate moiety [22,29]. However, phosphitylation becomes a crucial step, if β-cyanoethyl is used as the phosphate protecting group [27]. Using 2-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite for phosphitylation requires the presence of *N,N*-diisopropylethylamine (DIPEA) to neutralize HCl that is generated during the reaction. This, however, would lead to the removal of the β-cyanoethyl group at the phosphate moieties, which, due to the phosphorous atom in the oxidized state, is highly sensitive to basic agents and readily undergoes β-elimination [27].

An alternative reagent is 2-cyanoethyl-*N,N,N′,N′*-tetraisopropylphosphordiamidite in combination with tetrazole derivatives such as benzylmercaptotetrazole. Under those conditions, the phosphitylation proceeds with the production of one equivalent of diisopropylamine, which is neutralized by benzylmercaptotetrazole released back after the reaction. The tetrazole derivative is sufficiently acidic to act as a scavenger for diisopropylamine converting it into the ammonium salt. Thus, fully protected trimers can be converted to phosphoramidites without the loss of the β-cyanoethyl groups at the internucleotide phosphate linkages [27].

For the use in standard oligonucleotide synthesis, trinucleotide phosphoramidites have been dissolved in a mixture of acetonitrile and dichloromethane to a concentration of 0.1–0.15 M. The coupling yields are typically between 70–95%, preferentially with double or triple couplings, and a coupling time of 120 to 300 s [22,27,29].

Conclusion

The synthesis of fully protected trimers can be achieved in solution, on a solid phase or on soluble supports. The key element is the choice of a suitable set of orthogonal protecting groups to allow the selective deprotection of the functionality required for the reaction, while leaving all other protecting groups intact. The first trinucleotide synthesis was performed in solution using phosphotriester or phosphoramidite chemistry. More recently strategies for trimer assembly on a solid phase or soluble supports have been developed. Here, release of the synthesized trimer in fully protected form from the support is the crucial step. This has been convincingly achieved by using molecular entities linking the trimer to the support, which can be selectively cleaved either under reductive conditions (disulfide cleavage or hydrogenation) or under mild basic conditions leaving all protecting groups at the trimer undamaged.

In particular, soluble support strategies have great potential for an efficient large scale synthesis of fully protected trinucleotides. The essential feature here is that small molecular reagents can be easily removed after coupling and 5'-*O*-deprotection, by quantitative precipitation of the soluble support in a polar solvent, such as methanol.

With the developments in the field of biotechnology and protein engineering, the preparation of gene libraries has become a major issue. In this regard, the use of trinucleotide synthons for codon-based gene synthesis has high potential, as it allows the fully controlled total or partial randomization at any predefined number and position of codons of a given gene. Methods for their large scale preparation are available now.

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2.3 R. Suchsland, B. Appel, S. Müller, Synthesis of trinucleotide building blocks in solution and on solid phase. Curr. Protoc. Nucleic Acid Chem. 2018, 75, 1-26

Synthesis of Trinucleotide Building Blocks in Solution and on Solid Phase

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We have developed two methods, in solution and on solid phase, that give easy access to trinucleotide phosphoramidites capable of undergoing coupling reactions by the solid-phase phosphoramidite approach. The solution protocol is characterized by application of 5′-*O*-dimethoxytrityl (DMT) and 3′-*O*-*tert*-butyldimethylsilyl (TBDMS) as a pair of orthogonal protecting groups and 2-cyanoethyl (CE) for protection of the phosphate. Starting with suitably functionalized monomers, synthesis proceeds in the 3′- to 5′-direction, delivering the fully protected trinucleotide. The 3′-*O*-protecting group is cleaved followed by phosphitylation of the free 3′-OH group. The solid-phase protocol is based on standard phosphoramidite chemistry in conjunction with a dithiomethyl linkage connecting the 3′-starting nucleoside to the polymer. The disulfide bridge can be cleaved under neutral conditions for release of the trinucleotide from the support preserving all other protecting groups.

Keywords: codon • gene library • protein engineering • randomization • trinucleotide synthon

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INTRODUCTION

Protein engineering is a powerful technology that overcomes the restrictions of natural enzymes as biocatalysts (Davids, Schmidt, Bottcher, & Bornscheuer, 2013). Over the past several decades, interest in enzymes as biocatalysts for industrial use has strongly grown, as they enable new synthetic pathways with mild reaction conditions. In general, rational protein design and directed evolution are two alternative ways for the generation of enzymes with pre-deliberated, novel properties (Davids et al., 2013). For rational protein design, structural information is essential, but not always available, which is why directed evolution is often the method of choice (Shivange, Marienhagen, Mundhada, Schenk, & Schwaneberg, 2009). There are a number of techniques of combinatorial and evolutionary protein engineering available, which combine random mutagenesis or combinatorial gene synthesis with functional screening, at the phenotypic level, of the generated library of many structural variants. Successful screening relies on a library with a large number of potential hits and effective screening methods (Popova, Schubert, Bulla, Buchwald, & Kramer, 2015; Qu et al., 2018). Therefore, it is highly desirable not to waste a large proportion of a gene library on candidates that have little or no chance to pass the functional test. This means that randomization should be directed away from residues involved merely in the maintenance of scaffold structure and stability and towards residues that are expected to contribute to the envisaged new function. In addition,

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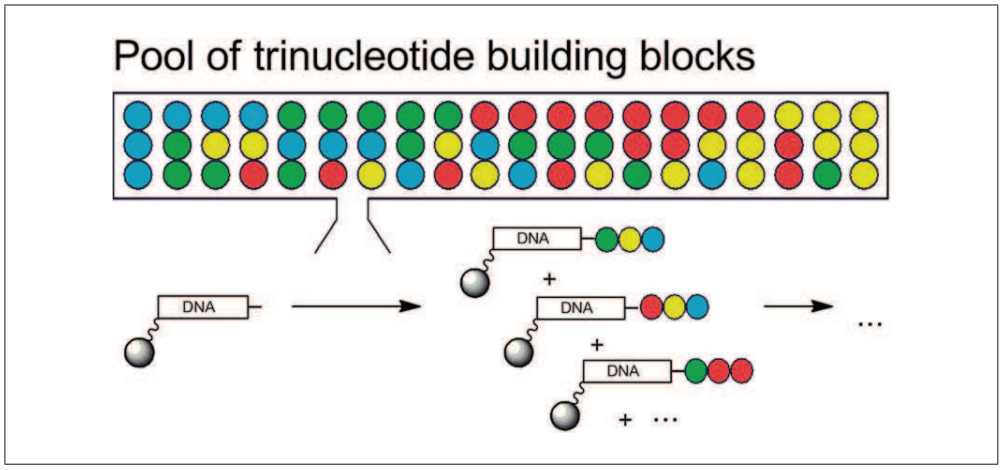

Figure 1 Scheme of DNA synthesis with trinucleotide synthons.

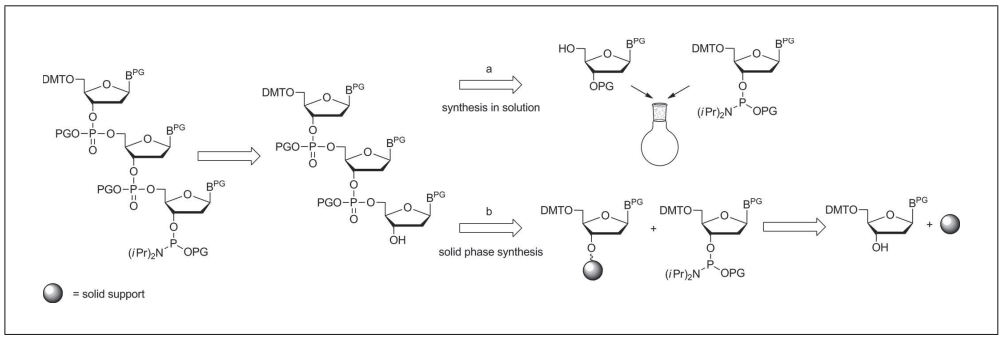

Figure 2 Retrosynthetic scheme of trinucleotide assembly in solution and on solid phase. Abbreviations: DMT, 4,4′-dimethoxytrityl; B^{PG}, protected base; PG, protecting group.

it is highly attractive to control not only the position but also the extent and quality of randomization. Among a host of related methods, the use of pre-defined mixtures of trinucleotide synthons representing the codons for the twenty canonical amino acids is a straightforward technique for codon-based synthesis of a gene segment (Fig. 1), fulfilling the requirements mentioned above (Arunachalam, Wichert, Appel, & Muller, 2012; Suchsland, Appel, & Muller, 2018). The attractiveness of the method lies in the fact that codon redundancy and stop codons are avoided, and that in contrast to all other methods, strictly controlled full or partial randomization at any arbitrarily chosen position of a given gene is possible. The advantage of using trinucleotide generated libraries has been demonstrated in the past mainly for randomization of immunoglobulins (Knappik et al., 2000) and more recently for tHisF of a hyperthermophile (Popova et al., 2015).

A number of ways for the preparation of trinucleotide phosphoramidites have been reported in the literature (recently reviewed in Suchsland et al., 2018). Whereas synthesis in solution has dominated the efforts in the past (extensively reviewed in Arunachalam et al., 2012) more recently trinucleotide synthesis on precipitative soluble supports (Jabgunde, Molina, Virta, & Lonnberg, 2015; Kungurtsev, Lonnberg, & Virta, 2016) or on solid phase have gained momentum.

This article describes two protocols for synthesis of trinucleotide synthons, in solution and on a solid support, as depicted in Figure 2.

CAUTION: All reactions must be run in a suitable fume hood with efficient ventilation. Safety glasses and reagent-impermeable protective gloves should be worn.

SYNTHESIS OF TRINUCLEOTIDE SYNTHONS IN SOLUTION

The synthesis of trinucleotides in solution requires the definition of a pair of orthogonal protecting groups for the 5′- and 3′-OH functionalities, in order to avoid side products by competition of the two hydroxyl groups during trinucleotide synthesis. Furthermore, permanent protection of the phosphate moieties as well as of the heterocyclic bases is required. The following protocol describes the synthesis of trinucleotide synthons in solution starting with an *N*-acyl-5′-*O*-DMT protected nucleoside-3′-*O*-CE-phosphoramidite **5**, which is coupled to an *N*-acyl-3′-*O*-TBDMS protected nucleoside **6**. The obtained dinucleotide **7** is extended in the 5′-direction by first removing the 5′-*O*-DMT group followed by coupling of another *N*-acyl-5′-*O*-DMT protected nucleoside-3′-*O*-CE-phosphoramidite to deliver the fully protected trinucleotide **9**. Finally, the 3′-*O*-TBDMS group is removed to generate the free 3′-OH function required for phosphitylation of the trimer **10** (Fig. 3).

Materials

Pyridine (stored over KOH, freshly distilled prior to use and kept at room temperature over 4 Å molecular sieves)
Desoxyadenosine (dA)
Desoxyguanosine (dG)

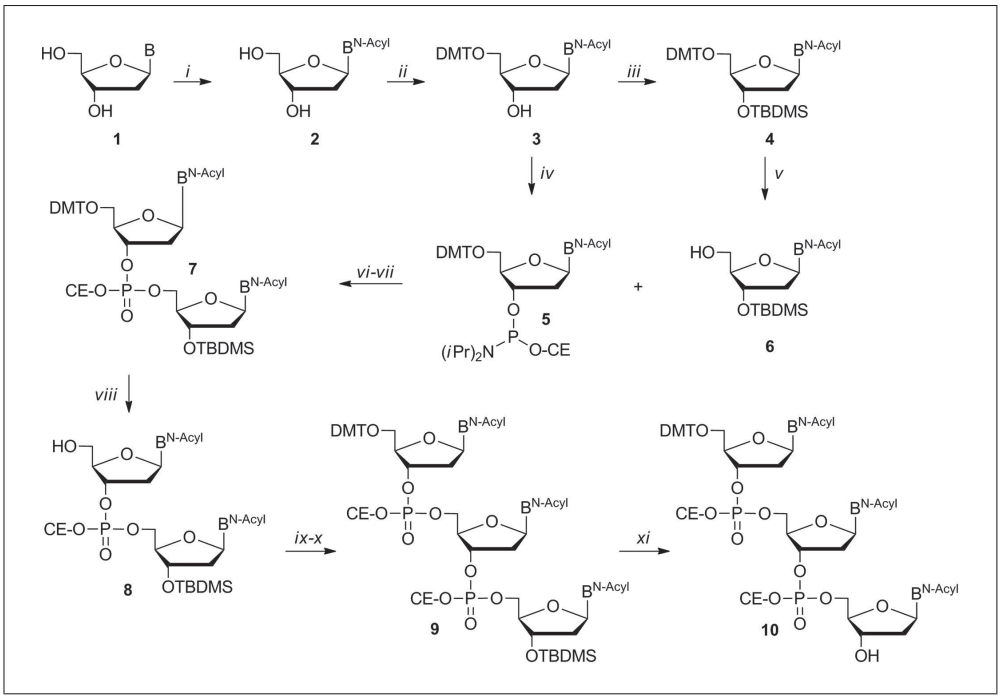

Figure 3 Reaction scheme for preparation of fully protected trinucleotides in solution. i) 1 eq. **1**, pyridine, 5 eq. TMSCl, 30 min, 5 eq. isobutyric anhydride (dG, dA) or 5 eq. BzCl (dC), 2 hr, H_2O, conc. aq. NH_3, 30 min, 0°C, H_2O; ii) 1 eq. **2**, dry pyridine, 1.5 eq. dry TEA, 0.05 eq. DMAP, 1.3 eq. DMTCl, rt, 3 hr; iii) 1 eq. **3**, 1.2 eq. TBDMSCl, 1.3 eq. $AgNO_3$, THF, 10% pyridine, rt, 10 hr; iv) 1 eq. **3**, 1.2 eq. 2-cyanoethyl-*N,N*-diisopropylphosphonamidic chloride, DIPEA, DCM, 5 min, rt; v) 1 eq. **4**, 3% TCA in DCM, 1 min, rt; vi) 1.3 eq. **5**, 1 eq. **6**, 10 eq. 5-benzylmercaptotetrazole, MeCN, 5 min, rt; vii) 0.2 M iodine solution (pyridine/THF/H_2O, 2:2:1), 5 min, rt; viii) 1 eq. **7**, 5% TCA in DCM, 5 min, rt; ix) 1 eq. **8**, 1.5 eq. **5**, 10 eq. 5-benzylmercaptotetrazole, MeCN, 5 min, rt; x) 0.2 M iodine solution (pyridine/THF/H_2O, 2:2:1), 5 min, rt; xi) 1 eq. **9**, TEA·3HF, DMF, rt, 2 hr. Abbreviations: eq., equivalent; rt, room temperature; TMSCl, trimethylsilyl chloride; BzCl, benzoyl chloride; TEA, triethylamine; DMAP, dimethylaminopyridine; TBDMSCl, *tert*-butyldimethylsilyl chloride; THF, tetrahydrofuran; DIPEA, *N,N*-diisopropylethylamine; DCM, dichloromethane; TCA, trichloroacetic acid; TEA·3HF, triethylamine trihydrofluoride; DMF, *N,N*-dimethylformamide; B, base; $B^{N\text{-}Acyl}$, N-acyl protected base; CE, 2-cyanoethyl; DMT, 4,4′-dimethoxytrityl.

Desoxycytidine (dC)
Thymidine
Trimethylsilyl chloride (TMSCl)
Isobutyric anhydride
Benzoyl chloride
Concentrated ammonia
Dimethylaminopyridine (DMAP)
Triethylamine (TEA)
4,4′-Dimethoxytrityl chloride (DMTCl)
Diethyl ether
tert-Butyldimethylsilyl chloride (TBDMSCl)
Silver nitrate ($AgNO_3$)
Tetrahydrofuran, anhydrous (THF)
Sodium bicarbonate ($NaHCO_3$), saturated aqueous solution
Sodium sulfate (Na_2SO_4)
Dichloromethane (DCM), anhydrous
3% trichloroacetic acid (TCA) in dichloromethane (v/v)
N,N-Diisopropylethylamine (DIPEA; stored over calcium hydride and freshly distilled)
2-Cyanoethyl-*N,N*-diisopropylchlorophosphoramidite
Methanol (MeOH), anhydrous
Ethyl acetate
Saturated aqueous sodium chloride solution (brine)
Acetonitrile, anhydrous
5-Benzylmercaptotetrazole (BMT)
0.2 M iodine solution (iodine in 2:2:1 pyridine/THF/H_2O, w/v/v/v)
6% aqueous sodium bisulfite solution ($NaHSO_3$; w/v)
Anhydrous dimethylformamide (DMF)
Triethylamine trihydrofluoride (TEA·3HF)
Silica gel for column chromatography (0.063-0.200 mm)
HPLC Buffer A: 5% acetonitrile in deionized water (w/w)
HPLC Buffer B: 70% acetonitrile in deionized water (w/w)

Magnetic stir plate
Magnetic stir bar
250-, 100-, and 50-mL round bottom flasks
25-, 50-, and 250-mL Schlenk flasks
250- and 50-mL measuring cylinders
20-, 10-, 5-, and 1-mL syringes with cannula (0.5 mL internal diameter)
Rubber septa
Rotary evaporator
Chromatography columns
TLC plate (Macherey-Nagel Alugram Sil G/UV_{254})
Ultraviolet light (254 nm)
ÄKTA Purifier (GE Healthcare)
RP-HPLC Column EC 250/4 Nucleodur 100-5 C18 ec (Macherey-Nagel)

N-acylation of 2′-deoxyadenosine, 2′-deoxycytidine, and 2′-deoxyguanosine (2)

1. Evaporate 10 mmol nucleoside twice with 50 mL dry pyridine in a 250-mL round bottom flask.
2. Add 100 mL dry pyridine.
3. Cool in an ice bath 10 min and add 5 mL trimethylchlorosilane (39.3 mmol) dropwise from a syringe.

4. Stir for 30 min.
5. Add 5 equivalents of the acylation reagent:

   8.5 mL isobutyric anhydride (51.3 mmol) for dA and dG
   6 mL benzoyl chloride (52.1 mmol) for dC).
6. Stir for 2 hr.
7. Add 20 mL ice cold water.
8. Add 20 mL concentrated ammonia.
9. Stir in an ice bath 30 min.
10. Concentrate residue *in vacuo* and add to the oily residue 50 mL water.
11. Wash aqueous layer with small amount of ether.

    dG crystallization in water starts within a few minutes.
12. Purify by silica chromatography to remove inorganic salts (eluant: DCM/MeOH, 8:2) to generate the products in 90% to 96% yield.
13. Characterize products by TLC (on silica, see Meyers & Meyers, 2008).

    Rf ≈ 0.5 (DCM/MeOH, 8:2).

5′-O-Dimethoxytritylation of 2′-deoxynucleosides (3)

14. Evaporate 10 mmol thymidine or *N*-acylated nucleoside **2** twice with 50 mL dry pyridine in a 250-mL Schlenk flask.

    Dry the flask prior to use with a heat gun and let it cool down to room temperature under vacuum. Use argon atmosphere during the whole experiment. Avoid any moisture.
15. Add 100 mL dry pyridine.
16. Add 61 mg DMAP (0.05 mmol) and 2 mL dry TEA (15 mmol).
17. Add 4.4 g DMTCl (1.3 equivalents) and stir 4 hr at room temperature.
18. Add 200 mL water and extract product with ether.
19. Remove solvent *in vacuo*.
20. Purify with silica gel chromatography (DCM/MeOH, gradient 99:1 to 95:5) to generate, after evaporation of the appropriate fractions, the desired products in 86% to 97% yield.
21. Characterize products by TLC (on silica).

    Rf ≈ 0.5 (DCM/MeOH, 9:1).

3′-O-Silylation of 5′-O-dimethoxytritylated desoxynucleosides (4)

22. Dissolve 5 mmol 5′-*O*-dimethoxytritylated desoxynucleoside **3** in a mixture of 20 mL anhydrous THF and 3 mL anhydrous pyridine in a 50-mL round bottom flask.
23. Add 1.01 g $AgNO_3$ (5.9 mmol) and 0.99 g TBDMSCl (6.6 mmol), and stir overnight.
24. Add saturated $NaHCO_3$ solution and extract product with DCM.
25. Wash with saturated $NaHCO_3$ solution and dry organic layer with Na_2SO_4.
26. Filter off Na_2SO_4 and remove solvent *in vacuo*.

The yield is nearly quantitative. Nucleosides can be used for the following syntheses without further purification.

27. Characterize products by TLC (on silica).

    Rf ≈ 0.8 (DCM/MeOH, 95:5).

Synthesis of nucleoside phosphoramidites (5)

28. Evaporate 1 mmol 5′-*O*-DMT protected nucleosides **3** twice with 5.5 mL of a mixture of dry DCM and dry pyridine (20:1).

29. Dissolve nucleoside in 10 mL dry DCM in a 50-mL Schlenk flask.

    Dry the flask prior to use with a heat gun and let it cool down to room temperature under vacuum. Use argon atmosphere during the whole experiment. Avoid any moisture.

30. Add 1 mL freshly distilled DIPEA (5.74 mmol).

31. Add 0.27 mL 2-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite (1.2 mmol) slowly while stirring.

32. Stir at least 5 min and check for complete conversion via TLC (DCM/ethyl acetate/TEA, 45:45:10). Add 1 mL MeOH.

33. Stir for 5 min. Add 4 mL TEA and 10 mL ethyl acetate.

34. Wash twice with saturated $NaHCO_3$ solution and brine.

35. Dry organic layer with Na_2SO_4, filter off the desiccant, and remove solvent *in vacuo.*

36. Purify by silica gel chromatography (eluent A: EtOAc/TEA, 9:1; eluent B: DCM/EtOAc/TEA 5:4:1).

    Start with A/B 1:1 and increase amount of B in 5% steps until phosphoramidite elution is completed.

37. Co-evaporate phosphoramidite with 5 mL dry DCM at least five times.

    This step is absolutely necessary for entire removal of triethylamine.

38. Characterize products by TLC (on silica).

    Rf ≈ 0.9 (DCM/ethyl acetate/TEA, 45:45:10; for dG = 0.4).

Removal of the 5′-O-DMT group (6)

39. Dissolve 5 mmol 3′-*O*-silylated-5′-*O*-dimethoxytritylated desoxyribonucleosides **4** in 100 mL DCM containing 3% trichloroacetic acid.

40. Stir for 10 min.

41. Pour reaction mixture into saturated aqueous $NaHCO_3$ solution in a separatory funnel.

42. Wash organic phase with saturated aqueous $NaHCO_3$ solution and dry organic layer with Na_2SO_4.

43. Filter off Na_2SO_4 and remove solvent *in vacuo.*

44. Purify with silica gel chromatography (DCM/MeOH, gradient 99:1 to 95:5) to generate, after evaporation of the appropriate fractions, the desired products in 84% to 90% yield.

45. Characterize products by TLC (on silica).

    Rf ≈ 0.5 (DCM/MeOH, 9:1).

Table 1 MS Data and Yield of 5′-*O*-DMT-3′-*O*-TBDMS Dinucleotides 7

5′-DMT-3′-OTBDMS dinucleotide **7**	Mass as Na-peak (*m/z*)	Calculated mass (*m/z*)	Yield (%)
AA	1196	1173	79
AC	1206	1183	83
AG	1212	1189	76
AT	1117	1094	89
CA	1206	1183	90
CC	1216	1193	87
CG	1222	1199	79
CT	1127	1104	87
GC	1222	1199	80
GG	1228	1205	65
GT	1133	1110	82
TA	1117	1094	80
TC	1127	1104	83
TG	1133	1110	81
TT	1038	1015	90

Coupling reaction I: Synthesis of dinucleotides (7)

46. Dissolve 1 mmol deoxynucleoside phosphoramidite **5** and 0.8 mmol 5′-*O*-deprotected deoxynucleoside **6** in 10 mL anhydrous acetonitrile in a 25-mL Schlenk flask.

    Dry the flask prior to use with a heat gun and let it cool down to room temperature under vacuum. Use argon atmosphere during the whole experiment. Avoid any moisture.

47. Add 1.92 g 5-benzylmercaptotetrazole (10 mmol) and stir 30 min.

48. Add 0.2 M iodine solution to the reaction mixture until no discoloration of the iodine is detectable.

49. Remove solvent *in vacuo* and dissolve residue in 50 mL DCM.

50. Wash organic layer with 50 mL aqueous 6% $NaHSO_3$ solution, then 50 mL saturated aqueous $NaHCO_3$ solution, and then 50 mL water.

51. Dry organic layer with Na_2SO_4, filter desiccant, and remove solvent *in vacuo.*

52. Purify with silica gel chromatography (DCM/MeOH, 9:1) to generate, after evaporation of the appropriate fractions, the desired products in 74% to 90% yield (Table 1).

53. Characterize products by TLC (on silica) and MS (MALDI; Table 1).

    Rf ≈ 0.6 (DCM/MeOH, 9:1)

Removal of the 5′-O-DMT group (8)

54. Dissolve dinucleotide **7** in 10 mL DCM containing 3% trichloroacetic acid (v/v).

55. Stir for 10 min.

56. Wash with saturated $NaHCO_3$ solution and dry organic layer with $NaSO_4$.

Table 2 MS Data and Yield of 5′-OH-3′-*O*-TBDMS Dinucleotides 8

5′-OH-3′-TBDMS dinucleotide **8**	Mass as Na-peak (*m/z*)	Calculated mass (*m/z*)	Yield (%)
AA	894	871	81
AC	904	881	85
AG	910	887	80
AT	815	792	82
CA	904	881	91
CC	914	891	86
CG	920	897	88
CT	825	802	85
GC	920	897	89
GG	926	903	81
GT	831	808	87
TA	815	792	81
TC	825	802	86
TG	831	808	82
TT	736	713	88

57. Filter off $NaSO_4$, remove solvent *in vacuo*, and purify with silica gel chromatography (DCM/MeOH, gradient 99:1 to 99:5) to generate yields from 80% to 91% (Table 2).

58. Characterize products by TLC (on silica) and MS (MALDI; Table 2).

    Rf ≈ 0.4 (DCM/MeOH, 9:1).

Coupling II (9)

59. Dissolve 1.2 mmol deoxynucleoside phosphoramidite **5** in 10 mL anhydrous acetonitrile in a 25-mL Schlenk flask.

    Dry the flask prior to use with a heat gun and let it cool down to room temperature under vacuum. Use argon atmosphere during the whole experiment. Avoid any moisture.

60. Add 1 mmol 5′-*O*-unprotected dinucleotide **8** and stir 2 min.

61. Add 1.92 g 5-benzylmercaptotetrazole (10 mmol) and stir 1 hr.

62. Add 0.2 M iodine solution until no discoloration of the iodine is detectable.

63. Remove solvent *in vacuo* and dissolve residue in 50 mL DCM.

64. Wash organic layer consecutively with 50 mL 6% $NaHSO_3$ solution, 50 mL saturated $NaHCO_3$ solution, and 50 mL water.

65. Dry organic layer with Na_2SO_4, filter off desiccant, and remove solvent *in vacuo*.

66. Purify with silica gel chromatography (DCM/MeOH, gradient: 99:1 to 99:5) to generate, after evaporation of the appropriate fractions, the desired products in 59% to 71% yield (Table 3).

67. Characterize products by TLC (on silica) and MS (MALDI; Table 3).

    Rf ≈ 0.7 (DCM/MeOH, 95:5).

Table 3 MS Data and Yield of 5′-*O*-DMT-3′-*O*-TBDMS Trinucleotides 9

5′-*O*-DMT-3′-*O*-TBDMS trinucleotide 9	Mass as Na-peak (*m/z*)	Calculated mass (*m/z*)	Yield (%)
AAA	1633	1610	63
ACC	1653	1630	71
ATC	1563	1540	71
ATG	1569	1546	75
CAC	1654	1629	68
CAG	1658	1635	73
CAT	1563	1540	75
CCA	1652	1629	59
CTG	1579	1556	71
GAT	1569	1546	71
GCA	1658	1635	69
GCG	1674	1651	65
GCT	1580	1557	56
GGC	1674	1651	64
GGT	1586	1563	69
GTA	1569	1546	66
GTT	1491	1468	58
TGG	1586	1563	56
TTC	1484	1461	70
TTT	1395	1372	55

Deprotection of the 3′-OH group (10)

68. Dissolve 0.5 mmol 3′-*O*-TBDMS protected trinucleotide **9** in 10 mL anhydrous DMF.
69. Add 0.25 mL TEA·3HF and stir overnight.
70. Dilute with 50 mL saturated $NaHCO_3$ solution and extract product with 30 mL DCM.
71. Dry organic layer with Na_2SO_4, filter off desiccant, and remove solvent *in vacuo.*
72. Purify by RP-HPLC using buffer A at a flow rate of 0.5 mL/min and the following gradient of buffer B:

    0% buffer B for 5 min,
    0% to 100% buffer B over 23.5 min,
    100% buffer B for 30 min.

73. Characterize products by MS (MALDI) and ^{31}P NMR (Table 4).

SYNTHESIS OF TRINUCLEOTIDE SYNTHONS ON SOLID SUPPORT

BASIC PROTOCOL 2

Fully protected trinucleotides can also be synthesized on solid support, which is advantageous in terms of avoiding time consuming purification steps. Care has to be taken so that the starting nucleoside is anchored to the support via a linker that allows cleavage of the synthesized trinucleotide from the support under conditions that leave all other

Table 4 MS and ^{31}P-NMR Data of 5′-*O*-DMT-3′-OH Trinucleotides 10

Trinucleotide 5′-DMT 3′-OH[a] 10	Mass as Na-peak (*m/z*)	Calculated mass (*m/z*)	^{31}P-NMR[b] δ (ppm)
AAA	1519	1496	−2.78, −2.74, −2.58 (br)
ACC	1539	1516	−2.572 (br), −2.467, −2.435
ATC	1450	1427	−2.62, −2.60, −2.55, −2.42
ATG	1456	1433	−2.64 (br), −2.56 (br)
CAC	1540	1516	−2.83, −2.73, −2.42, −2.40
CAG	1545	1522	−2.85, −2.79, −2.53 (br)
CAT	1450	1426	−2.83, −2.74, −2.46 (br)
CCA	1539	1516	−2.68 (br), −2.60 (br)
CTG	1466	1442	−2.71, −2.62, −2.54 (br)
GAT	1456	1433	−3.14 (br), −2.85 (br)
GCA	1545	1522	−3.69, −3.65, −2.70, −2.60
GCG	1562	1537	−2.84, −2.74, −2.56, −2.45
GCT	1466	1443	−2.67 (br), −2.45 (br)
GGC	1562	1538	−2.69, 2.65, −2.47, −2.39
GGT	1472	1449	−2.70 (br), −2.51, −2.49
GTA	1456	1433	−2.70, −2.64, −2.58 (br)
GTT	1377	1354	−2.70 (br), −2.51, −2.49
TGG	1472	1449	−2.80, −2.66, −2.65, −2.50
TTC	1371	1348	−2.70, −2.52, −2.46, −2.43
TTT	1281	1258	2.71, −2.53, −2.48, −2.45

[a] Yield was virtually quantitative in all cases.
[b] 300 MHz, DMSO-d_6.

protecting groups intact. The following protocol describes the loading of a polymer (most suitably amino-functionalized cross-linked polystyrene/divinylbenzene) with the starting nucleosides followed by trinucleotide assembly (Fig. 4). To ensure orthogonality of cleavage conditions to the protecting groups of the trinucleotide **16**, a disulfide linkage was chosen for connecting the starting nucleoside via the 3′-hydroxyl function with the amino groups of the polymer. First, *N*-formamidine-5′-*O*-DMT protected nucleoside **12** is converted to the 3′-*O*-methylthiomethyl (MTM) derivative **13**, which is then reacted with 2-mercaptopropionic acid to form the disulfide bridged species **14**. This is immobilized on the support by amide bond formation to give **15**, which serves as the starting nucleoside for assembly of the trinucleotide **16** under standard DNA synthesis conditions using 3′-*O*-methyl-phosphoramidites of *N*-acyl-5′-*O*-DMT protected nucleosides. Reductive cleavage of the disulfide bridge and spontaneous decay of the initially formed *S,O*-hemiacetal delivers the fully protected trimer **17**, ready for phosphitylation.

Materials

Thymidine
Desoxyadenosine (dA or 2′-deoxyadenosine)
Desoxyguanosine (dG)
Desoxycytidine (dC)
Dichloromethane (DCM), anhydrous
Methanol (MeOH), anhydrous
Dimethylformamide (DMF), anhydrous

N,N-Dimethylformamide dimethylacetal
N,N-Dibutylformamide dimethylacetal (see recipe)
Pyridine (stored over KOH, freshly distilled over molecular sieve)
4,4′-Dimethoxytrityl chloride (DMTCl)
Sodium bicarbonate ($NaHCO_3$), saturated solution
Sodium sulfate (Na_2SO_4)
Saturated NaCl solution (brine)
Dimethyl sulfoxide, anhydrous (DMSO)
Acetic anhydride
Glacial acetic acid
Triethylamine (TEA)
Sulfuryl chloride (freshly distilled and colorless)
Potassium *p*-toluenethiosulfonate (potassium thiotosylate)
2-Mercaptopropanoic acid
N,N,N′,N′-Tetramethyl-*O*-(*N*-succinimidyl)uronium tetrafluoroborate (TSTU)
Custom Primer Support Amino 200 (GE Healthcare)
Acetonitrile (99+%, extra pure, ACROS Organics, 10265290)
Acetone
Ethyl acetate
Argon gas, anhydrous
5′-*O*-DMT-*N*-benzoyl-2′-deoxyadenosine-3′-methoxy-*N,N*-(*i*Pr)$_2$-phosphoramidite (ChemGenes)
5′-*O*-DMT-thymidine-3′-methoxy-*N,N*-(*i*Pr)$_2$-phosphoramidite (ChemGenes)
Acetonitrile, 99.9%, extra dry over molecular sieves (Acros)
0.3 M 5-benzylmercaptotetrazole (BMT) solution in anhydrous acetonitrile (emp Biotech)
3 Å molecular sieves, activated, under argon (Roth, N893.1))
Capping reagent A (20% *N*-methylimidazole in acetonitrile, v/v)
Capping reagent B (acetonitrile/acetic anhydride/2,4,6-collidine, 5:2:3, v/v/v)
3% trichloroacetic acid in 1,2-dichloroethane (DCE; w/v)
0.01 M iodine solution (iodine in 1:5:11 2,4,6-collidine/H_2O/MeCN, w/v/v/v)
Tris(2-carboxyethyl)phosphine (TCEP)
4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) buffer, pH 7
NaOH solution (aqueous, concentrated)

Magnetic stir plate and magnetic stir bar
Rotary evaporator (equipped to vent to a nitrogen atmosphere)
Chromatography columns
Silica gel for column chromatography (0.063-0.200 mm)
TLC plate (Macherey-Nagel, Alugram Sil G/UV_{254})
Ultraviolet light (254 nm)
Lyophilizer
Glass frit (Robu Glass Filter Por 3, 25 mL)
Pharmacia Gene Assembler Plus
Synthesizer vials with caps
Plastic tube (2 mL)
Vortex mixer
Table centrifuge
UV/Vis-spectrophotometer (Ultro spec 2100 pro, Amersham Bioscience)

N-protection of 2′-deoxyadenosine (11, dA)

1. Co-evaporate 0.45 g (1.8 mmol) 2′-deoxyadenosine three times with dry DCM/MeOH in a 50-mL round bottom flask.

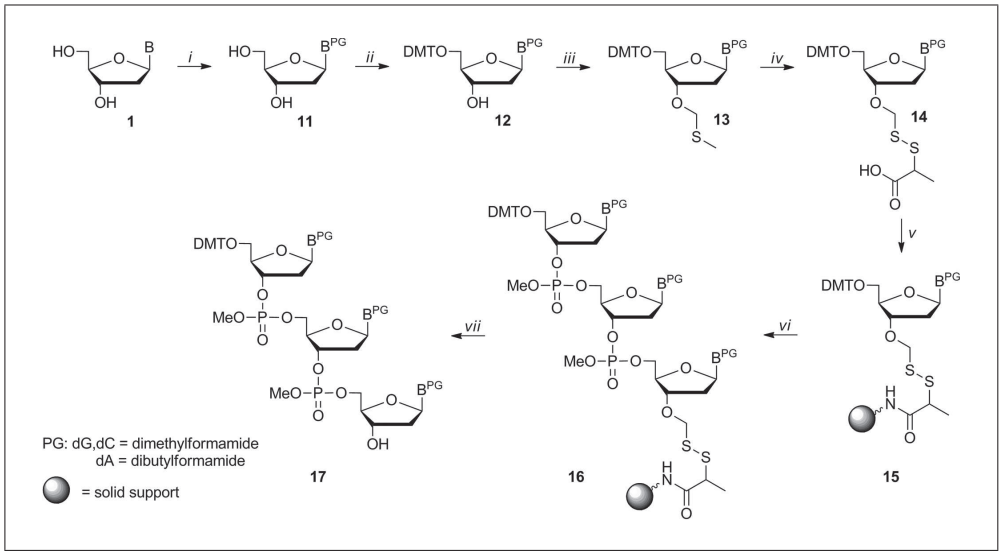

Figure 4 Reaction scheme for preparation of fully protected trinucleotides on solid phase. i) for 1 eq. dG or dC (1): 5 eq. dimethylformamide-dimethylacetal, 1 hr, 50°C, DMF; for 1 eq. dA (1): 1.4 eq. dibutylformamide-dimethylactal, rt, overnight, then 1 hr, 80°C; ii) 1 eq. **11**, addition of 1.3 eq. DMTCl at 0°C, stirring for 15 min in pyridine, 3 hr up to overnight rt; iii) 1 eq. **12**, 70 eq. DMSO, 53 eq. acetic anhydride, 53 eq. acetic acid, 20 hr, rt; iv) 1 eq. **13**, 3 eq. TEA, 1 eq. sulfuryl chloride, 1.5 eq. potassium thiotosylate, 2 eq. 2-mercaptopropionic acid, DCM, 2 hr, rt; v) 1 eq. **14**, 3 eq. TSTU, 5 eq. TEA, amino-polystyrene, 48 hr, DMF:water or DCM:DMF:water, rt; vi) automated synthesis via DNA Synthesizer Gene Assembler Special (Amersham Bioscience) with **15** as solid support and *p*-methoxy phosphoramidite; vii) 1 eq. **16**, 6.6 eq. TCEP, HEPES buffer (pH 7), MeCN, 2 × ultrasonic bath overnight, 2 × 55°C, 6 hr. Abbreviations: eq., equivalent; rt, room temperature; DMF, *N,N*-dimethylformamide; DMTCl, 4,4′-dimethoxytrityl chloride; DCM, dichloromethane; TSTU, *N,N,N′,N′*-tetramethyl-*O*-(*N*-succinimidyl)uronium tetrafluoroborate; TEA, triethylamine; TCEP, tris(2-carboxyethyl)phosphine; B, base; B^{PG}, protected base.

2. Add 10 mL dry DMF.
3. Add 0.51 g (2.52 mmol) *N,N*-dibutylformamide dimethylacetal, stir overnight at room temperature, and then at 80°C, 1 hr.
4. Evaporate solvent *in vacuo*.
5. Purify residue by column chromatography on silica gel using DCM/MeOH (gradient: 98:2 to 8:2), collect fractions containing desired product, and evaporate to dryness to give the product in 71% yield.
6. Characterize product by ^{1}H NMR.

N^6-Dibutylformamidine-2′-deoxyadenosine:

*^{1}H NMR (300 MHz, DMSO-d_6): δ [ppm] = 8.93 (s, 1H, dibutylformamide N=C-H); 8.45 and 8.40 (2 s, 2H, H8 and H2); 6.40 (t, J = 6.78 Hz, 1H, H1′); 5.32 (d, J = 3.93 Hz, 1H, 3′-OH); 5.14 (t, 1H, 5′-OH); 4.43 (m, 1H, H3′); 3.89 (m, 1H, C4′); 3.58 (m, 4H, -N-(**H**$_2$-CH$_2$-CH$_2$-CH$_3$)$_2$); 3.43 (m, 2H, H5′(a), H5′(b)); 2.73 (m, 1H, H2′′); 2.29 (m, 1H, H2′); 1.59 (m, 4H, -N-(CH$_2$-**H**$_2$-CH$_2$-CH$_3$)$_2$); 1.31 (m, 4H, N-(CH$_2$-CH$_2$-**H**$_2$-CH$_3$)$_2$); 0.92 (m, 6H, N-(CH$_2$-CH$_2$-CH$_2$-**H**$_3$)$_2$).*

N-protection of 2′-deoxycytidine and 2′-deoxyguanosine (11, dC, dG)

7. Co-evaporate 2′-deoxynucleoside (2.64 mmol) three times with dry DCM/MeOH in a 100-mL round bottom flask.
8. Add 20 mL dry DMF.

9. Add 1.75 mL (13.2 mmol) *N,N*-dimethylformamide dimethylacetal and stir overnight at room temperature.
10. Evaporate solvent *in vacuo*.
11. Purify residue by crystallization from DCM in an ice bath.
12. Filter off solid product and dry in the lyophilizer to give the products in 86% to 100% yield.
13. Characterize products by ^{1}H NMR.

N^4-Formamidine-2′-deoxycytidine:

^{1}H NMR (300 MHz, DMSO-d_6): δ [ppm] = 8.61 (s, 1H, formamidine N=C-H); 8.01 (d, J = 7.19 Hz, 1H, C6); 6.15 (dd, J = 6.69 Hz, J = 6.40 Hz, 1H, C1′); 5.96 (d, J = 7.19 Hz, 1H, C5); 5.20 (d, J = 4.15, 1H, 3′-OH); 5.00 (t, J = 5.14 Hz, 1H, 5′-OH); 4.21 (m, 1H, H4′); 3.8 (m, 1H, H3′); 3.57 (m, 2H, H2′(a), H2′(b)); 3.16 (s, 3H, CH_3-formamidine); 3.03 (s, 3H, CH_3-formamidine); 2.07 (m, 2H, 5′/5′′).

N^2-Formamidine-2′-deoxyguanosine:

^{1}H NMR (300 MHz, DMSO-d_6): δ [ppm] = 11.30 (s, 1H, NH); 8.55 (s, 1H, formamidine N=C-H); 8.04 (s, 1H, H8); 6.24 (t, J = 6.21 Hz, 1H, H1′); 5.30 (d, J = 3.51 Hz, 1H, 3′-OH); 4.94 (t, J = 5.42 Hz, 1H, 5′-OH); 4.38 (m, 1H, H3′); 3.83 (m, 1H, C4′); 3.54 (m, 2H, H5′(a), H5′(b)); 3.16 (s, 3H, CH_3-formamidine); 3.03 (s, 3H, CH_3-formamidine); 2.59 (m, 1H, H2′(a)); 2,22 (m, 1H, H2′(b)).

5′-O-DMT-protection (12)

14. Evaporate thymidine or *N*-protected 2′-deoxynucleoside **11** (1.9 mmol) three times with dry pyridine in a 50-mL round bottom flask.
15. Transfer dry nucleoside in a Schlenk round bottom flask under argon atmosphere.

    Dry the flask prior to use with a heat gun and let it cool down to room temperature under vacuum. Use argon atmosphere during the whole experiment. Avoid any moisture.

16. Add 9.5 mL dry pyridine and stir 10 min in an ice bath.
17. Add 0.84 g DMTCl (2.47 mmol) and stir 30 min in an ice bath.
18. Stir 3 to 12 hr at room temperature and monitor by TLC.
19. Evaporate solvent *in vacuo*.
20. Dissolve residue in DCM and wash with saturated $NaHCO_3$ then saturated NaCl solution.
21. Dry organic layer with Na_2SO_4, filter off the desiccant, and remove solvent *in vacuo*.
22. Purify residue by column chromatography on silica gel using DCM/MeOH (gradient: 99:1 to 9:1), collect appropriate fractions containing product, and remove solvent *in vacuo* to give the product in 47% to 80% yield.

    Store DCM over $NaHCO_3$.

23. Characterize products by ^{1}H NMR.

5′-O-Dimethoxytrityl-N^6-dibutylformamidine-2′-deoxyadenosine:

^{1}H NMR (300 MHz, DMSO-d_6): δ [ppm] = 8.91 (s, 1H, dibutylformamide N=C-H); 8.35 (2 br s, 2H, H8 and H2); 7.37-7.12 (m, 9H, Ar-DMT); 6.83-6.71 (m, 4H, Ar-DMT); 6.42 (t, J = 6.41 Hz, 1H, H1′); 5.36 (m, 1H, 3′-OH); 4.49 (m, 1H, H3′); 3.99 (quart,

*J = 4.14 Hz, 1H, H4′);3.71 (s, 6H, DMT-methoxy); 3.59 (t, 2H, -N-(C**H₂**-CH₂-CH₂-CH₃)₂); 3.43 (t, 2H, -N-(C**H₂**-CH₂-CH₂-CH₃)₂); 3.16 (m, 2H, H5′(a), H5′(b)); 2.90 (m, 1H, H2′(a)); 2.23 (m, 1H, H2′(b)); 1.59 (m, 4H, -N-(CH₂-C**H₂**-CH₂-CH₃)₂); 1.32 (m, 4H, N-(CH₂-CH₂-C**H₂**-CH₃)₂); 0.92 (dt, 6H, N-(CH₂-CH₂-CH₂-C**H₃**)₂).*

5′-O-Dimethoxytrityl-N⁴-formamidine-2′-deoxycytidine:

¹H NMR (300 MHz, DMSO-d₆): δ [ppm] = 8.62 (s, 1H, formamidine N=C-H); 7.82 (d, J = 7.18 Hz, 1H, H6); 7.42-7.21 (m, 9 H, DMT-Ar); 6.94-6.86 (m, 4 H, DMT-Ar); 6.16 (t, J = 6.24 Hz, 1H, H1′); 5.74 (d, J = 7.18 Hz, 1H, H5); 5.31 (d, J = 4.55, 1H, 3′-OH); 4.27 (m, 1H, H4′); 3.91(m, 1H, H3′); 3.74 (s, 6H, DMT-methoxy); 3.24 (m, 2H, H2′(a), H2′(b)); 3.16 (s, 3H, CH₃-formamidine); 3.03 (s, 3H, CH₃-formamidine); 2.15 (m, 2H, H5′(a), H5′(b)).

5′-O-Dimethoxytrityl-N²-formamidine-2′-deoxyguanosine:

¹H NMR (300 MHz, DMSO-d₆): δ [ppm] =11.34 (s, 1H, NH); 8.51 (s, 1H, formamidine N=C-H); 7.92 (s, 1H, 8); 7.39-7.15 (m, 9H, Ar-DMT); 6.81 (m, 4H, Ar-DMT); 6.31 (m, 1H, H1′); 5.37 (d, J = 4.36 Hz, 1H, 3′-OH); 4.42 (m, 1H, H3′); 3.94 (m, 1H, H4′); 3.72 (s, 6H, DMT-methoxy); 3.54 (m, 2H, H5′(a), H5′(b)); 3.11 (s, 3H, CH₃-formamidine); 3.03 (s, 3H, CH₃-formamidine); 2.68 (m, 1H, H2′(a)); 2.31 (m, 1H, H2′(b)).

5′-O-Dimethoxytrityl-2′-deoxythymidine:

¹H NMR (300 MHz, DMSO-d₆): δ [ppm] = 11.27 (s, 1H, NH); 7.5 (s, 1H, H6); 7.42-7.20 (m, 9H, Ar-DMT); 6.95-6.85 (m, 4H, Ar-DMT); 6.21 (t, J = 6.57 Hz, 1H, H1′); 5.32 (s, 1H, 3′-OH); 4.3 (m, 1H, 4′H); 3.88 (m, 1H, H3′); 3.73 (s, 6H, methoxy-DMT); 3.19 (m, 2H, H5′(a), H5′(b)); 2.20 (m, 2H, C2′/C2′′-H); 1.45 (s, 3H, CH₃).

Functionalize 3′-O-MTM (13)

24. Evaporate 5′-*O*-dimethoxytrityl-2′-deoxynucleoside **12** (0.82 mmol) three times with dry DCM in a 50-mL round bottom flask.

25. Transfer the dry starting material to a 25-mL Schlenk flask.

    Dry the flask prior to use with a heat gun and let it cool down to room temperature under vacuum. Use argon atmosphere during the whole experiment. Avoid any moisture.

26. Add 4.1 mL dry dimethyl sulfoxide (57.4 mmol).

27. While stirring, add first 4.1 mL acetic anhydride (43.4 mmol) and then dropwise 2.5 mL glacial acetic acid (43.4 mmol).

28. Stir at room temperature overnight.

29. Wash with saturated aqueous $NaHCO_3$ followed by saturated NaCl solution.

30. Dry organic layer with Na_2SO_4, filter off the desiccant, and remove solvent *in vacuo*.

31. Purify residue by column chromatography on silica gel using DCM/MeOH (gradient: 99:1 to 95:5), collect appropriate fractions containing product, and remove solvent *in vacuo* to give the product in 52% to 68% yield.

    Store DCM over $NaHCO_3$.

32. Characterize products by ^{1}H NMR and ^{13}C NMR.

    3′-O-Methylthiomethyl-5′-O-dimethoxytrityl-N⁶-dibutylformamidine-2′-deoxyadenosine:

    *¹H NMR (300 MHz, DMSO-d₆): δ [ppm] = 8.91 (s, 1H, dibutylformamide N=C-H); 8.65-8.32 (br s, 2H, H8 and H2); 7.39-7.13 (m, 9H, Ar-DMT); 6.87-6.74 (m, 4H, Ar-DMT); 6.38 (t, J = 6.76 Hz, 1H, H1′); 4.73 (s, 2H, MTM-CH₂); 4.70 (m, 1H, H3′); 4.10 (m, 1H, H4′); 3.72 (s, 6H, DMT-methoxy); 3.58 (t, 2H, -N-(C**H₂**-CH₂-CH₂-CH₃)₂); 3.43*

*(t, 2H, -N-(C**H**$_2$-CH$_2$-CH$_2$-CH$_3$)$_2$); 3.21 (m, 2H, H5′(a), H5′(b)); 3.05 (m, 1H, H2′(a)); 2.48 (m, J 1H, H2′(b)); 2.05 (s, 3H, S-CH$_3$); 1.59 (m, 4H, -N-(CH$_2$-C**H**$_2$-CH$_2$-CH$_3$)$_2$); 1.31 (m, 4H, N-(CH$_2$-CH$_2$-C**H**$_2$-CH$_3$)$_2$); 0.92 (m, 6H, N-(CH$_2$-CH$_2$-CH$_2$-C**H**$_3$)$_2$).*

*^{13}C NMR (300 MHz, DMSO-d$_6$) δ [ppm] = 159.34 (1C, C6); 158.01 (1C, DMT –C-O); 157.79 (1C, DMT –C-O); 151.81 (1C, dibutylformamide-CH-); 151.36 (1C, C4); 151.10 (1C, C2); 149.61 (1C, C5); 144.74 (1C, -DMT-Ar); 141.28 (1C, C8); 135.47 (2C, DMT-Ar); 129.51 (2C,CH, DMT-Ar); 127.65 (6C, DMT-Ar); 126.60 (1C, CH, DMT-Ar); 113.05 (4C, DMT-Ar); 85.52 (1C, DMT, tertiary C); 83.71 (1C, C1′); 83.06 (1C, C4′); 76.15 (1C, C3′); 72.86 (1C, MTM-CH$_2$); 63.44 (1C, C5′); 54.95 (2C, DMT-methoxy); 50.88 (1C, -N-(**C**H$_2$-CH$_2$-CH$_2$-CH$_3$)$_2$); 44.1 (1C, -N-(**C**H$_2$-CH$_2$-CH$_2$-CH$_3$)$_2$); 35.18 (1C, C2′); 30.44 (1C, -N-(CH$_2$-**C**H$_2$-CH$_2$-CH$_3$)$_2$); 28.64 (1C, -N-(CH$_2$-**C**H$_2$-CH$_2$-CH$_3$)$_2$); 19.61 (1C, -N-(CH$_2$-CH$_2$-**C**H$_2$-CH$_3$)$_2$); 19.11 (1C, -N-(CH$_2$-CH$_2$-**C**H$_2$-CH$_3$)$_2$); 13.71 (1C, -N-(CH$_2$-CH$_2$-CH$_2$-**C**H$_3$)$_2$); 13.52 (1C, -N-(CH$_2$-CH$_2$-CH$_2$-**C**H$_3$)$_2$); 13.21 (1C, MTM-CH$_3$).*

3′-O-Methylthiomethyl-5′-O-dimethoxytrityl-N^4-formamidine-2′-deoxycytidine:

^{1}H NMR (300 MHz, DMSO-d$_6$): δ [ppm] = 8.63 (s, 1H, formamidine N=C-H); 7.81 (d, J = 7.18 Hz, 1H, H6); 7.41-7.21 (m, 9 H, DMT-Ar); 6.93-6.86 (m, 4 H, DMT-Ar); 6.12 (t, J = 6.36 Hz, 1H, H1′); 5.76 (d, J = 7.20 Hz, 1H, H5); 4.68 (m, 2H, -CH$_2$-S-); 4.53 (m, 1H, H4′); 4.02 (m, 1H, C3′); 3.74 (s, 6H, DMT-methoxy); 3.27 (m, 2H, C2′(a), C2′(b)); 3.16 (s, 3H, CH$_3$-formamidine); 3.03 (s, 3H, CH$_3$-formamidine); 2.28 (m, 2H, H5′(a), H5′(b)); 2,00 (s, 3H, -S-CH$_3$).

^{13}C NMR (300 MHz, DMSO-d$_6$) δ [ppm] = 170.09 (1C, C2); 162.25 (1C, C4); 160.90 (1C, DMF N=C-N); 158.15 (2C, DMT –C-O); 144.38 (1C, -DMT-Ar); 135.44 (2C, DMT-Ar); 129.85 (2C,CH, DMT-Ar); 127.88 (4C, DMT-Ar); 127.70 (2C, DMT-Ar); 126.81 (1C, CH, DMT-Ar); 113.22 (4C, DMT-Ar); 86.02 (1C, DMT, tertiary C); 83.15 (1C, C1′); 82.67 (1C, C4′); 74.66 (1C, C3′); 73.00 (1C, MTM-CH$_2$); 66.68 (1C, C6); 66.05 (1C, C5); 65.70 (1C, C5′); 54.99 (2C, DMT-methoxy); 45.06 (1C, DMF-CH$_3$); 37.71 (1C, C2′); 24.29 (1C, DMF-CH$_3$); 13.20 (1C, MTM-CH$_3$).

3′-O-Methylthiomethyl-5′-O-dimethoxytrityl-N^2-formamidine-2′-deoxyguanosine:

^{1}H NMR (300 MHz, DMSO-d$_6$): δ [ppm] = 11.35 (s, 1H, NH); 8.51 (s, 1H, formamidine N=C-H); 7.96 (s, 1H, H8); 7.37-7.16 (m, 9H, Ar-DMT); 6.82 (m, 4H, Ar-DMT); 6.25 (m, 1H, H1′); 4.70 (s, 2H, MTM-CH$_2$); 4.67 (m, 1H, H3′); 4.00 (m, J = 4.52 Hz, J = 3.95 Hz, J = 4.74 Hz, J = 4.88 Hz, 1H, C4′); 3.72 (s, 6H, DMT-methoxy); 3.21 (m, 2H, H5′(a), H5′(b)); 3,09 (s, 3H, CH$_3$-formamidine); 3.02 (s, 3H, CH$_3$-formamidine); 2.85 (m, 1H, H2′); 2.44 (m, 1H, H2′′); 2.01 (s, 3H, S-CH$_3$).

^{13}C NMR (300 MHz, DMSO-d$_6$) δ [ppm] = 158.03 (2C, DMT –C-O); 157.76 (1C, DMF-CH-); 157.55 (1C, C4); 157.13 (1C, C6); 149.57 (1C, C2); 144.74 (1C, -DMT-Ar); 136.61 (1C, C8); 135.44 (2C, DMT-Ar); 129.58 (2C,CH, DMT-Ar); 127.68 (6C, DMT-Ar); 126.65 (1C, CH, DMT-Ar); 119.91 (1C, C5); 113.07 (4C, DMT-Ar); 85.61 (1C, DMT, tertiary C); 82.99 (1C, C1′); 82.75 (1C, C4′); 75.85 (1C, C3′); 72.89 (1C, MTM-CH$_2$); 63.76 (1C, C5′); 54.97 (2C, DMT-methoxy); 35.98 (1C, DMF-CH$_3$); 34.62 (1C, C2′); 25.45 (1C, DMF-CH$_3$); 13.14 (1C, MTM-CH$_3$).

5′-O-Dimethoxytrityl-3′-O-methylthiomethyl-2′-deoxythymidine:

^{1}H NMR (300 MHz, DMSO-d$_6$): δ [ppm] = 11.34 (s, 1H, NH); 7.5 (s, 1H, C6H); 7.43-7.2 (m, 9H, Ar-DMT); 6.93-6.86 (m, 4H, Ar-DMT); 6.17 (t, 1H, H1′); 4.68 (s, 2H, MTM-CH$_2$); 4.58 (m, 1H, H4′); 4.00 (m, 1H, H3′); 3.74 (s, 6H, methoxy-DMT); 3.23 (m, 2H, H5′(a), H5′(b)); 2.32 (m, 2H, H2′(a), H2′(b)); 2.03 (s, 3H, S-CH$_3$); 1.46 (s, 3H, CH$_3$).

^{13}C NMR (300 MHz, DMSO-d$_6$) δ [ppm] = 168.6 (1C, C4); 158.04 (2C, DMT –C-O); 150.26 (1C, C2); 144.7 (1C, DMT-Ar); 135.3 (1C, C6); 135.2 (2C, DMT-Ar); 129 (2C,CH, DMT-Ar); 127.8 (2C, DMT-Ar); 127.6 (4C, DMT –Ar); 126.8 (1C, CH, DMT-Ar); 113.4 (4C, DMT –Ar); 109.7 (1C, C5); 86.1 DMT, quartet C); 83.9 (1C, C1′); 82.7 (1C, C4′), 75.6 (1C, C3′); 72.7 (1C, MTM-CH$_2$); 63.5 (1C, C5′); 54.98 (2C, DMT-methoxy); 36.35 (1C, C2′); 13,17 (1C, MTM-methyl); 11.61 (1C, C5-CH3).

Disulfide formation (14)

33. Evaporate 5′-*O*-DMT-3′-*O*-methylthiomethyl-2′-deoxynucleoside **13** (0.6 mmol) three times with dry DCM in a 25-mL round bottom flask.
34. Transfer dry nucleoside under inert conditions in a dry Schlenk flask.

    Dry the flask prior to use with a heat gun and let it cool down to room temperature under vacuum. Use argon atmosphere during the whole experiment. Avoid any moisture.

35. Add 2.4 mL dry DCM (37.8 mmol).
36. Add 0.18 mL TEA (1.79 mmol) and stir in an ice bath.
37. Mix 0.05 mL sulfuryl chloride (0.6 mmol) with 0.46 mL DCM. Add solution slowly and dropwise to the reaction mixture. Stir 10 min in an ice bath.

    The sulfuryl chloride should be freshly distilled prior to use.

38. Mix 0.17 g potassium thiotosylate (0.9 mmol) with 0.56 mL dry DMF. Add solution dropwise to the reaction mixture at room temperature. Stir 10 min at room temperature.
39. Add 0.13 mL 2-mercaptopropanoic acid (1.3 mmol). Stir 90 min at room temperature.
40. Wash with saturated aqueous $NaHCO_3$ and brine.
41. Dry organic layer with Na_2SO_4, filter off desiccant, and remove solvent *in vacuo*.
42. Use the residue directly for coupling to amino-functionalized solid support without further purification.

Immobilize on solid support (15)

43. Dissolve all of the 3′-*O*-functionalized nucleoside **14** obtained from step 42 in DCM (1.4 mL) and DMF (0.3 mL) in a 10-mL round bottom flask.
44. Add 0.54 g TSTU (1.8 mmol) and 0.4 mL TEA (3 mmol).
45. Add amino polystyrene (Custom Primer Support Amino 200; 355 mg/mmol nucleoside **14**) and stir at room temperature 48 hr.
46. Wash solid support consecutively with EtOH, DCM, and water (5 to 6 mL of each solvent) in a glass frit.
47. Dry solid support in vacuum desiccator under vacuum.

Determine loading density

48. Take an aliquot of support loaded with nucleoside (~5 mg).
49. Add 1 mL 3% dichloroacetic acid in dichloroethane.
50. Measure absorption of the supernatant at 498 nm with an UV spectrometer.

    Dilute the supernatant until absorbance values <1 are obtained. Measure each sample at least three times and calculate the average value.

51. Calculate loading density with regression line of the standard curve (see Support Protocol 1) and the absorbance value. Determine loading densities of supports with:

dA: 113 μmol/g
dC: 58.82 μmol/g
dG: 51.56 μmol/g
T: 58.97 μmol/g.

Cap free amino groups on solid support

52. Add capping reagent A and B in equal amounts to each solid support.

    For ~300 mg solid support use 1 mL of each capping reagent.

53. Stir 30 min at room temperature.

54. Wash solid support with 5 mL acetonitrile in a glass frit and dry it *in vacuo*.

Synthesize trinucleotide synthons on MTM-functionalized support for 2′-deoxyadenosine and thymidine (16)

Here, the solid-phase synthesis of trinucleotides **17** is described in an exemplary manner for the synthons ATT, AAT, TTT, and AAA using a Gene Assembler Special DNA synthesizer from Amersham Bioscience. A standard column reactor is used with columns for 0.2 and 1.3 μmol scale standard syntheses. For scaling up to 10 μmol, the tubes from valve 1 to column reactor and from column reactor to valve 2 were replaced with longer ones (110 cm and 129 cm, both with an inner diameter of 0.5 cm). With this modification the cycle volume for coupling is increased to ~960 μl.

55. Prepare 0.1 M solutions of 5′-*O*-DMT-*N*-benzoyl-2′-deoxyadenosine-3′-methoxy-*N,N*-(*i*Pr)$_2$-phosphoramidite and 5′-*O*-DMT-thymidine-3′-methoxy-*N,N*-(*i*Pr)$_2$-phosphoramidite in appropriate synthesizer vials.

56. Prepare 0.3 M solution of BMT in acetonitrile in appropriate synthesizer vials.

    Avoid moisture. Use gentle argon flush during the filling of the bottles. Add molecular sieves to solutions and store overnight.

57. Fill designated synthesizer bottles with all reagents and solutions.

58. Weigh out the amount of solid support (**15**, dA or T) required for a 10 μmol synthesis, and add to a standard column for 1 μmol synthesis (inner volume 200 μl).

59. Place column into column holder.

60. Purge all solvents and BMT solution with the standard purge program of the Gene Assembler Special.

61. Purge all amidite solutions manually.

    This step is done manually to avoid wasting p-methoxy-amidites.

62. Program desired sequence.

63. Start synthesis without final detritylation (DMT-on modus).

    Use standard submethod for detritylation at the DNA 10 μmol scale. For coupling reaction of 3′-methoxy-N,N-(iPr)$_2$-phosphoramidite use method shown in Table 5.

64. When synthesis is complete, remove column from the synthesizer and dry column with argon.

Cleave trinucleotide (17)

65. Dissolve 5.99 g HEPES with 25 mL water and adjust to pH 7 with concentrated aqueous NaOH solution.

66. Dissolve 300 μmol TCEP in 600 μl acetonitrile and 400 μl HEPES buffer.

Table 5 Coupling Cycle for Trinucleotide Assembly on a Gene Assembler

Listing		Function		Description	
1	1.50	VALVE POS	2.3	Set valve 2 to position 3	Acetonitrile wash
2	2.50	INTEGRATE	0	Stop integration	Peak data from detritylation can be displayed on the screen
3	3.50	AUTOZERO			
4	4.50	ML/MIN	0.00	Stop the flow	
5	4.50	LOOP TIMES	4	Start of loop	Set valve 3 in recycle position
6	4.50	VALVE POS	1.8	Set valve 1 to position 8	Addition of 75 µl BMT solution
7	4.55	ML/MIN	0.75	Set flow rate 0.75 mL/min	
8	4.65	ML/MIN	0.00	Stop the flow	
9	4.65	VALVE POS	1.6	Set valve 1 to position x	Addition of 100 µl amidite solution
10	4.70	ML/MIN	1.00	Set flow rate 1.0 mL/min	
11	4.80	ML/MIN	0.00	Stop the flow	
12	4.80	VALVE POS	1.8	Set valve 1 to position 8	Addition of 75 µl BMT solution
13	4.85	ML/MIN	0.75	Set flow rate 0.75 mL/min	
14	4.95	ML/MIN	0.00	Stop the flow	
15	4.95	END OF LOOP			
16	4.95	VALVE POS	1.1	Set valve 1 to position 1	Push BMT and amidite solution into recycle loop with acetonitrile
17	5.00	ML/MIN	1.00	Set flow rate 1.0 mL/min	
18	5.10	ML/MIN	0.00	Stop the flow	
19	5.10	LOOP TIMES	7	Start of loop	Set valve 3 in recycle position
20	5.10	STEP VALVE	3	Step valve 3 to next position	
21	5.10	END OF LOOP			
22	5.15	ML/MIN	2.50	Set flow rate 2.5 mL/min	Coupling for 3 min
23	8.15	ML/MIN	0.00	Stop the flow	
24	8.15	STEP VALVE	3	Step valve 3 to next position	Set valve 3 in flow through position

(Continued).

Table 5 *(Continued).*

Listing		Function		Description	
25	8.20	ML/MIN	2.5	Set flow rate 2.5 mL/min	Acetonitrile wash 30 sec
26	8.70	ML/MIN	0.00	Stop the flow	
27	8.75	VALVE POS	2.5	Set valve 2 to position 5	
28	8.80	ML/MIN	1.00	Set flow rate 1.0 mL/min	
29	8.80	LOOP TIMES	6	Start of loop	
30	8.80	VALVE POS	2.5	Set valve 2 to position 5	Capping A 100 µl
31	8.90	VALVE POS	2.6	Set valve 2 to position 6	Capping B 100 µl
32	9.00	END OF LOOP			
33	9.00	ML/MIN	0.00	Stop the flow	
34	9.05	VALVE POS	2.3	Set valve 2 to position 3	Acetonitrile wash for 30 sec
35	9.05	ML/MIN	2.5	Set flow rate 2.5 mL/min	
36	9.55	CALL METHOD		Set valve 2 to position 4	Oxidation for 1 min (2.5 mL)
37	10.55	VALVE POS	2.3	Set valve 2 to position 3	Acetonitrile wash for 1.2 min
38	12.15	ML/MIN	0.00	Stop the flow	
39	12.15	LOOP TIMES	7	Start of loop	Set valve 3 in recycle position
40	12.15	STEP VALVE	3	Step valve 3 to next position	Set valve 3 in flow through position
41	12.15	END OF LOOP			

67. Remove trinucleotide-carrying polymer from the column and add to a 2-mL plastic tube.
68. Add TCEP solution.
69. Shake 8 hr (using a shaker) and then overnight in an ultrasonic bath.
70. Filter using a glass frit and transfer supernatant in a new 2-mL tube.
71. Wash support consecutively with MeCN, EtOH, acetone, THF, and ethyl acetate (350 µl of each solvent) in a glass frit.
72. Evaporate collected supernatants *in vacuo*.
73. Precipitate trinucleotide from cold water.
74. Characterize products by MS (MALDI; Table 6).

Table 6 MS Data of 5′-*O*-DMT-3′-OH Trinucleotides 17

Trinucleotide **17**	Mass (*m/z*)	Calculated mass (*m/z*)
ATT	1284.84 (Na-peak)	1260.14
AAT	1385.82 (2× Na peak)	1339.24
TTT	1180.30	1180.34
AAA	1448.22 (2× Na peak)	1402.50

SUPPORT PROTOCOL 1

RECORDING OF A STANDARD CURVE FOR LOADING DENSITY DETERMINATION

For determination of the support loading density (see Basic Protocol 2, steps 48 to 51 above), a standard curve is needed, showing the absorption at 498 nm, depending on the dimethoxytrityl concentration. To this end, defined amounts of DMTCl are dissolved in the detritylation agent (3% dichloroacetic acid), the absorption of the resulting solutions is measured and plotted against DMT concentration. From the resulting curve, the amount of 5'-O-DMT protected starting nucleoside on the support can be calculated.

Materials

4,4′-Dimethoxytrityl chloride (DMTCl)
3% dichloroacetic acid in 1,2-dichloroethane (DCE; v/v)

UV/Vis-spectrophotometer (Ultro spec 2100 pro, Amersham Bioscience)

1. Dissolve 25 mg DMTCl in 40 mL 3% dichloroacetic acid in DCE (v/v).
2. Prepare a 1:200 dilution.
3. From the step 2 dilution, prepare a dilution series with 19 successive 1:10 dilutions to obtain 20 samples for measuring the absorption.
4. Measure the absorption of each diluted sample at 498 nm three times with a UV spectrophotometer.
5. Generate a standard curve.

BASIC PROTOCOL 3

PHOSPHITYLATION OF TRINUCLEOTIDE

To convert fully protected trimers to synthons for use in oligonucleotide synthesis, phosphitylation of the 3′-OH group is required. This can be carried out under standard conditions with the typical reagents. However, in the case of trimers, where the phosphate moieties are protected with the 2-cyanoethyl (CE) group, particular care has to be taken regarding reaction conditions, in order to prevent premature cleavage of the phosphate protecting group. This is achieved only with 2-cyanoethyl-*N,N,N′,N′*-tetraisopropylphosphorodiamidite in combination with 5-benzylmercaptotetrazole as phosphitylation agent (Fig. 5) as described in Janczyk, Appel, Springstubbe, Fritz, & Muller, 2012. We describe a protocol that considers the more labile nature of the 2-cyanoethyl group but can be applied to trimers with the more robust methyl protecting group on the phosphate moieties as well.

Materials

3′-*O*-unprotected 5′-*O*-, *N*-, and P-protected trinucleotide **10** or **17**
Dichloromethane (DCM), anhydrous
2-Cyanoethyl *N,N,N′,N′*-tetraisopropylphosphorodiamidite
5-Benzylmercaptotetrazole (BMT)

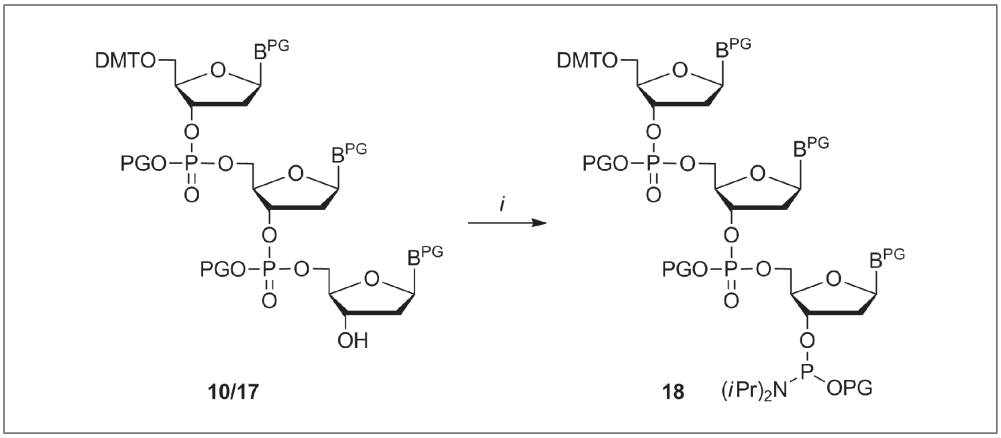

Figure 5 Phosphitylation of fully protected trinucleotides. i) 1 eq. **10** or **17**, DCM, 1.1 eq. 2-cyanoethyl *N,N,N′,N′*-tetraisopropylphosphorodiamidite, 1.2 eq. 5-benzylmercaptotetrazole, 2 hr, rt. Abbreviations: DCM, dichloromethane; B^{PG}, protected base; PG, protecting group; DMT, 4,4′-dimethoxytrityl.

Magnetic stir plate with magnetic stir bar
25-mL Schlenk flask
TLC plates (Macherey-Nagel Alugram Sil G/UV_{254})
Ultraviolet light lamp (254 nm)
Oil pump

1. Dissolve 0.5 mmol 3′-*O*-unprotected trinucleotide **10** or **17** in 5 mL anhydrous DCM in a 25-mL Schlenk flask.

   Dry the flask prior to use with a heat gun and let it cool down to room temperature under vacuum. Use argon atmosphere during the whole experiment. Avoid any moisture.

2. Slowly add 0.18 mL 2-cyanoethyl *N,N,N′,N′*-tetraisopropylphosphorodiamidite (0.55 mmol) and 0.12 g 5-benzylmercaptotetrazole (0.6 mmol) while stirring at room temperature.
3. Stir 2 hr at room temperature.
4. Remove solvent *in vacuo.*
5. Add 2 mL anhydrous DCM and evaporate again *in vacuo*.
6. Dry under vacuum overnight using an oil pump.
7. Store at 4°C.

COUPLING OF TRINUCLEOTIDE PHOSPHORAMIDITES

SUPPORT PROTOCOL 2

Successful phosphitylation of trinucleotide building blocks is tested directly by usage in coupling reactions on a DNA/RNA synthesizer.

Materials

Acetonitrile, 99.9%, extra dry over molecular sieves (Acros)
Trinucleotide phosphoramidite **18**
Deoxy-mononucleotide phosphoramidites:
5′-*O*-DMT-*N*-benzoyl-2′-deoxyadenosine-3′-CE-*N,N*-diisopropyl-phosphoramidite (LINK)
5′-*O*-DMT-*N*-acetyl-2′-deoxycytidine-3′-CE-*N,N*-diisopropyl-phosphoramidite (LINK)

5′-*O*-DMT-*N*-dmf-2′-deoxyguanosine-3′-CE-*N,N*-diisopropyl-phosphoramidite (LINK)
5′-*O*-DMT-thymidine-3′-CE-*N,N*-diisopropyl-phosphoramidite (LINK)
Solid support:
5′-*O*-DMT-*N*-benzoyl-2′-deoxyadenosine-3′-lcaa CPG 500 Å (ChemGenes)
5′-*O*-DMT-*N*-acetyl-2′-deoxycytidine-3′-lcaa CPG 500 Å (ChemGenes)
5′-*O*-DMT-*N*-dmf-2′-deoxyguanosine-3′-lcaa CPG 500 Å (ChemGenes)
5′-*O*-DMT-thymidine-3′-lcaa CPG 500 Å (ChemGenes)
5-Benzylmercaptotetrazole (BMT)
3 Å molecular sieves, activated, stored under argon (Roth)
Capping reagent A (20% *N*-methylimidazole in acetonitrile, v/v)
Capping reagent B (5:2:3 acetonitrile/acetic anhydride/2,4,6-collidine, v/v/v)
3% trichloroacetic acid in 1,2-dichloroethane (DCE, ≥99%, for synthesis, ROTH; w/v)
0.01 M iodine solution (iodine in 1:5:11 2,4,6-collidine/H_2O/MeCN, w/v/v/v)

Gene Assembler Special DNA Synthesizer (Pharmacia)
Plastic tube (2 mL)
Plastic tube with screw cap (2 mL)
Thermostatic mixer
Table centrifuge
Vacuum concentrator for 2 mL plastic vials

1. Prepare 0.1 M solutions of deoxy-mononucleotide phosphoramidites and acetonitrile in appropriate synthesizer vials.
2. Prepare 0.15 M solutions of trinucleotide phosphoramidite **18** and acetonitrile in appropriate synthesizer vial.
3. Prepare 0.3 M solution of BMT in acetonitrile in appropriate synthesizer vials.

   Avoid moisture. Use a gentle argon flush while filling the bottles. Add molecular sieves to solutions and store overnight.
4. Fill all reagents and solutions in the designated synthesizer bottles.
5. Weigh out amount of solid support (CPG 500 Å) required for 1 µmol synthesis scale into a standard column (inner volume 200 µl).
6. Place column into column holder.
7. Purge all solvents and BMT solution with standard program.
8. Purge all amidites manually.

   *This step is done manually to avoid wasting trinucleotide phosphoramidite **18**.*
9. Program sequence.

   *As an optimal procedure, hexanucleotides were synthesized by coupling two mononucleotide phosphoramidites to the starting nucleoside on the solid support prior to coupling of the trinucleotide phosphoramidite **18**.*
10. Start synthesis with final detritylation (DMT-off mode).

    Standard program for 1 µmol scale is used. See Table 7 for reagents and coupling times.
11. When the synthesis is complete, remove column from the synthesizer and dry column with argon.
12. Transfer column with the support into a 2-mL plastic vial with screw cap.

Table 7 Conditions for Coupling of Trinucleotide Phosphoramidites on the DNA Synthesizer

Step	Reagent	Time
5′-DMT-deprotection	3% Dichloroacetic acid in dichloroethane (v/v)	36 sec
Activation/coupling	Mononucleotide phosphoramidite: 0.1 M in MeCN Trinucleotide phosphoramidite: 0.15 M in MeCN 0.3 M BMT in MeCN	5 min
Capping	Cap A: *N*-methylimidazole/MeCN (1:5, v/v) Cap B: MeCN/Ac_2O/2,4,6-collidine/(5:2:3, v/v/v)	48 sec
Oxidation	0.01 M iodine in 2,4,6-collidine/water/MeCN (1:5:11; w/v/v/v)	18 sec

Table 8 Analysis of MS Data of Obtained Hexamers

Hexamer[a]	Mass as Na-peak (*m/z*)	Calculated mass (*m/z*)
AAATTA	1798	1775
ACCTTC	1726	1703
ATCTTA	1765	1732
ATGTTC	1781	1758
CACTTA	1750	1727
CAGTTC	1766	1743
CATTTA	1765	1742
CCATTC	1726	1703
CTGTTA	1781	1756
GATTTA	1805	1782
GCATTC	1766	1743
GCGTTC	1782	1759
GCTTTC	1757	1734
GGCTTA	1806	1783
GGTTTA	1821	1798
GTATTA	1805	1782
GTTTTC	1772	1749
TGGTTA	1821	1791
TTCTTA	1756	1733
TTTTTA	1771	1748

[a]Bold letters: Trinucleotide building block.

13. Add 1.5 mL concentrated ammonia solution and incubate vial 2 hr at 55°C in a thermostatic mixer.
14. After cooldown to room temperature, centrifuge vial and transfer supernatant into a new 2 mL plastic vial.
15. Wash column with the support twice with water (500 µl), centrifuge, and collect all fractions.
16. Concentrate combined solutions *in vacuo*.
17. Analyze by MALDI-MS.

Twenty hexamers were synthesized and analyzed by MALDI-MS (Table 8).

REAGENTS AND SOLUTIONS

For common stock solutions, see Current Protocols, 2001. Use deionized water in all recipes and protocol steps. Store dichloromethane (DCM) for column chromatography over potassium chloride.

N,N-Dibutylformamide-dimethylacetal

1. Mix 1 equivalent *N,N*-dibutylamine with 1.1 equivalents *N*-dimethylformamide-dimethylacetal.
2. Stir for 3 days under reflux at 100°C.
3. Purify via vacuum distillation.
4. Characterize by ^{1}H NMR.

*^{1}H NMR (300 MHz, DMSO-d_6): δ [ppm] = 4.52 (s, 1H, -C**H**-(O-CH_3)$_2$); 3.31 (s, 6H, (-OCH_3)$_2$); 2.61 (t, 4H, -N-(C**H$_2$**-CH_2-CH_2-CH_3)$_2$); 1,42 (m, 4H, -N-(CH_2-C**H$_2$**-CH_2-CH_3)$_2$); 1.31 (m, 4H, N-(CH_2-CH_2-C**H$_2$**-CH_3)$_2$); 0.91 (t, 6H, N-(CH_2-CH_2-CH_2-C**H$_3$**)$_2$).*

COMMENTARY

Background Information

The use of trinucleotide synthons for codon-based gene synthesis was suggested in 1992 (Sondek & Shortle, 1992). Since then a number of strategies have been published, using either phosphorus (V) or phosphorus (III) chemistry, mostly in solution (Suchsland et al., 2018). The key issue in trinucleotide synthesis is the definition of protecting groups that by orthogonality allow site-selective deblocking on one side, and preserve sufficient stability under conditions of trinucleotide synthesis and phosphitylation on the other. Thus, the published strategies for trinucleotide synthesis mainly differ in the nature of the protecting groups for the 3′-OH function and the phosphate moiety. Accordingly, the quality of products and the number of steps required for trinucleotide assembly varies between the strategies. Main problems with earlier attempts of trinucleotide synthesis were low yields of trinucleotides and side products resulting from insufficiently stable or even no protecting groups on the 3′-OH (and as a consequence, by reaction of unprotected 3′-OH groups with activated 3′-O-phosphoramidites or 3′-O-phosphodiesters the formation of, for example, 3′-3′-linked dimers and trimers; see Kayushin et al., 1996; Lyttle, Napolitano, Calio, & Kauvar, 1995). Likewise, protecting groups at the 3′-OH position had to be removed under rather harsh conditions, thus causing damage to other functionalities of the trinucleotide (Lyttle et al., 1995; Virnekas et al., 1994). Among the different 3′-*O*-protecting groups, 2-azidomethylbenzoyl and TBDMS stand out as the best performing functionalities (Janczyk et al., 2012; Yagodkin et al., 2007). The 2-azidomethylbenzoyl group can be selectively removed with triphenylphosphine/H_2O, inducing reduction of the azide to a primary amine followed by spontaneous intramolecular cyclization to release isoindoline-1-one and the free 3′-OH group. The TBDMS group is removed by treatment with fluoride. Therein, it is important to use a mild agent, such as 3HF/triethylamine, because the harder fluoride in *tert*-butylammonium fluoride (TBAF) would also cleave the phosphotriester bond. Both strategies, 3′-*O*-2-azidomethylbenzoyl and 3′-*O*-TBDMS protection, have been successfully used for preparation of trinuleotide synthons by phosphotriester chemistry or by the phosphoramidite approach (Janczyk et al., 2012; Yagodkin et al., 2007). Herein, the protocol using the 3′-*O*-TBDMS strategy is described.

As an alternative to synthesis in solution, trinucleotides can be assembled on solid phase. Here it is of utmost importance that cleavage of the trinucleotide from the support proceeds under conditions that leave all other protecting groups intact. Controlled pore glass (CPG) with the start nucleoside linked via an oxalyl anchor has been used in this regard and was found to be suitable for large scale production of trinucleotides (Kayushin, Korosteleva, & Miroshnikov, 2000). Recently, soluble supports were recognized as suitable alternatives to solid supports in trinucleotide synthesis (Kungurtsev et al., 2016). In general, supported syntheses are advantageous over solution chemistry in terms of efficiency, because they are less time consuming. On the other hand, chemistry in solution is better suited to large scale synthesis. Soluble supports combine the advantages of solution chemistry and solid-phase methods, however,

they require a completely novel reaction scheme composed of iterative cycles of detritylation, precipitation, coupling and precipitation. In contrast, trinucleotide assembly on solid support proceeds by standard DNA synthesis, and thus is a straight-forward procedure. Herein we describe a detailed protocol for trinucleotide assembly on cross-linked polystyrene/divinylbenzene, relying on linkage of the start nucleoside to the support via a disulfide bridge, and release of the fully protected trinucleotide from the support by reductive cleavage. It remains to be seen what strategy for trinucleotide synthesis-in solution, on solid support, or soluble support-will win the most recognition. The available strategies have been extensively reviewed in Arunachalam et al., 2012, Raetz, Appel, & Muller, 2016, and Suchsland et al., 2018.

Critical Parameters and Troubleshooting

Removal of the 3′-*O*-TBDMS group with 3HF/triethylamine requires quenching with saturated bicarbonate solution and pH control. Acidic conditions must be strictly avoided to prevent loss of the trinucleotide 5′-*O*-DMT group. Likewise, basic conditions must be avoided, because of the lability of the β-cyanoethyl (CE) group at the phosphate moiety.

Phosphitylation of trinucleotides with CE protected phosphate moieties requires careful control of pH and must proceed under conditions described above and in Janczyk et al., 2012. The standard reagent, 2-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite in the presence of diisopropylethyl amine (DIPEA), cannot be used due to the base-labile CE group on the phosphate. Alternatively, 2-cyanoethyl-*N,N,N′,N′*-tetraisopropylphosphordiamidite in combination with diisopropylammonium tetrazolide as an activator is a common reagent for phosphitylation, without requiring the presence of a strong base. This however is also not suitable for CE protected trinucleotides because reaction would produce two equivalents of DIPEA, harming the CE group. When diisopropylammonium tetrazolide is replaced with 5-benzylmercaptotetrazole, only one equivalent of DIPEA is produced upon reaction. 5-Benzylmercaptotetrazole is sufficiently acidic to neutralize DIPEA by conversion to its ammonium salt, acting as a scavenger for DIPEA, and thus ensuring CE groups at the internucleotide linkages are not cleaved (see Janczyk et al., 2012).

The introduction of the 3′-*O*-MTM group has to be performed under dry conditions and glacial acetic acid should be added slowly, in small portions with stirring, such that the acid-labile DMT group is harmed as little as possible. For the general strategy of introduction of the MTM group see Semenyuk et al., 2006.

For reductive cleavage of trinucleotides with methyl protection of the internucleotide phosphate linkages, DTT must be avoided. The thiol groups of DTT are soft nucleophiles and would attack the soft methyl electrophiles protecting the phosphates, causing demethylation; TCEP is a superior alternative.

In general, the methyl protecting group at the internucleotide phosphate provides the more robust alternative compared with the CE group. Accordingly, synthesis of trinucleotides using nucleoside methylphosphoramidites is the less challenging way and may be preferential. However, looking at synthesis economy, CE protected trinucleotide synthons when used in standard DNA synthesis are removed in one step together with protecting groups at the heterocyclic bases and cleavage of the oligomeric product from the support. When methyl protection at the phosphate is used, an extra step utilizing a thiol or thiolate for removal of the methyl group is required.

CPG was found less suitable as a support for solid-phase synthesis of trinucleotides because loading densities with starting nucleosides were significantly lower than for cross-linked polystyrene/divinylbenzene (Custom Primer Support Amino 200, GE Healthcare).

Anticipated Results

The solid-phase strategy described in this article is carried out at small scale to produce trinucleotides for initial testing. With appropriate equipment, assembly of trimers can be scaled up to prepare trinucleotides at gram scale, as reported for the strategy mentioned above using CPG, with the starting nucleoside linked via an oxalyl anchor (Kayushin et al., 2000).

Time Considerations

The time for synthesis of trinucleotides in solution is significantly longer than for solid-phase synthesis. In the latter, the time-limiting step is functionalization and immobilization of the starting nucleoside. However, sufficient amounts of support loaded with each of the four nucleosides can be prepared and stored until use.

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Article

Solid Phase Assembly of Fully Protected Trinucleotide Building Blocks for Codon-Based Gene Synthesis

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Abstract: The use of pre-formed trinucleotides, representing codons of the 20 canonical amino acids, for oligonucleotide-directed mutagenesis offers the advantage of controlled randomization and generation of "smart libraries". We here present a method for the preparation of fully protected trinucleotides on solid phase. The key issue of our strategy is the linkage of the starting nucleoside to the solid support via a traceless disulfide linker. Upon trinucleotide assembly, the disulfide bridge is cleaved under reducing conditions, and the fully protected trinucleotide is released with a terminal 3′-OH group.

Keywords: trinucleotide building block; gene library; protein engineering; codon; randomization

1. Introduction

A major goal of molecular biotechnology is the evolution/development of enzymes with desired and improved properties for industrial or pharmaceutical application [1,2]. Often the knowledge about structural details of the enzyme of interest is missing, such that rational design is not an option [2]. On the contrary, directed evolution offers the possibility, via random mutagenesis, of generating large protein libraries, which are screened for functionality, such that detailed structural information is not required [2]. Furthermore, combinatorial gene synthesis offers the option of restricting structural variations to residues of interest, i.e., those that are involved in a certain function such as catalysis. Nevertheless, there are a number of hurdles to be considered. For example, the size of gene libraries resulting from large random mutagenesis would be too big for practical screening [3]. Other challenges are the structure of the genetic code, where some amino acids are encoded by up to six triplets and others by just one, leading to a bias of the mutation, favoring those amino acids with multiple codons [4]. On the methodological side, directed evolution by error-prone PCR is a widely used strategy, which however, generates bias of mutation depending on the polymerase used [4]. Furthermore, the generation of stop codons cannot be prevented, which leads to abortion fragments instead of the full-length enzymes [5]. A possibility to overcome these problems is the use of trinucleotide building blocks (Figure 1), which represent codons of the canonical amino acids and thus permit the controlled synthesis of a fully or partially randomized gene library without stop codons or codon bias [6]. There are several but not easy ways to synthesize such trinucleotide building blocks, either in solution [7] or, as a more recent addition to the field, on soluble support [8,9]. We have previously developed a strategy for synthesis in solution of fully protected trinucleotide phosphoramidites [6] and, more recently, have succeeded in synthesizing trinucleotides on solid support. The preparation of

trinucleotides in solution can be carried out at a larger scale. However, it requires careful design of the strategy and orthogonal protection of individual groups. Thus, a pair of orthogonal protecting groups for the 5′- and 3′-OH functionalities needs to be designed, in order to avoid side reactions during trinucleotide synthesis. Stable protection of the phosphate moieties as well as of the heterocyclic bases throughout trinucleotide synthesis is essential. After each reaction step, isolation and purification of intermediate products is required, making trinucleotide assembly in solution a rather tedious and time-consuming procedure. Syntheses on support are advantageous in terms of efficiency. Products bound to the support and removal of excess reagents by simple washing facilitates the process and saves time. The future may lie in the usage of soluble polymers [8,9]. Soluble supports combine the advantages of solution chemistry and solid phase methods. However, they require a reaction scheme optimized for liquid phase assembly, which currently is still under investigation. Trinucleotide assembly on solid support can proceed by standard phosphoramidite chemistry and, thus, is a valuable strategy for the preparation of fully protected trinucleotides, until the major hurdles of supported oligonucleotide assembly on soluble polymers have been overcome. Our initial protocol for solid phase trinucleotide assembly has been described recently [10]. We here wish to report our studies regarding the choice of a suitable polymer, efficient conjugation chemistry for linking the starting nucleoside to the support, and detachment of the fully protected trinucleotide.

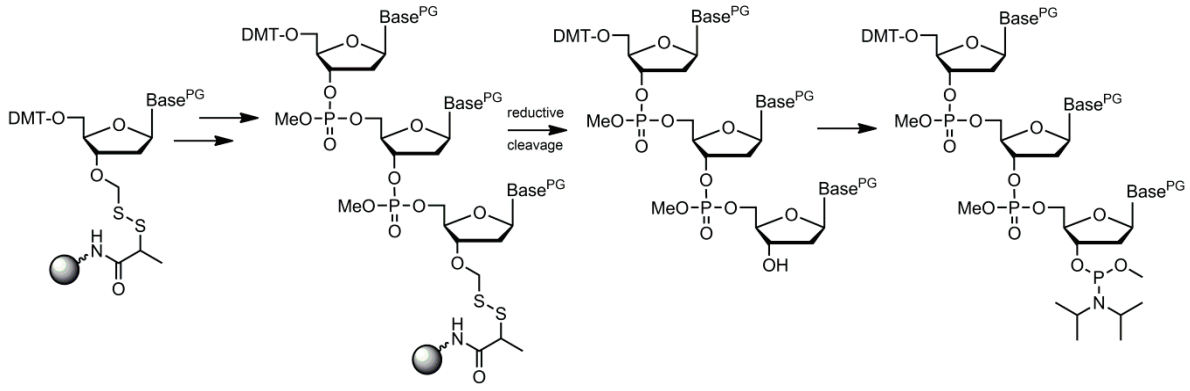


Figure 1. Reaction scheme for preparation of fully protected trinucleotide phosphoramidites.

2. Materials and Methods

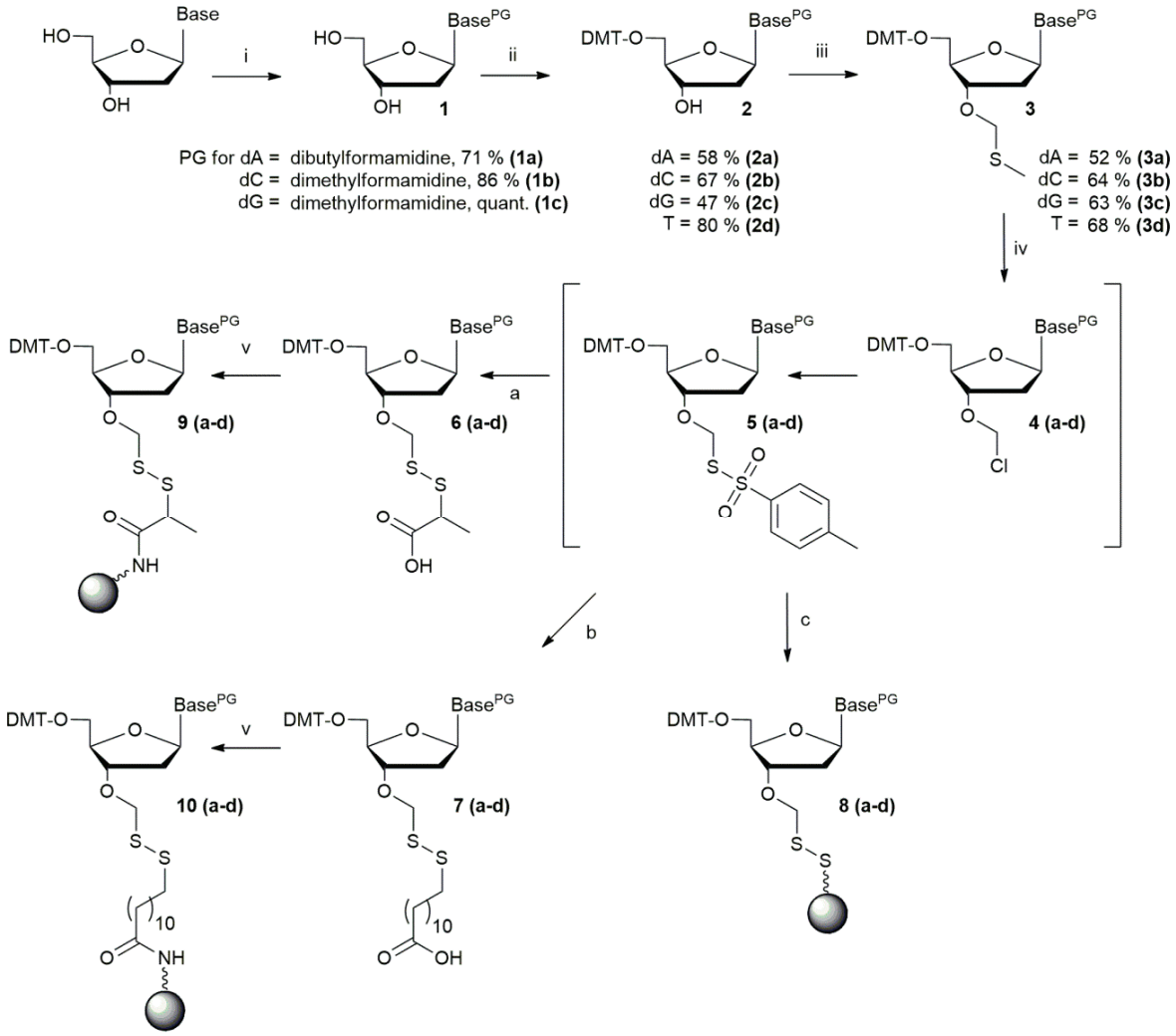
2.1. General Information

Chemicals were purchased from commercial suppliers (Roth, Sigma Aldrich, Merck, TCI). Organic solutions were concentrated by rotary evaporation at 40 °C. Pyridine was stored over KOH, distilled prior to use and stored over a molecular sieve. Buffers for the purification of trinucleotides were prepared with micro-pore water (Nanopore, Thermo Scientific). Products were visualized by thin layer chromatography (TLC) on aluminum sheets covered with silica gel 60 F254 (Merck) at 254 nm. Unless otherwise described, the standard TLC solvent system dichloromethane/methanol = 95:5 was used. Column chromatography was carried out on silica gel from Merck (Geduran Si 60, 0.063–0.200 mm). Solvents for column chromatography were purified by rotary evaporation. All buffers for HPLC analyses were filtered through a 0.2 µm acetonitrile resistant membrane and degassed in the ultrasonic bath for 15 min. MALDI-TOF analyses were done with a Bruker Daltonics Microflex in either positive or negative mode. For this, 1 µL sample solution was mixed with 1 µL hydroxypicolinic acid solution (in H_2O/acetonitrile = 1:1) on a plate (MSP 96 target ground steel) and dried to crystallization [11]. Determination of support loading densities was carried out by detritylation of the support-linked nucleoside and UV/VIS absorption measurements. Therefore, 3% dichloroacetic acid was added to the support. The absorption of the DMT-cation was measured at 498 nm, and the corresponding DMT concentration was determined by comparison to a standard curve. For the detailed procedure see Suchsland et al. [10] and Supplementary Materials S1.

List of solid supports used in this work: **A** = Amino CPG (controlled pored glass), 500 Ångström (105 µmol/g) from Chem Genes, (USA); **B** = Polystyrene A SH, 200–400 mesh (850 µmol/g) from RAPP Polymere (Germany); **C** = Polystyrene AM NH_2, 200–400 mesh (980 µmol/g, high loaded) from RAPP Polymere (Germany); **D** = Custom Primer Support Amino, high cross-linked polystyrene/divinylbenzene (200 µmol/g), from GE Healthcare (Sweden).

2.2. Synthesis of 5′-O-Dimethoxytrityl-3′-O-Methylthiomethyl-2′-Deoxynucleosides

The synthesis of 3′-*O*-thiomethyl-modified nucleosides (in Scheme 1(3a–d)) is described in detail in Suchsland et al. [10]. See Supplementary Materials S2 for ^{1}H and ^{13}C NMR spectra.



Scheme 1. Reaction scheme of nucleoside functionalization and immobilization. Nucleotides are linked to the support via a disulfide bridge, with (**a**) short linker (sec. carbon), (**b**) long linker (prim. carbon), (**c**) direct connection; (i) dC/dG: 5 eq. dimethylformamide-dimethylacetal, 1 h, 50 °C, in dimethylformamide; dA: 1.4 eq. dibutylformamide-dimethylacetal, rt, overnight, 1 h, 80 °C, in dimethylformamide; (ii) 1.3 eq. 4,4′-dimethoxytrityl chloride, 3 h up to overnight in pyridine; (iii) 70 eq. dimethyl sulfoxide, 53 eq. acetic acid, 53 eq. acetic anhydride, rt, 20 h; (iv) 3 eq. triethylamine, 1 eq. sulfuryl chloride, 1.5 eq. potassium thiotosylate in dichloromethane. (**a**) 2 eq. 2-mercapto propionic acid, rt, 2 h; (**b**) 2 eq. 12-mercaptododecanoic acid, rt, 2 h; (**c**) solid support B, rt, 1 h; (v) 2 eq. TSTU, 5 eq. triethylamine, solid support (A/C/D), rt, overnight in dimethylformamide/dichloromethane/water.

2.3. Loading of Support

2.3.1. Thymidine as Starting Nucleoside

Loading of Polystyrene A SH (B)

5′-*O*-Dimethoxytrityl-3′-*O*-methylthiomethyl-thymidine (**3d**) (0.85 mmol) was coevaporated three times with dry dichloromethane and dissolved under dry conditions in dry dichloromethane (53.55 mmol, 3.42 mL). Triethylamine (2.55 mmol, 0.35 mL) was added and the solution stirred in an ice bath. Freshly, up to colorlessness, distilled sulfuryl chloride (0.85 mmol, 0.07 mL) was mixed with dichloromethane (10.2 mmol, 0.65 mL), and this solution was added slowly and dropwise to the reaction mixture, which turned orange. After 10 min in the ice bath, the solution was stirred for 5 min at room temperature. Potassium thiotosylate (1.28 mmol, 0.243 g) was mixed with dimethylformamide (10.2 mmol, 0.78 mL), and this solution was added to the reaction mixture dropwise, changing the color from orange to yellow. Formation of the intermediate product **5d** was observed by TLC. After stirring for 10 min, 820 mg solid support **B** (polystyrene A SH) was added, and the reaction mixture was stirred for 20 h at room temperature. Upon removal of the liquid, the support was washed with dichloromethane and methanol and dried in vacuo [12,13]. The loading density of the support **8d** was 238.43 µmol/g.

Loading of Amino-Functionalized Supports (A, C, D)

- Preparation of 2′-*O*-linker modified thymidine derivatives **6d** and **7d**

5′-*O*-Dimethoxytrityl-3′-*O*-methylthiomethyl-thymidine (**3d**) (2.32 mmol) was dissolved in dry dichloromethane (9.33 mL). Triethylamine (6.96 mmol, 0.96 mL) was added, and the reaction mixture was cooled in an ice bath. Freshly distilled sulfuryl chloride (2.32 mmol, 0.19 mL) in dichloromethane (1.77 mL) was added dropwise to the reaction mixture. The solution was stirred for 10 min in the ice bath. After warming up to room temperature, a solution of potassium thiotosylate (3.4 mmol, 0.65 g) in dry dimethylformamide (2.17 mL) was added dropwise to the reaction mixture. Formation of the intermediate product **5d** was observed by TLC. After stirring for 10 min 2-mercaptopropanoic acid (4.64 mmol, 0.38 mL) was added. The color of the reaction mixture turned to light red due to the loss of traces of the DMT protecting group. After 20 min the color of the solution changed to yellow. After stirring for 1.5 h the reaction mixture was washed with saturated aqueous sodium bicarbonate solution and brine. After drying over Na_2SO_4 and removing the solvent under vacuum, the residue (**6d**) was used for in situ coupling to solid support (**A**, **C**, and **D**) [12,13]. For synthesis of compound **7d**, mercaptododecanoic acid was used instead of 2-mercaptopropanoic acid. Synthesis was conducted in the same way as described above for 2-mercaptopropanoic acid, although at smaller scale (0.45 mmol of **3d** and 0.9 mmol of mercaptododecanoic acid).

- Loading of solid support: Amino CPG (**A**)

One eighth of the reaction mixture from above containing 5′-*O*-dimethoxytrityl-3′-*O*-(propanoic acid-2-dithiomethyl)-thymidine (**6d**) was dissolved in dimethylformamide (0.4 mL), dichloromethane (0.8 mL) and H_2O (0.1 mL). Triethylamine (2.3 mmol, 0.32 mL) and 2-succinimide-1,1,3,3-tetramethyluronium tetrafluoroborate (TSTU) (2.5 mmol, 0.72 g) were added. Afterward solid support **A** (60 mg, Amino CPG) was added, and the reaction mixture was stirred for 48 h at room temperature. The loaded solid support (**9d**) was washed with ethanol, dichloromethane, and water and dried in vacuo [14]. The loading was 10.5 µmol/g. The coupling of 5′-*O*-dimethoxytrityl-3′-*O*-(dodecanic acid-12-dithiomethyl)-thymidine (**7d**) to Amino CPG was done under the same conditions. The loading of the resulting material, **10d**, was 7.9 µmol/g.

- Loading of solid support: Polystyrene AM NH_2 (**C**)

Loading of polystyrene support **C** with 2-mercaptopropanoic acid or 12-mercaptododecanoic acid was done as described for the loading of Amino CPG (**A**) with one sixth of the 5′-*O*-dimethoxytrityl-3′-*O*-(propanoic acid-2-dithiomethyl)-thymidine (**6d**) or 5′-*O*-di-methoxytrityl-3′-*O*-(dodecanoic acid-12-dithiomethyl)-thymidine (**7d**) reaction mixture and 145 mg Polystyrene AM NH_2 (**C**) (for loading densities see Table 1).

- Loading of solid support: Custom Primer Support Amino (**D**)

Loading of polystyrene support **D** with 2-mercaptopropanoic acid or 12-mercaptododecanoic acid was equally done with one third of the 5′-*O*-dimethoxytrityl-3′-*O*-(propanoic acid-2-dithiomethyl)-thymidine reaction mixture (**6d**) and 230 mg Custom Primer Support Amino (**D**) or with one sixth of the 5′-*O*-dimethoxytrityl-3′-*O*-(dodecanoic acid-12-dithiomethyl)-thymidine reaction mixture (**7d**) and 150 mg Custom Primer Support Amino (**D**) (for loading densities see Table 1).

Table 1. Loading densities of various supports and trinucleotide recovery upon reductive cleavage with TCEP.

Entry #	Solid Support	Linker	Starting Nucleoside	Loading [μg/mol]	Recovery of Trinucleotide
1	A Amino CPG	12-mercaptododecanoic acid	Thymidine	7.9	quantitative
2	A Amino CPG	2-mercaptopropanoic acid	Thymidine	10.5	quantitative
3	B Thiol PS	-	Thymidine	238.4	<10%
4	C Amino PS	12-mercaptododecanoic acid	Thymidine	245.0	<10%
5	C Amino PS	2-mercaptopropanoic acid	Thymidine	57.8	<10%
6	D Amino PS (hcl)	12-mercaptododecanoic acid	Thymidine	89.3	quantitative
7	D Amino PS (hcl)	2-mercaptopropanoic acid	Thymidine	58.9	quantitative
			Deoxyguanosine	51.6	quantitative
			Deoxycytidine	58.8	quantitative
			Deoxyadenosine	113.0	quantitative

CPG—controlled pored glass, PS—Polystyrene, hcl—highly crosslinked.

Capping of Unreacted Amino or Thiol Groups on Solid Supports A, B, C, and D

The unreacted free amino-groups on the solid supports **A**, **C**, and **D** as well as the free thiol-groups on solid support **B** were capped with capping reagents A and B (Cap A: 0.67 mL *N*-methylimidazole, 3.4 mL acetonitrile; Cap B: 0.84 mL acetic anhydride, 2.1 mL acetonitrile, 1.26 mL trimethylpyridine) while stirring for 15 min. After capping, the solid support was washed with acetonitrile and dried in vacuo.

2.3.2. Loading of 2′-Deoxyguanosine on Solid Support D

Starting with the 3′-*O*-methylthiomethyl functionalized guanosine derivative (**3c**) (0.6 mmol, 0.41 g) the synthesis of acid-modified nucleoside **6c** using 2-mercaptopropanoic acid (1.2 mmol, 0.13 mL) was carried out under the same condition as described for the thymidine derivative **6d** (see above). The resulting crude compound **6c** was used for in situ coupling to solid support. It was dissolved in dimethylformamide/dichloromethane = 1:4. TSTU (3 eq.) and triethylamine (5 eq.) were added to the reaction mixture. Afterward, 285 mg solid support **D** was added to the reaction mixture and stirred for 48 h. Upon removal of the liquid, the solid support was washed with ethanol, dichloromethane, and water, and dried in vacuo. The loading was 51.56 μmol/g.

2.3.3. Loading of 2′-Deoxyadenosine on Solid Support D

Starting with the 3′-*O*-methylthiomethyl functionalized adenosine derivative (**3a**) (0.12 mmol, 0.09 g) the synthesis of acid-modified nucleoside **6a** using 2-mercaptopropanoic acid (0.24 mmol, 0.02 mL was carried out under the same condition as described for the thymidine derivative **6d** (see above). Half

of the resulting crude compound **6a** was used for in situ coupling to solid support. It was dissolved in dimethylformamide/dichloromethane/water = 5:1:1.25. TSTU (3 eq.) and triethylamine (5 eq.) were added to the reaction mixture. Afterward, 43 mg solid support **D** was added to the reaction mixture and stirred for 48 h. Upon removal of the liquid, the solid support was washed with ethanol, dichloromethane, and water, and dried in vacuo. The loading was 113 μmol/g.

2.3.4. Loading of 2′-Deoxycytidine on Solid Support D

Starting with 3′-*O*-methylthiomethyl functionalized cytidine derivative (**3b**) (0.28 mmol, 0.19 g) the synthesis of acid-modified nucleoside **6b** using 2-mercaptopropanoic acid (0.56 mmol, 0.047 mL was carried out under the same condition as described for the thymidine derivative **6d** (see above). The resulting crude compound **6b** was used for in situ coupling to solid support. It was dissolved in dimethylformamide/dichloromethane = 1:4. TSTU (3 eq.) and triethylamine (5 eq.) were added to the reaction mixture. Afterwards, 136 mg solid support **D** was added to the reaction mixture and stirred for 48 h. Upon removal of the liquid, the solid support was washed with ethanol, dichloromethane, and water, and dried in vacuo. The loading was 58.82 μmol/g.

2.3.5. Capping of Unreacted Amino Groups on Solid Supports D

The free amino groups of the solid support were capped with capping reagents A and B (Cap A: 0.8 mL *N*-methylimidazole, 4.1 mL acetonitrile; Cap B: 1 mL acetic anhydride, 2.5 mL acetonitrile, 1.5 mL trimethylpyridine) while stirring for 30 min. After capping, the solid support was washed with acetonitrile and dried in vacuo.

2.4. Cleavage from Support

Choice of Cleavage Reagent

Two reagents, DTT and TCEP, were tested as reducing agents for the cleavage of the disulfide bridge linking the nucleotide to the support. To this end, loaded solid support **B** was incubated with either DTT or TCEP at 55 °C in 0.5 mL phosphate buffer (9.6 mM, pH 7) overnight. For results see Table 1. For details see Supplementary Materials S3.

2.5. Synthesis and Purification of Trinucleotides

Trinucleotides were synthesized by phosphoramidite chemistry in 1 μmol scale with *β*-cyanoethyl- or methyl-protected standard phosphoramidites from LGC Link, Scotland and benzylmercaptotetrazole as activator [10]. Syntheses were carried out with a Gene Assembler Special from Amersham Biosciences, with a coupling time of 1.5 min and in DMT-on mode.

2.5.1. Trinucleotide Cleavage from Solid Support

Trinucleotides were cleaved from support with 300 μmol TCEP in 600 μL acetonitrile and 400 μL HEPES-buffer (100 mM, pH 7) at 55 °C in an ultrasonic bath overnight. Afterward the solid supports were treated with 3% dichloroacetic acid to check if cleavage of the trinucleotide was completed. Eventually the remaining trinucleotides would be detected by the red color of the cleaved-off DMT cation.

2.5.2. Precipitation of Trinucleotides

Upon cleavage, the solid support was washed with water, acetonitrile, ethanol, acetone, tetrahydrofurane, and ethylacetate. Washing solutions were combined and, after evaporation, fully protected trinucleotides were precipitated from water.

3. Results and Discussion

Due to the obvious advantages of solid phase synthesis over solution chemistry, we sought to develop a strategy that allows the assembly of fully protected trinucleotides on solid support. The key issue is the detachment of the trinucleotide from the support maintaining all protecting groups. Reductive cleavage is a suitable strategy, as it is orthogonal to the acid labile DMT group, as well as to nucleophilic removal of the phosphate and nucleobase protecting groups (Figure 1) [15–17]. Thus, we took an example from a previously published strategy for the synthesis of RNA using 2′-*O*-*tert*-butyldithiomethyl-protected building blocks [18], and developed a protocol for trinucleotide assembly with the starting nucleoside connected to the solid support by a dithiomethyl linkage (Figure 1). Reductive cleavage of the disulfide bond would produce a *S*,*O*-hemiacetal, which spontaneously decomposes into alcohol and thioformaldehyde, thus yielding the free 3′-OH group [18], which subsequently can be phosphitylated as described in our earlier work on the preparation of trinucleotide synthons [6].

For immobilization of the starting nucleoside, first the 3′-OH group needed to be appropriately functionalized (Scheme 1). This was achieved by the site-specific introduction of a methylthiomethyl group, followed by activation of the obtained 3′-*O*-methylthiomethyl (3′-*O*-MTM) derivatives **3a–d** by treatment with sulfuryl chloride to produce the 3′-*O*-chloromethyl ether (**4a–d**) in a Pummerer rearrangement [19,20]. The chloromethylether reacts with potassium thiotosylate forming the reactive compound **5a–d**, which subsequently is linked to the polymer via a disulfide bridge [12,13]. 5′-*O*-Dimethoxytritylated nucleosides were used as starting compounds requiring careful control of reaction conditions (pH), in particular during the first step of MTM introduction.

3′-*O*-MTM derivatives **3a–d** of all four deoxynucleotides were prepared with satisfying yields (52%–68% [10]) and linked either directly to thiol-derivatized polystyrene **B** (**8a–d**) or, via a bifunctionalized linker, to amino-derivatized polystyrene **C** and **D** (**10a–d**) or amino-derivatized CPG **A** (**9a–d**). As a bifunctionalized linker, we tested 2-mercaptopropionic acid or, as a longer variant, 12-mercaptodecanoic acid. Both linker molecules comprise a thiol group for disulfide formation, however in the shorter variant it is connected to a secondary carbon and in the longer variant it is connected to a primary carbon atom. Thus, the formation of the disulfide bridge, its stability during trinucleotide assembly, and disulfide cleavage can be studied depending on linker lengths and type of the thiol. After attachment of the linker to nucleoside derivatives (**6a–d**, **7a–d**), immobilization to aminopolystyrene or Amino CPG was achieved by amide bond formation upon activation of the linker carboxyl group with TSTU [14].

Seven types of support, each consisting of either polystyrene or CPG, linked directly or via a bifunctionalized linker to thymidine as the starting nucleoside, were prepared for initial testing (Figure 2, Table 1). The loading was determined by removal of the DMT group under acidic conditions according to Tang et al. [11] (for the detailed procedure see Suchsland et al. [10]). In general, the loading of polystyrene (**B**, **C**, **D**) was found to be higher (58–245 µmol/g) than that of Amino CPG **A** (8–11 µmol/g). Removal of the nucleoside from the support was tested by treatment with either a buffered solution of 1,4-dithiothreitol (DTT, pH7) at 50 °C or tris-(2-carboxyethyl)-phosphine (TCEP, pH 7) at 55 °C in the ultrasonic bath for 20 h. The same assay as used above for the determination of support loading with nucleoside was applied for analyzing nucleoside/trinucleotide recovery. To this end, upon reductive cleavage of the disulfide bridge linking the starting nucleoside to the support, and extensive washing, the support was treated with 3% dichloroacetic acid, and the absorption at 498 nm was measured to detect any remaining nucleoside by detritylation. Interestingly, there was always DMT detectable when polystyrene **B** and **C** were treated with 3% dichloroacetic acid after disulfide cleavage (Table 1, entry #3, #4, and #5). This may be the result of either inefficient cleavage of the disulfide bridge, such that trinucleotides remained covalently linked to the polymer, or, trinucleotides upon disulfide cleavage non-specifically interact with the polystyrene, such that they cannot be washed off completely. This was different for CPG and highly crosslinked (hcl) polystyrene (Table 1, entries #1, #2, #6, and #7), which are the typical supports used in oligonucleotide synthesis, and thus, not surprisingly, showed best results.

TCEP was found being more efficient (see Supplementary Materials S3) [21] and, due to the intended use of methyl-protected phosphoramidites (see below), it was the better choice for the cleavage of the synthesized trinucleotides from the support. Overall, highly crosslinked polystyrene showed the best features in terms of nucleoside loading and TCEP-mediated detachment, independent of the linker connecting the starting nucleoside to the amino-functionalized polymer. Thus, hcl polystyrene would be the best choice for trinucleotide assembly, in particular, also when the process needs to be carried out at a larger scale.

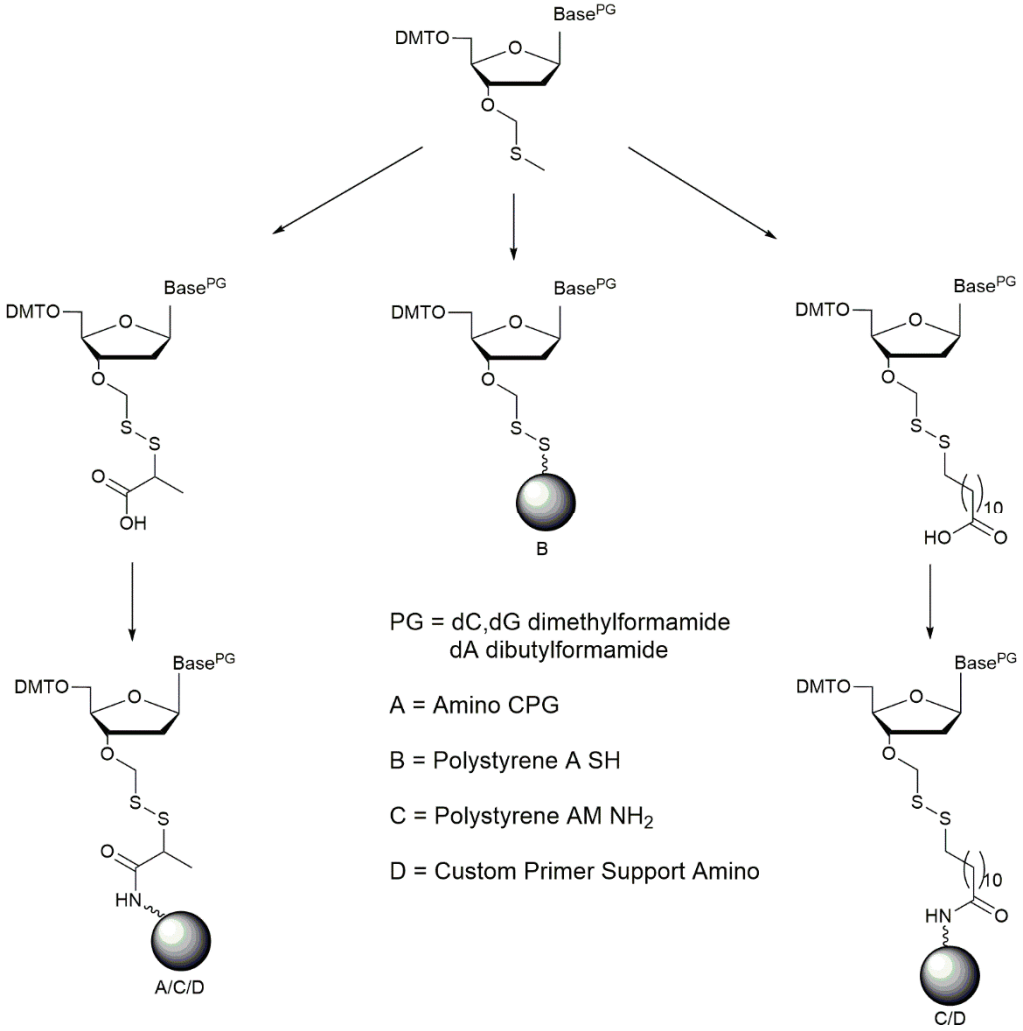


Figure 2. Overview of supports loaded with starting nucleoside.

In previous experiments we had investigated the influence of the nature of the linker thiol being primary, secondary, or tertiary. The stability of a disulfide bridge depends on the nature of the bridge-forming thiol, with a tertiary thiol being most stabilizing due to the electron-donating effect of the tertiary alkyl group. In our preliminary experiments, we found the disulfide bridge formed with the primary thiol was clearly more labile than those formed with the secondary and tertiary thiols, when it was cleaved with DTT (see Supplementary Materials S4 for details). Under the conditions of disulfide cleavage used here (TCEP, over 20 h), this stability difference could not been observed, since cleavage from support **A** and **D**, with either 12-mercaptododecanoic acid or 2-mercaptopropanoic acid as linker was completed after a rather long incubation time. Nevertheless, based on our previous results with DTT, we decided to use 2-mercaptopropanoic acid as linker for our further studies, because of the higher stability of the formed disulfide bridge and, hence, less risky trinucleotide assembly. Thus, in addition to T, the 5′-*O*-DMT-3′-*O*-MTM-functionalized derivatives of dA, dC, and dG (**3a–c**, Scheme 1), were also

linked to 2-mercaptopropionic acid followed by immobilization on amino-functionalized polystyrene (**D**) (Table 1).

Before use of the loaded supports, free amino groups were capped by treatment with acetic anhydride. Then, trinucleotides were synthesized by coupling of either *β*-cyanoethyl- or methyl-protected phosphoramidites of *N*-acyl-5′-*O*-DMT-protected 2′-deoxynucleosides. Previously, we had used *β*-cyanoethyl-protected phosphoramidites for trinucleotide assembly in solution and found that the *β*-cyanethyl group was suitable for phosphate protection. It was stable throughout trinucleotide assembly and phosphitylation, provided that particular attention was paid to reaction and purification conditions, in particular to the pH of reaction media, solvents, eluents, and buffers. In order to circumvent these challenges and to allow easier handling of the fully protected trinucleotides during purification and in further reactions, we later switched to the more stable methyl group for phosphate protection. Altogether, ten different trinucleotides were synthesized basically following the standard protocol for DNA synthesis [10]. For initial testing, syntheses were carried out at 1 µmol scale. Three trimeric oligodeoxyribonucleotides were assembled from *β*-cyanoethyl phosphoramidites, and seven were assembled from methyl phosphoramidites (Table 2). For oxidation of the phosphite, a diluted solution of iodine (0.02 M) was used, because of the previously observed partial cleavage of the disulfide bond when treated with 0.1 M iodine solution [18,22].

Table 2. Overview of synthesized trinucleotides.

Phosphate Protecting Group	Trinucleotide	Mass Calculated	Mass Found	Cleavage Reagent
Me	ATT	1260.14	1284.84 (+Na)	TCEP
Me	AAT	1339.24	1385.82 (+2Na)	TCEP
Me	TTT	1180.34	1180.30	TCEP
Me	AAA	1402.50	1448.22 (+2Na)	TCEP
Me	GGA	1449.49	1472.49 (+Na)	DTT
Me	GGC	1459.47	1482.34 (+Na)	DTT
Me	GGT	1370.44	1393.38 (+Na)	DTT
β-cyanoethyl	TTT	1258.37	1281.29 (+Na)	DTT
β-cyanoethyl	TTA	1337.42	1360.34 (+Na)	DTT
β-cyanoethyl	TTC	1347.39	1370.38 (+Na)	DTT

Upon cleavage from the support with a buffered solution of DTT, the quality of fully protected trinucleotides TTT, TTA, and TTC carrying the *β*-cyanoethyl group at the phosphates was confirmed by HPLC and MALDI-TOF MS (Table 2 and Supplementary Materials S5). However, when using methyl-protected phosphoramidites, partial loss of the methyl group was observed upon removal of trinucleotides from the support with DTT, due to nucleophilic attack of the DTT–SH group onto the methyl group carbon (Supplementary Materials S6). On the contrary, when trinucleotides were removed by disulfide cleavage with TCEP under the conditions mentioned above, the methyl protection at the phosphates remained intact (Supplementary Materials S7). Upon cleavage from the support, fully protected trinucleotides were precipitated from water, dried, and stored under vacuum.

Trinucleotides should be phosphitylated directly prior to use in DNA synthesis. Particular care has to be taken when *β*-cyanoethyl-protected trinucleotides are phosphitylated, as we have described recently [6]. Here, the lability of the *β*-cyanoethyl group under basic conditions requires careful choice of the phosphitylation reagent and control of reaction conditions. The same optimized conditions can be applied to methyl-protected phosphoramidites, although the methyl group at the phosphorous is less labile.

4. Conclusions

In conclusion, assembly of trinucleotides on solid support with the starting nucleoside being linked to the polymer via a disulfide bridge is a straightforward strategy for the preparation of

fully protected trimeric oligodeoxyribonucleotides. Upon cleavage of the disulfide under reducing conditions, the trimer with all protecting groups at the nucleobases, the phosphates, and the terminal 5′-OH-group remaining intact is released into solution. Following evaporation of the solvent, trimers are precipitated from water, dried, and stored until use in standard DNA synthesis, starting with phosphitylation as described [6]. The solid phase strategy nicely adds to our previously developed approach for the preparation of trinucleotide synthons in solution and to the more recently published methods for trinucleotide assembly on a precipitative soluble support [8,23]. So far, syntheses were carried out at 1 µmol scale to demonstrate the suitability of the method. In future work, fully protected trimers will be prepared at a larger scale to produce sufficient amounts for further evaluation of the trimers in gene library synthesis.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-3417/9/11/2199/s1, Figure S1. Standard curve of DMT-Cl for calculation of loading density, Figure S2. Synthesis of 5′-*O*-dimethoxytrityl-3′-*O*-dithiomethyl-*N*-benzoyl-2′-deoxycytidine with primary, secondary, or tertiary disulfide bridge (S-prim/sec/tert), Figure S3. Synthesized trinucleotides with primary, secondary, and tertiary disulfide bridge ATC-S_{prim}, CAC-S_{sec}, TTC-S_{tert} (* calculated mass), Figure S4. Reductive cleavage of the 3′-disulfide bridge of trinucleotides ATC-S_{prim}, CAC-S_{sec}, and TTC-S_{tert}. Shown are the mass spectra of trinucleotides after 30 min and 9 h of treatment with DTT, Figure S5. MS-Spectra of trinucleotides after cleavage from the support, Figure S6. RP-HPLC of TTT-3′-OH after cleavage from solid support, buffer A: 5% acetonitrile, buffer B: 70% acetonitrile, VP 125/21 Nucleosil 120-10 C18, Figure S7. RP-HPLC TTA-3′-OH after cleavage from solid support, buffer A: 5% acetonitrile, buffer B: 70% acetonitrile, VP 125/21 Nucleosil 120-10 C18, Figure S8. RP-HPLC TTC-3′-OH after cleavage from solid support, buffer A: 5% acetonitrile, buffer B: 70% acetonitrile, VP 125/21 Nucleosil 120-10 C18, Figure S9. MS-Spectra of trinucleotides with methyl/protected phosphate after cleavage with DTT from the support. The difference of 14 indicates loss of a methyl group at the phosphate, Figure S10. MS-Spectra of trinucleotides with methyl-protected phosphate after cleavage with TCEP from the support, Table S1. Cleavage conditions, Table S2. 3′-OMT-S-prim/sec/tert yields and masses.

Author Contributions: Conceptualization, S.M. and B.A.; methodology, M.J., R.S., B.A., and S.M.; validation, M.J., R.S., and S.M.; investigation, M.J. and R.S.; resources, S.M.; data curation, R.S. and S.M.; writing—original draft preparation, R.S. and B.A.; writing—review and editing, S.M.; supervision, S.M.; project administration, S.M.; funding acquisition, R.S. and S.M.

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Conflicts of Interest: There are no conflicts to declare.

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Supplementary Material

Solid Phase Assembly of Fully Protected Trinucleotide Building Blocks for Codon-Based Gene Synthesis

S1. Determination of the Loading

Determination of support loading densities was carried out by UV absorption measurements. Therefore 3% dichloroacetic acid was added to the support. The DMT concentration was measured at 498 nm and compared to a standard curve. For the detailed procedure see Suchsland et al. [11].

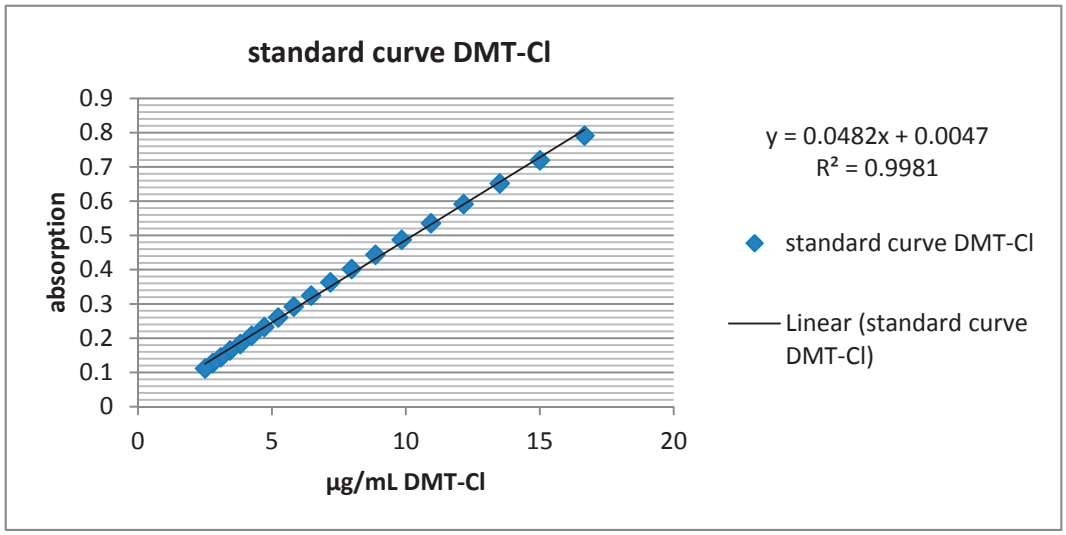


Figure S1. Standard curve of DMT-Cl for calculation of loading density

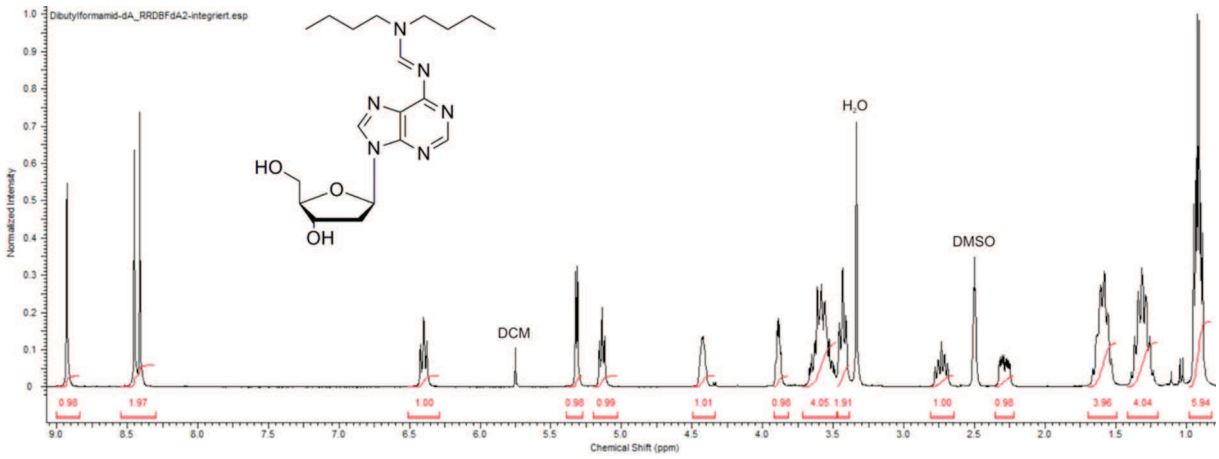
The same assay was used for checking completeness of nucleoside cleavage from the support. To this end, upon reductive cleavage of the disulfide bridge linking the nucleoside to the support, and extensive washing, the support was treated with 3% dichloroacetic acid, and the absorption at 498 nm was measured to detect any remaining nucleoside by detritylation.

S2. ^{1}H- and ^{13}C-Spectra of Final and Intermediate Products of the Synthesis of 5′-*O*-Dimethoxytrityl-3′-*O*-Methylthiomethyl-2′-Deoxynucleosides

The synthesis of 3′-*O*-methylthiomethyl-modified nucleosides (**3a–d** in Scheme 1 of the main manuscript) has been described in detail in Suchsland et al. [11]. ^{1}H and ^{13}C NMR spectra were measured in deuterated DMSO with a Bruker 300 MHz NMR spectrometer. Original spectra are shown here. See Suchsland et al. [11] for the list of analytical data and interpretation.

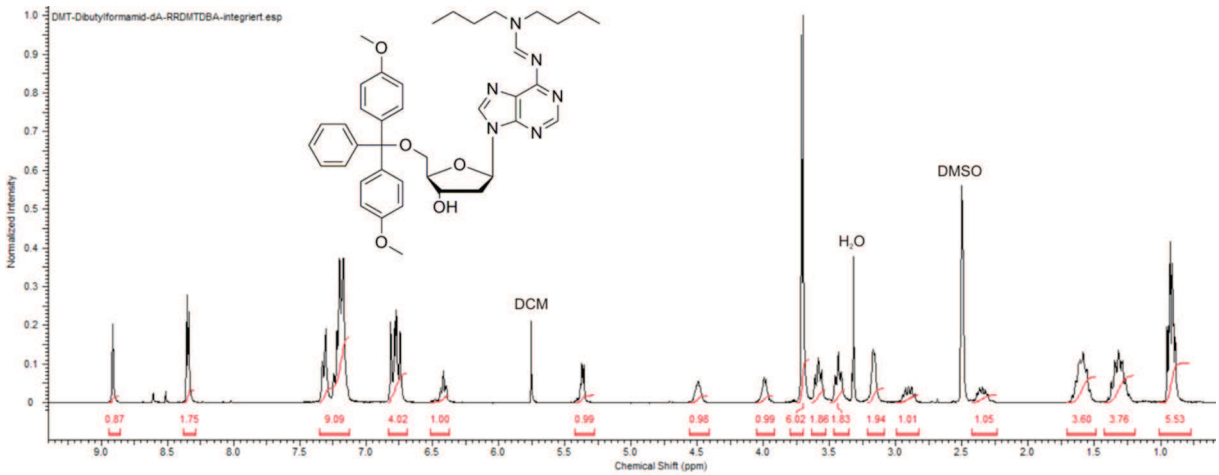
2′-Deoxyadenosine

^{1}H NMR spectrum, *N*-6-[(dibutylamino)methylene]-2′-deoxyadenosine (**1a**)



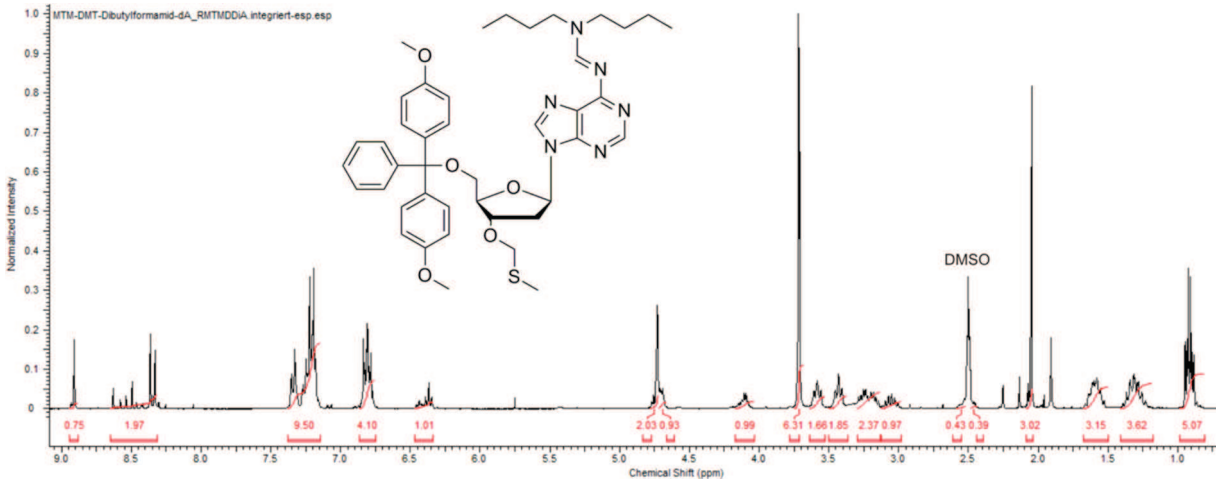
δ [ppm] = 8.93 (s, 1H, dibutylformamide N = C–H); 8.45 and 8.40 (2 s, 2H, H8 and H2); 6.40 ("t", *J* = 6.78 Hz, 1H, H1′); 5.32 (d, *J* = 3.93 Hz, 1H, 3′-OH); 5.14 (t, 1H, 5′-OH); 4.43 (m, 1H, H3′); 3.89 (m, 1H, H4′); 3.58 (m, 4H, –N–(C**H**$_2$–CH$_2$–CH$_2$–CH$_3$)$_2$); 3.43 (m, 2H, H5′(a), H5′(b)); 2.73 (m, 1H, H2′(a)); 2.29 (m, 1H, H2′(b)); 1.59 (m, 4H, –N–(CH$_2$–C**H**$_2$–CH$_2$–CH$_3$); 1.31 (m, 4H, N–(CH$_2$–CH$_2$–CH$_2$–CH$_3$)$_2$); 0.92 (m, 6H, N–(CH$_2$–CH$_2$–CH$_2$–C**H**$_3$)$_2$).

^{1}H NMR spectrum, 5′-*O*-(4,4′-dimethoxytrityl)-*N*-6-[(dibutylamino)methylene]- 2′-deoxyadenosine (**2a**)



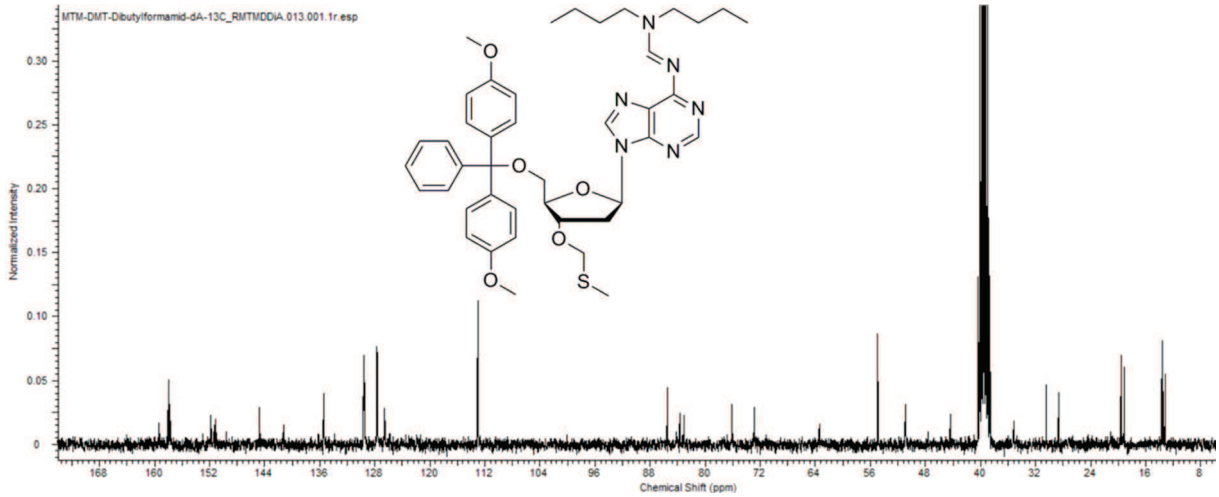
δ [ppm] = 8.91 (s, 1H, dibutylformamide N = C–H); 8.35 (2 br s, 2H, H8 and H2); 7.37–7.12 (m, 9H, Ar–DMT); 6.83–6.71 (m, 4H, Ar–DMT); 6.42 ("t", *J* = 6.41 Hz, 1H, H1′); 5.36 (m, 1H, 3′-OH); 4.49 (m, 1H, H3′); 3.99 (quart, *J* = 4.14 Hz, 1H, H4′); 3.71 (s, 6H, DMT–methoxy); 3.59 (m, 2H, –N–(C**H**$_2$–CH$_2$–CH$_2$–CH$_3$)$_2$); 3.43 (m, 2H, –N–(C**H**$_2$–CH$_2$–CH$_2$–CH$_3$)$_2$); 3.16 (m, 2H, H5′(a), H5′(b)); 2.90 (m, 1H, H2′(a)); 2.23 (m, 1H, H2′(b)); 1.59 (m, 4H, –N–(CH$_2$–C**H**$_2$–CH$_2$–CH$_3$)$_2$); 1.32 (m, 4H, N–(CH$_2$–CH$_2$–C**H**$_2$–CH$_3$)$_2$); 0.92 (m, 6H, N–(CH$_2$–CH$_2$–CH$_2$–C**H**$_3$)$_2$).

^{1}H NMR spectrum, 5′-*O*-(4,4′-dimethoxytrityl)-3′-*O*-methylthiomethyl-*N*-6-[(dibutylamino)methylene]- 2′-deoxyadenosine (**3a**)



δ [ppm] = 8.91 (s, 1H, dibutylformamide N = C–H); 8.65–8.32 (br s, 2H, H8 and H2); 7.39–7.13 (m, 9H, Ar–DMT); 6.87–6.74 (m, 4H, Ar–DMT); 6.38 ("t", *J* = 6.76 Hz, 1H, H1′); 4.73 (s, 2H, MTM–CH_2); 4.70 (m, 1H, H3′); 4.10 (m, 1H, H4′); 3.72 (s, 6H, DMT–methoxy); 3.58 (m, 2H, –N–(C**H**$_2$–CH_2–CH_2–CH_3)$_2$); 3.43 (m, 2H, –N–(C**H**$_2$–CH_2–CH_2–CH_3)$_2$); 3.21 (m, 2H, H5′(a), H5′(b)); 3.05 (m, 1H, H2′(a)); 2.48 (m, 1H, H2′(b)); 2.05 (s, 3H, S–CH_3); 1.59 (m, 4H, –N–(CH_2–C**H**$_2$–CH_2–CH_3)$_2$); 1.31 (m, 4H, N–(CH_2–CH_2–C**H**$_2$–CH_3)$_2$); 0.92 (m, 6H, N–(CH_2–CH_2–CH_2–C**H**$_3$)$_2$).

^{13}C NMR spectrum, 5′-*O*-(4,4⊛dimethoxytrityl)-3′-*O*-methylthiomethyl-*N*-6-[(dibutylamino)methylene]- 2′-deoxyadenosine (**3a**)

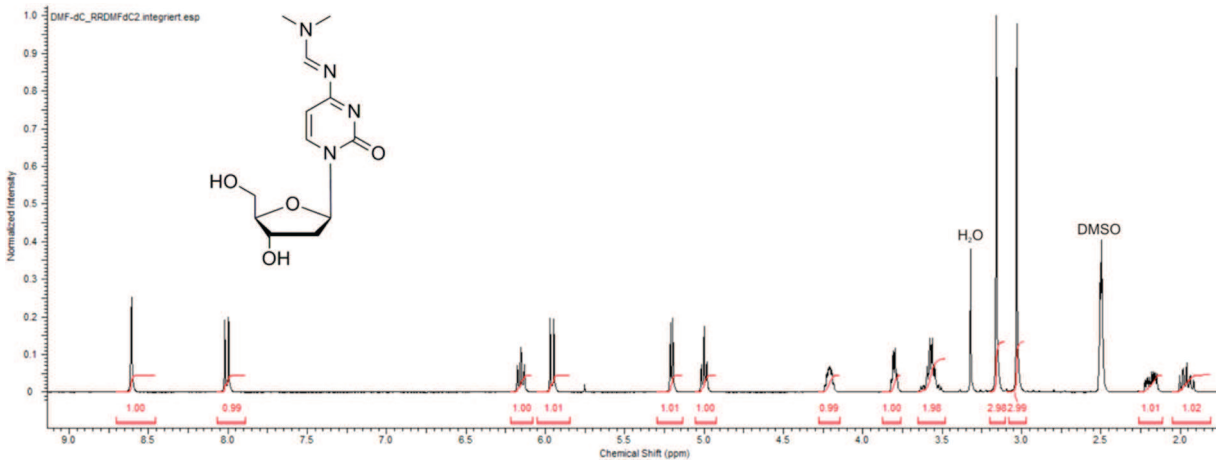


δ [ppm] = 159.34 (1C, C6); 158.01 (1C, DMT–CO); 157.79 (1C, DMT–C–O); 151.81 (1C, dibutylformamide–CH–); 151.36 (1C, C4); 151.10 (1C, C2); 149.61 (1C, C5); 144.74 (1C, –DMT–Ar); 141.28 (1C, C8); 135.47 (2C, DMT–Ar); 129.51 (2C,CH, DMT–Ar); 127.65 (6C, DMT–Ar); 126.60 (1C, CH, DMT–Ar); 113.05 (4C, DMT–Ar); 85.52 (1C, DMT, tertiary C); 83.71 (1C, C1′); 83.06 (1C, C4′); 76.15 (1C, C3′); 72.86 (1C, MTM–CH2); 63.44 (1C, C5′); 54.95 (2C, DMT–methoxy); 50.88 (1C, –N–(**C**H_2–CH_2–CH_2–CH_3)$_2$); 44.1 (1C, –N–(**C**H_2–CH_2–CH_2–CH_3)$_2$); 35.18 (1C, C2′); 30.44 (1C, –N–(CH_2–**C**H_2–CH_2–CH_3)$_2$); 28.64 (1C, –N–(CH_2–**C**H_2–CH_2–CH_3)$_2$); 19.61 (1C, –N–(CH_2–CH_2–**C**H_2–CH_3)$_2$); 19.11 (1C, –N–(CH_2–CH_2–**C**H_2–CH_3)$_2$); 13.71 (1C, –N–(CH_2–CH_2–CH_2–**C**H_3)$_2$); 13.52 (1C, –N–(CH_2–CH_2–CH_2–**C**H_3)$_2$); 13.21(1C, MTM–CH_3).

m/z calculated: 752.4, found: 775.1 (+Na)

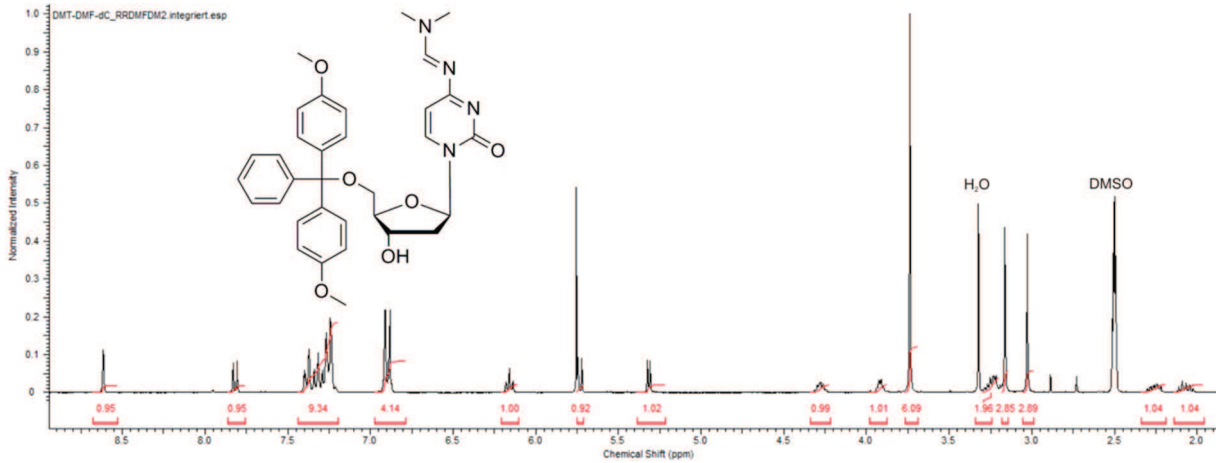
2′-Deoxycytidine

^{1}H NMR spectrum, *N*-4-dimethylformamidyl-2′-deoxycytidine (**1b**)



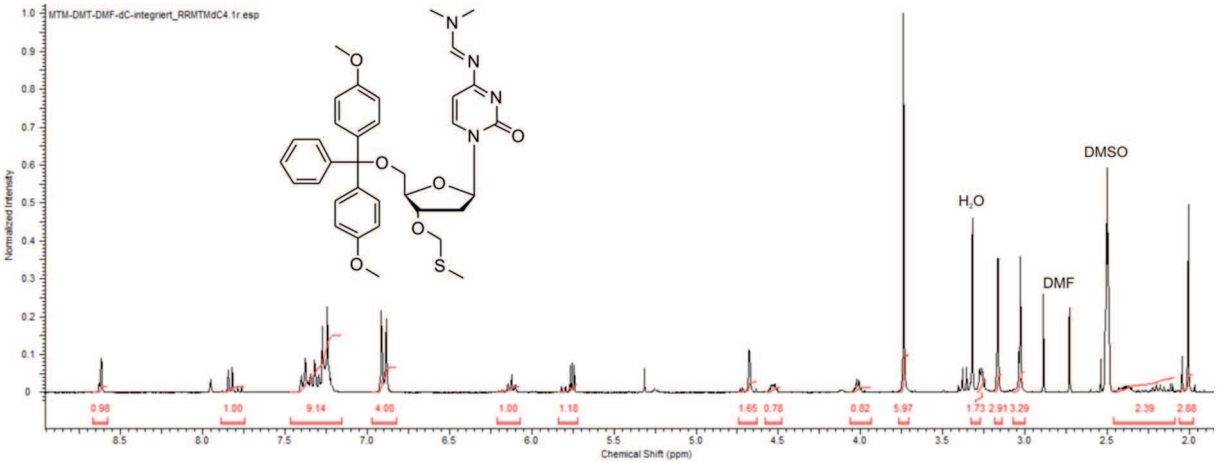
δ [ppm] = 8.61 (s, 1H, formamidine N = C–H); 8.01 (d, *J* = 7.19 Hz, 1H, C6); 6.15 (dd, *J* = 6.69 Hz, *J* = 6.40 Hz, 1H, C1′); 5.96 (d, *J* = 7.19 Hz, 1H, H5); 5.20 (d, *J* = 4.15, 1H, 3′-OH); 5.00 (t, *J* = 5.14 Hz, 1H, 5′-OH); 4.21 (m, 1H, H4′); 3.8 (m, 1H, H3′); 3.57 (m, 2H, H2′(a), H2′(b)); 3.16 (s, 3H, CH_3–formamidine); 3.03 (s, 3H, CH_3–formamidine); 2.07 (m, 2H, H5′(a)/H5′(b)).

^{1}H NMR spectrum, 5′-*O*-(4,4′-dimethoxytrityl)-*N*-4-dimethylformamidyl-2′-deoxycytidine (**2b**)



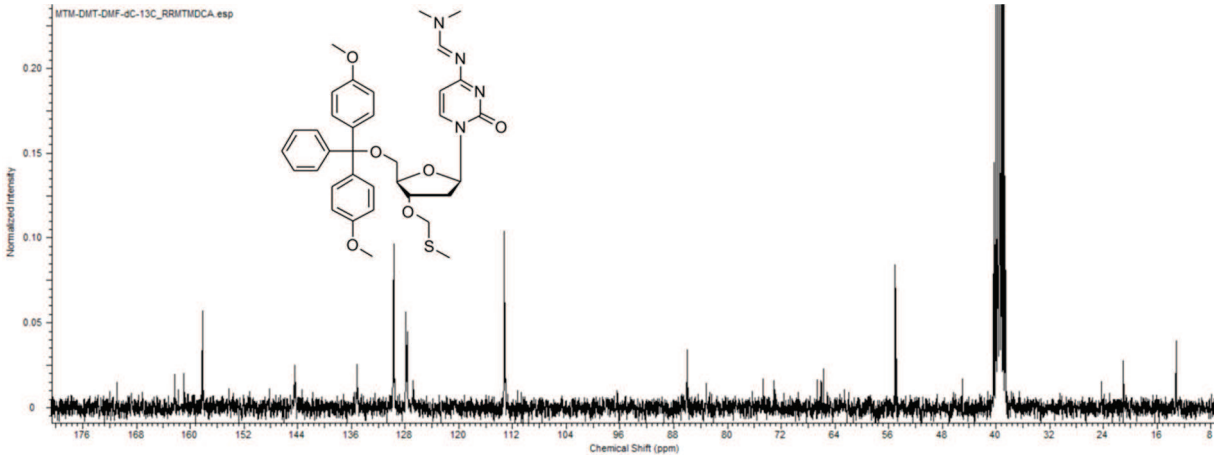
δ [ppm] = 8.62 (s, 1H, formamidine N = C–H); 7.82 (d, *J* = 7.18 Hz, 1H, H6); 7.42–7.21 (m, 9 H, DMT–Ar); 6.94–6.86 (m, 4 H, DMT–Ar); 6.16 ("t", *J* = 6.24 Hz, 1H, H1′); 5.74 (d, *J* = 7.18 Hz, 1H, H5); 5.31 (d, *J* = 4.55, 1H, 3′-OH); 4.27 (m, 1H, H4′); 3.91 (m, 1H, H3′); 3.74 (s, 6H, DMT–methoxy); 3.24 (m, 2H, H2′(a), H2′(b)); 3.16 (s, 3H, CH_3–formamidine); 3.03 (s, 3H, CH_3–formamidine); 2.15 (m, 2H, H5′(a), H5′(b)).

^{1}H NMR spectrum, 5′-*O*-(4,4′-dimethoxytrityl)-3′-*O*-methylthiomethyl-*N*-4-dimethylformamidyl-2′-deoxycytidine (**3b**)



δ [ppm] = 8.63 (s, 1H, formamidine N = C–H); 7.81 (d, *J* = 7.18 Hz, 1H, H6); 7.41–7.21 (m, 9 H, DMT–Ar); 6.93–6.86 (m, 4 H, DMT–Ar); 6.12 ("t", *J* = 6.36 Hz, 1H, H1′); 5.76 (d, *J* = 7.20 Hz, 1H, H5); 4.68 (m, 2H, –CH2–S–); 4.53 (m, 1H, H4′); 4.02 (m, 1H, C3′); 3.74 (s, 6H, DMT–methoxy); 3.27 (m, 2H, H2′(a), H2′(b)); 3.16 (s, 3H, CH_3–formamidine); 3.03 (s, 3H, CH_3–formamidine); 2.28 (m, 2H, H5′(a), H5′(b)); 2,00 (s, 3H, –S–CH_3).

^{13}C NMR spectrum, 5′-*O*-(4,4′-dimethoxytrityl)-3′-*O*-methylthiomethyl-*N*-4-dimethylformamidyl-2′-deoxycytidine (**3b**)

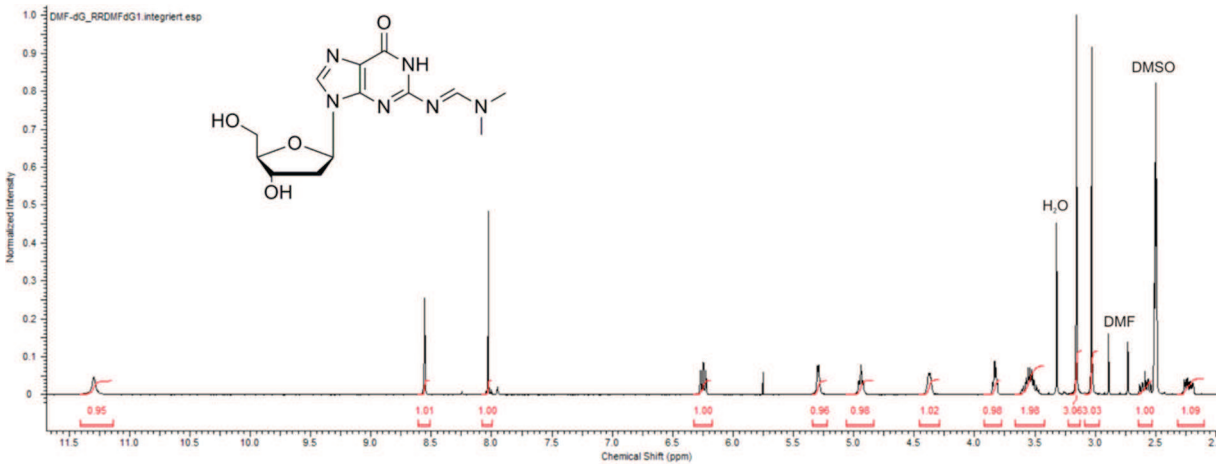


δ [ppm] = 170.09 (1C, C2); 162.25 (1C, C4); 160.90 (1C, DMF N = C–N); 158.15 (2C, DMT –C–O); 144.38 (1C, –DMT–Ar); 135.44 (2C, DMT–Ar); 129.85 (2C,CH, DMT–Ar); 127.88 (4C, DMT–Ar); 127.70 (2C, DMT–Ar); 126.81 (1C, CH, DMT–Ar); 113.22 (4C, DMT–Ar); 86.02 (1C, DMT, tertiary C); 83.15 (1C, C1′); 82.67 (1C, C4′); 74.66 (1C, C3′); 73.00 (1C, MTM–CH_2); 66.68 (1C, C6); 66.05 (1C, C5); 65.70 (1C, C5′); 54.99 (2C, DMT–methoxy); 45.06 (1C, DMF–CH_3); 37.71 (1C, C2′); 24.29 (1C, DMF–CH_3); 13.20 (1C, MTM–CH_3).

m/z calculated: 644.3, found: 645.2

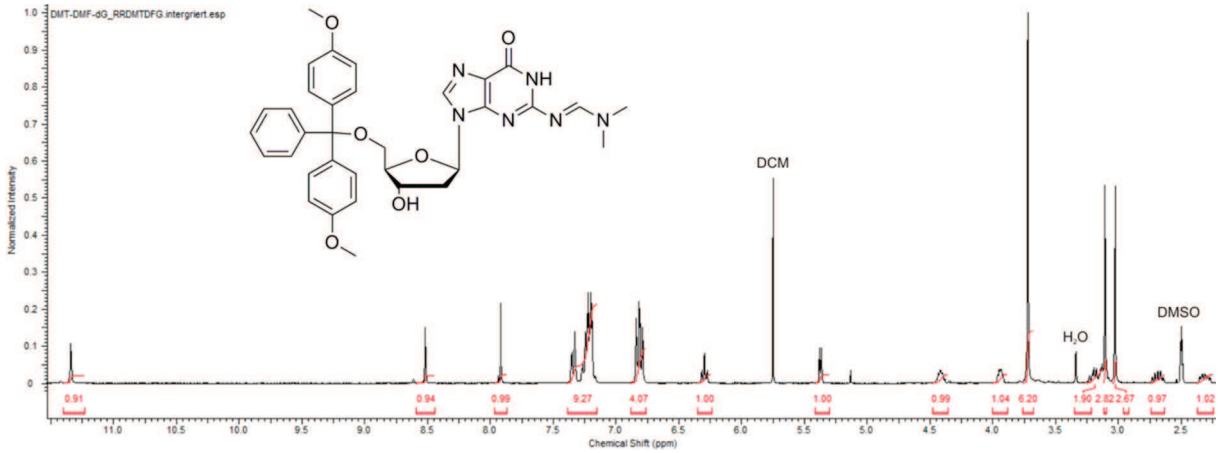
2′-Deoxyguanosine

^{1}H NMR spectrum, *N*-2-dimethylformamidyl-2′-deoxyguanosine (**1c**)



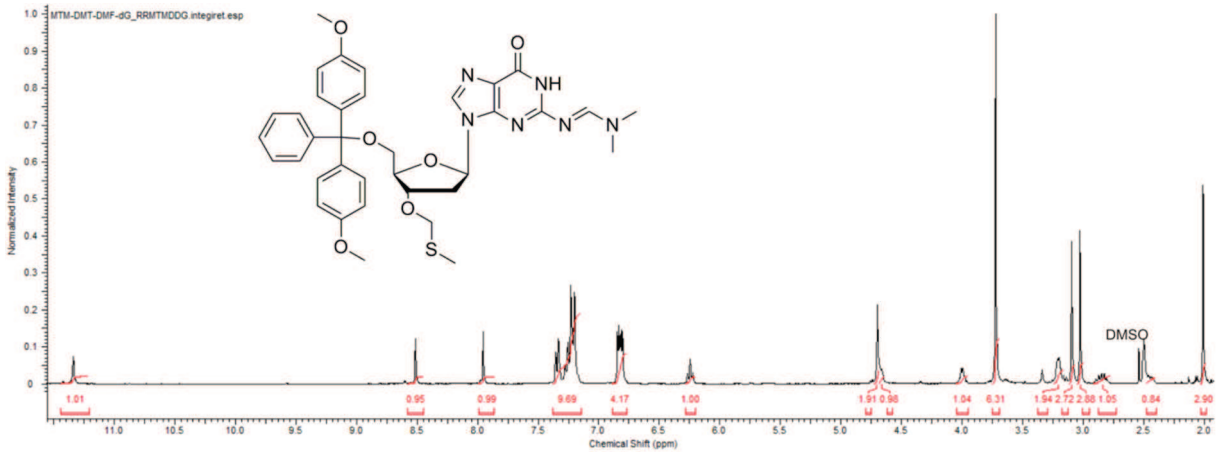
δ [ppm] = 11.30 (s, 1H, NH); 8.55 (s, 1H, formamidine N = C–H); 8.04 (s, 1H, H8); 6.24 ("t", *J* = 6.21 Hz, 1H, H1′); 5.30 (d, *J* = 3.51 Hz, 1H, 3′-OH); 4.94 (t, *J* = 5.42 Hz, 1H, 5′-OH); 4.38 (m, 1H, H3′); 3.83 (m, 1H, H4′); 3.54 (m, 2H, H5′(a), H5′(b)); 3.16 (s, 3H, CH_3–formamidine); 3.03 (s, 3H, CH_3–formamidine); 2.59 (m, 1H, H2′(a)); 2,22 (m, 1H, H2′(b)).

^{1}H NMR spectrum, 5′-*O*-(4,4′-dimethoxytrityl)- *N*-2-dimethylformamidyl-2′-deoxyguanosine (**2c**)



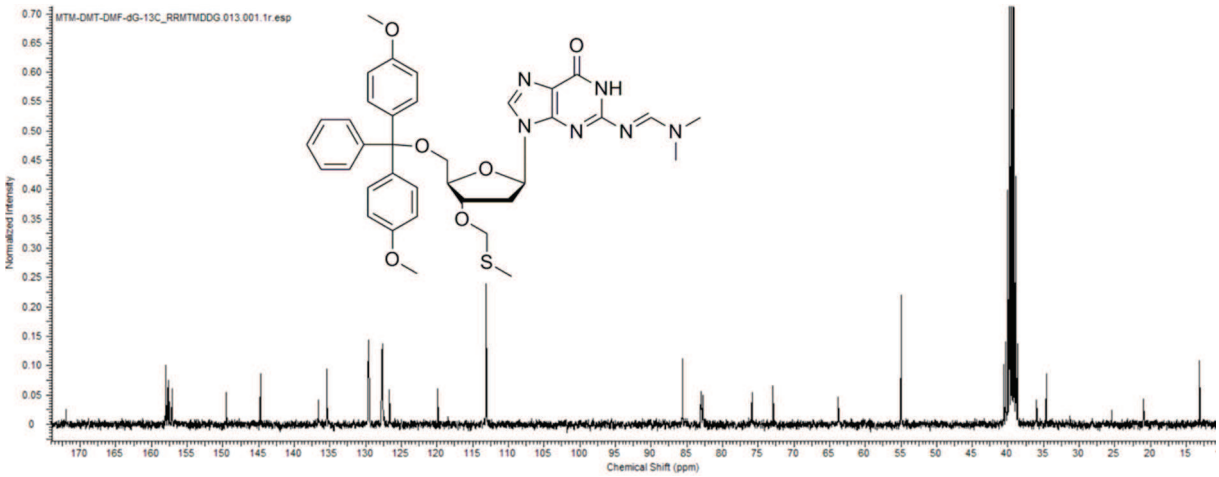
δ [ppm] =11.34 (s, 1H, NH); 8.51 (s, 1H, formamidine N = C–H); 7.92 (s, 1H, H8); 7.39–7.15 (m, 9H, Ar–DMT); 6.81 (m, 4H, Ar–DMT); 6.31 (m, 1H, H1′); 5.37 (d, *J* = 4.36 Hz, 1H, 3′-OH); 4.42 (m, 1H, H3′); 3.94 (m, 1H, H4′); 3.72 (s, 6H, DMT–methoxy); 3.54 (m, 2H, H5′(a), H5′(b)); 3.11 (s, 3H, CH_3–formamidine); 3.03 (s, 3H, CH_3–formamidine); 2.68 (m, 1H, H2′(a)); 2.31 (m, 1H, H2′(b)).

^{1}H NMR spectrum, 5′-*O*-(4,4′-dimethoxytrityl)-3′-*O*-methylthiomethyl-*N*-2-dimethylformamidyl-2′-deoxyguanosine (**3c**)



δ [ppm] = 11.35 (s, 1H, NH); 8.51 (s, 1H, formamidine N = C–H); 7.96 (s, 1H, H8); 7.37–7.16 (m, 9H, Ar–DMT); 6.82 (m, 4H, Ar–DMT); 6.25 (m, 1H, H1′); 4.70 (s, 2H, MTM–CH_2); 4.67 (m, 1H, H3′); 4.00 (m, 1H, C4′); 3.72 (s, 6H, DMT–methoxy); 3.21 (m, 2H, H5′(a), H5′(b)); 3,09 (s, 3H, CH_3–formamidine); 3.02 (s, 3H, CH_3–formamidine); 2.85 (m, 1H, H2′(a)); 2.44 (m, 1H, H2′(b)); 2.01 (s, 3H, S–CH_3).

^{13}C NMR spectrum, 5′-*O*-(4,4′-dimethoxytrityl)-3′-*O*-methylthiomethyl-*N*-2-dimethylformamidyl-2′-deoxyguanosine (**3c**)

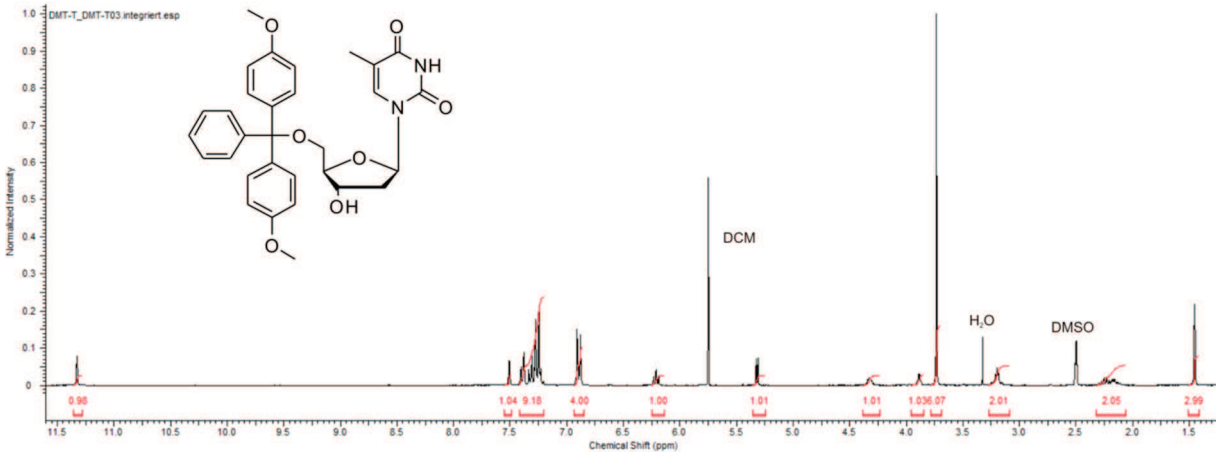


δ [ppm] = 158.03 (2C, DMT–C–O); 157.76 (1C, DMF–CH–); 157.55 (1C, C4); 157.13 (1C, C6); 149.57 (1C, C2); 144.74 (1C, –DMT–Ar); 136.61 (1C, C8); 135.44 (2C, DMT–Ar); 129.58 (2C,CH, DMT–Ar); 127.68 (6C, DMT–Ar); 126.65 (1C, CH, DMT–Ar); 119.91 (1C, C5); 113.07 (4C, DMT–Ar); 85.61 (1C, DMT, tertiary C); 82.99 (1C, C1′); 82.75 (1C, C4′); 75.85 (1C, C3′); 72.89 (1C, MTM–CH_2); 63.76 (1C, C5′); 54.97 (2C, DMT–methoxy); 35.98 (1C, DMF–CH_3); 34.62 (1C, C2′); 25.45 (1C, DMT–CH_3); 13.14 (1C, MTM–CH_3).

m/z calculated: 684.3, found: 684.2

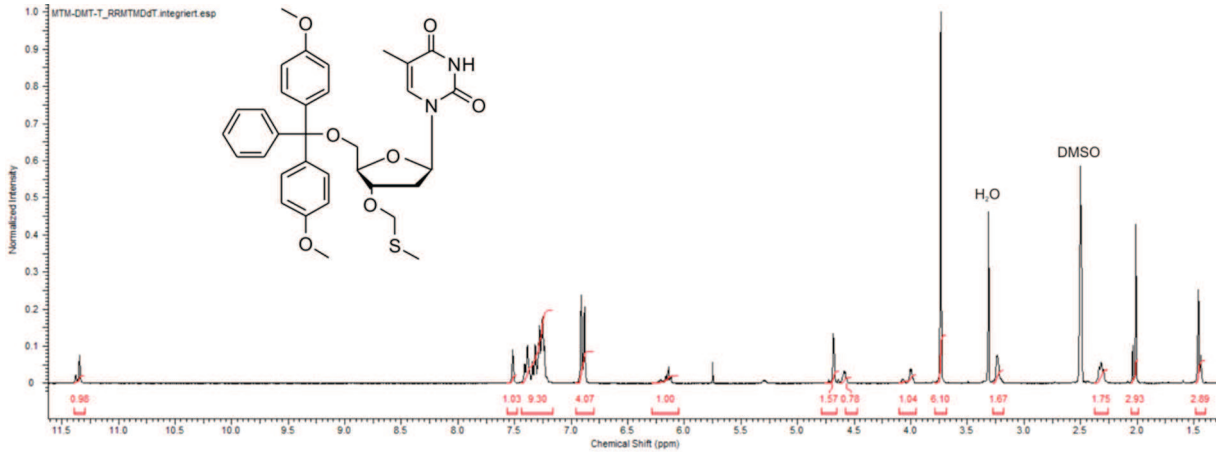
Thymidine

^{1}H NMR spectrum, 5′-*O*-(4,4′-dimethoxytrityl)-thymidine (**2d**)



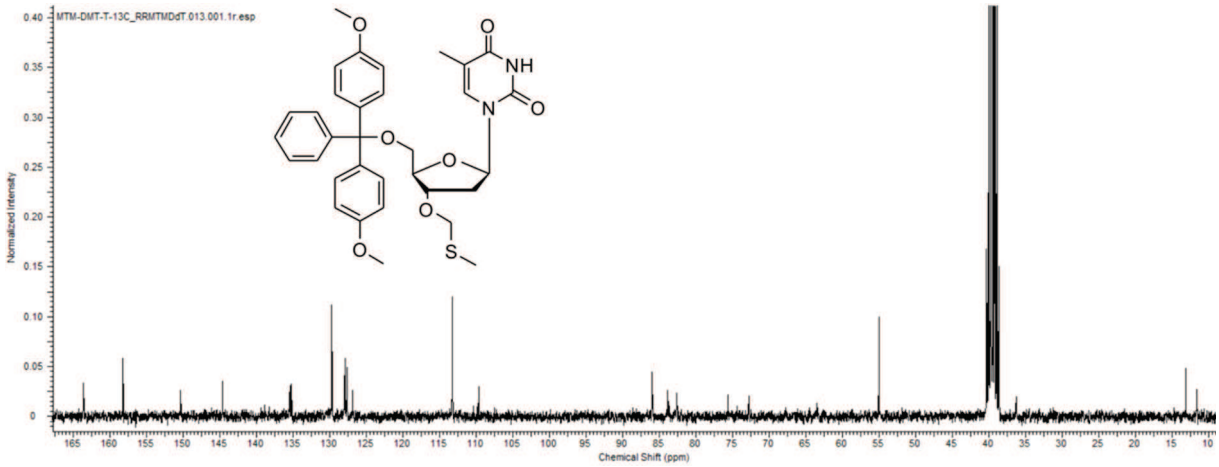
δ [ppm] = 11.27 (s, 1H, NH); 7.5 (s, 1H, H6); 7.42–7.20 (m, 9H, Ar–DMT); 6.95–6.85 (m, 4H, Ar–DMT); 6.21 ("t", *J* = 6.57 Hz, 1H, H1′); 5.32 (s, 1H, 3′-OH); 4.3 (m, 1H, 4′H); 3.88 (m, 1H, H3′); 3.73 (s, 6H, methoxy–DMT); 3.19 (m, 2H, H5′(a), H5′(b)); 2.20 (m, 2H, H2′(a),H2′(b)); 1.45 (s, 3H, CH_3).

^{1}H NMR spectrum, 5′-*O*-(4,4′-dimethoxytrityl)-3′-*O*-methylthiomethyl-thymidine (**3d**)



δ [ppm] =11.34 (s, 1H, NH); 7.5 (s, 1H, H6); 7.43–7.2 (m, 9H, Ar–DMT); 6.93–6.86 (m, 4H, Ar–DMT); 6.17 (m, 1H, H1′); 4.68 (s, 2H, MTM–CH_2); 4.58 (m, 1H, H4′); 4.00 (m, 1H, H3′); 3.74 (s, 6H, methoxy–DMT); 3.23 (m, 2H, H5′(a), H5′(b)); 2.32 (m, 2H, H2′(a), H2′(b)); 2.03 (s, 3H, S–CH_3); 1.46 (s, 3H, CH_3).

^{13}C NMR spectrum, 5′-*O*-(4,4′-dimethoxytrityl)-3′-*O*-methylthiomethyl-thymidine (**3d**)



δ [ppm] = 168.6 (1C, C4); 158.04 (2C, DMT–CO); 150.26 (1C, C2); 144.7 (1C, DMT–Ar); 135.3 (1C, C6); 135.2 (2C, DMT–Ar); 129 (2C,CH, DMT–Ar); 127.8 (2C, DMT–Ar); 127.6 (4C, DMT–Ar); 126.8 (1C, CH, DMT–Ar); 113.4 (4C, DMT–Ar); 109.7 (1C, C5); 86.1 DMT, quartet C); 83.9 (1C, C1'); 82.7 (1C, C4'), 75.6 (1C, C3'); 72.7 (1C, MTM–CH_2); 63.5 (1C, C5'); 54.98 (2C, DMT–methoxy); 36.35 (1C, C2'); 13,17 (1C, MTM–CH_3); 11.61 (1C, C5–CH_3).

m/z calculated: 604.2, found: 607.4 (+Na)

S3. Cleavage of the Disulfide Bridge

To compare TCEP and DTT as reducing agents for cleaving the disulfide bridge between nucleosides and solid support the following procedure was carried out:

10.3 mg of solid support B loaded with protected dC (loading density 167.5 μmol/g) was incubated with 6.7 μmol TCEP and 0.5 mL phosphate buffer (9.6 mM, pH 7) for 20 h at 50 °C. 10.4 mg solid support **B** with protected dC (loading density 167.5 μmol/g) was incubated with 6.7 μmol DTT and 0.5 mL phosphate buffer (9.6 mM, pH 7) for 20 h at 50 °C. After centrifugation, the supernatant was removed and a triple determination of UV absorption at 260 nm was measured in triplets with NanoDrop-ND 1000. Because of the missing extinction coefficient for the modified nucleoside, these values have to be seen as qualitative results.

Table 1. Cleavage conditions.

Cleavage reagent	A260-average	Average related to 10 mg solid support
TCEP (10.3 mg solid support)	5.56	5.39
DTT (10.4 mg solid support)	2.67	2.57

S4. Stability Test of Disulfides from Primary, Secondary, and Tertiary thiols

The three trinucleotides ATC-S_{prim}, CAC-S_{sec}, TTC-S_{tert} were synthesized in solution (see Jancyck et al. [6] and Suchsland et al. [11]) starting with 5′-dimethoxytrityl-3′-*O*-methylthiomethyl-*N*-benzoyl-2′-deoxycytidine (3′-OMTM cytidine).

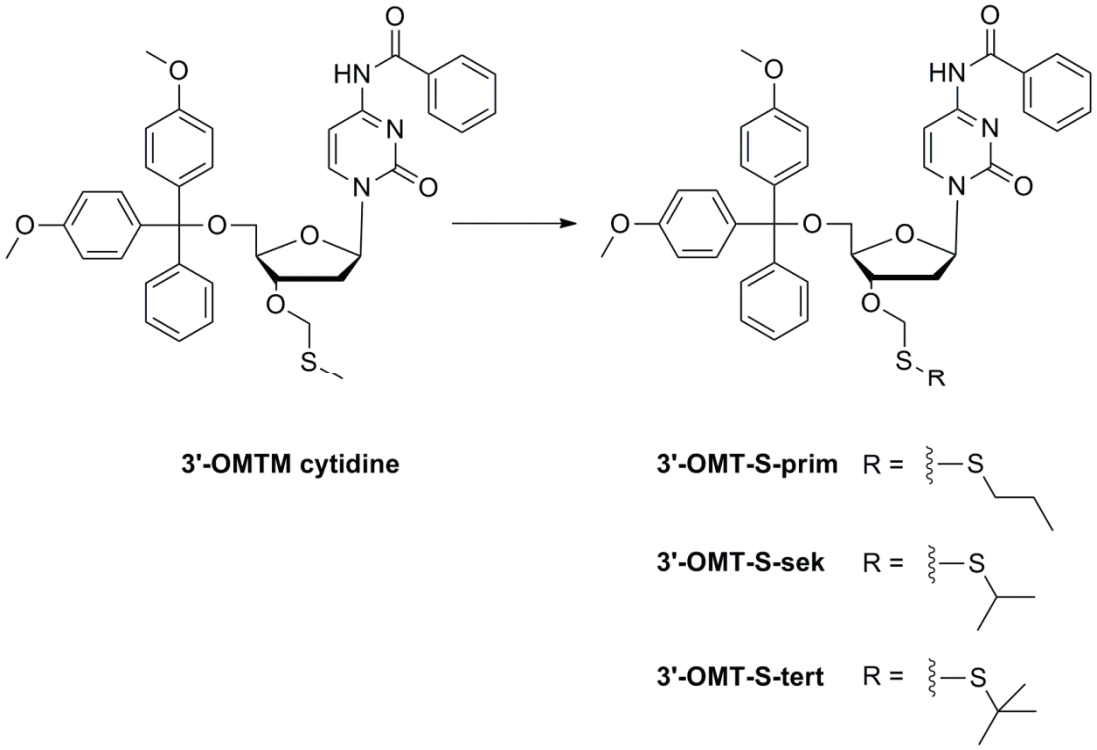


Figure 2. Synthesis of 5′-O-dimethoxytrityl-3′-O-dithiomethyl-N-benzoyl-2′-deoxycytidine with primary, secondary, or tertiary disulfide bridge (S-prim/sec/tert).

A solution of 5′-*O*-dimethoxytrityl-3′-*O*-methylthiomethyl-*N*-benzoyl-2′-deoxycytidine (3′-OMTM cytidine, 10 mmol) in dry dichloromethane (100 mL) was prepared. Triethylamine (1 eq., 1.4 mL) was added at 0 °C. After stirring for 10 min, sulfuryl chloride (1 eq., 0.81 mL in dichloromethane, 10 mL) was added dropwise over 10 min. Afterward the solution was warmed up to room temperature, and, after 15 min, a solution of potassium thiotosylate (2 eq., 4.5 g in dimethylformamide, 50 mL) was added. After stirring for 10 min, 5 eq. of thiol compound (1-propanethiol, 2-propanethiol, or *tert*-butylmercaptane) were added. Reaction was complete after stirring for 10 min at room temperature. The reaction mixture was washed with saturated sodium hydrogen carbonate solution. After phase separation, the organic phase was dried over sodium sulfate, and, after filtration, the solvent was removed in vacuo. Purification was carried out by silica gel column chromatography (dichloromethane/methanol = 95:5, R_f = 0.7). For yields and MALDI-TOF analyses, see table.

Table 2. 3′-OMT-S-prim/sec/tert yields and masses.

Thiol compound	MT cytidine derivative	Yield [%]	Calculated mass [g/mol]	Found mass [g/mol] (Na-peak)
1-propanethiol	3′-OMT-S-*prim*	56	753	776.1
2-propanethiol	3′-OMT-S-*sec*	62	753	776.4
tert-butylmercaptane	3′-OMT-S-*tert*	66	768	790.3

Synthesis of Trinucleotides ATC-S_{prim}, CAC-S_{sec}, TTC-S_{tert}

Synthesis was carried out under standard conditions (see Jancyck et al. [6] and Suchsland et al. [10]) of trinucleotide synthesis in solution starting with detritylation of *5′-O*-dimethoxytrityl-3′-*O*-dithiomethyl-*N*-benzoyl-2′-deoxycytidine (with S-prim/sec/tert) with trichoroacetic acid. Coupling reactions were carried out with cyanoethyl-protected 3′-*O*-phosphoramidites of *N*-acyl-5′-*O*-DMT/protected nucleosides and benzylmercaptotetrazole as activator in dry acetonitrile. Oxidation was carried out with 0.2 M iodine solution in pyridine/tetrahydrofurane/water (2:2:1). Fully protected trinucleotides were precipitated from water.

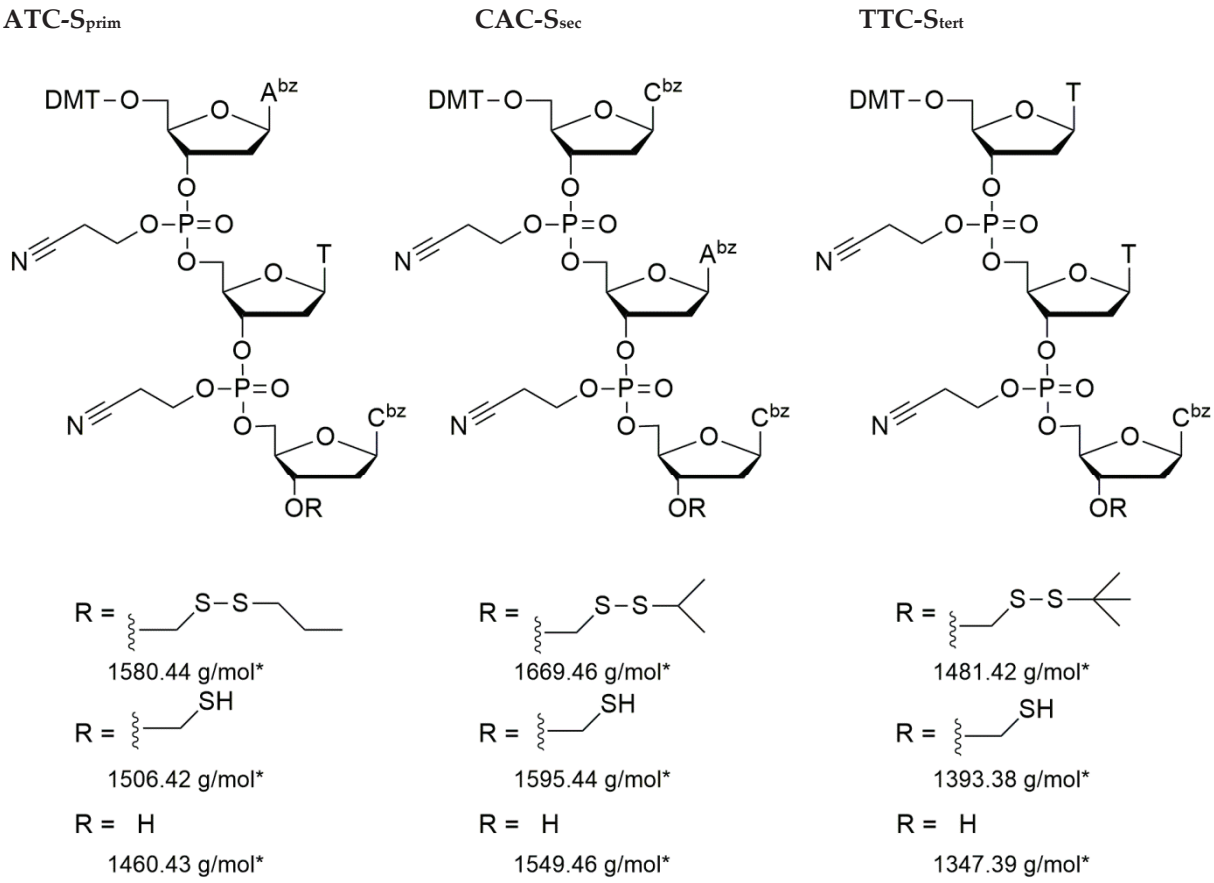


Figure 3. Synthesized trinucleotides with primary, secondary, and tertiary disulfide bridge ATC-S_{prim}, CAC-S_{sec}, TTC-S_{tert} (* calculated mass).

Disulfide Cleavage

The trinucleotides (0.1 mmol) were dissolved in dry dimethylformamide (4 mL) at room temperature. DTT (77 mg) was added and the mixture was stirred at 45 °C for at least 9 h. After 30 min and 9 h, the cleavage efficiency was analyzed by MALDI-TOF MS. The results indicate a complete cleavage of the disulfide bridge with the primary thiol, as the mass peak of trinucleotide ATC-S_{prim} disappeared after 30 min; though the thiomethyl residue was still detectable. After 9 h, only the final product with free 3′-OH was detectable. The disulfide bridge of the secondary thiol of CAC-S_{sec} was still detectable after 30 min, but disappeared after 9 h. The disulfide bridge with the tertiary thiol TTC-S_{tert} was still detectable after 9 h. Only a small signal for 3′-*O*-deprotected TTC trinucleotide was observed. These results underline the higher stability of disulfide bridges formed from tertiary thiols as compared to disulfide bridges formed from primary thiols.

ATC-S_{prim} **CAC-S_{sec}** **TTC-S_{tert}**

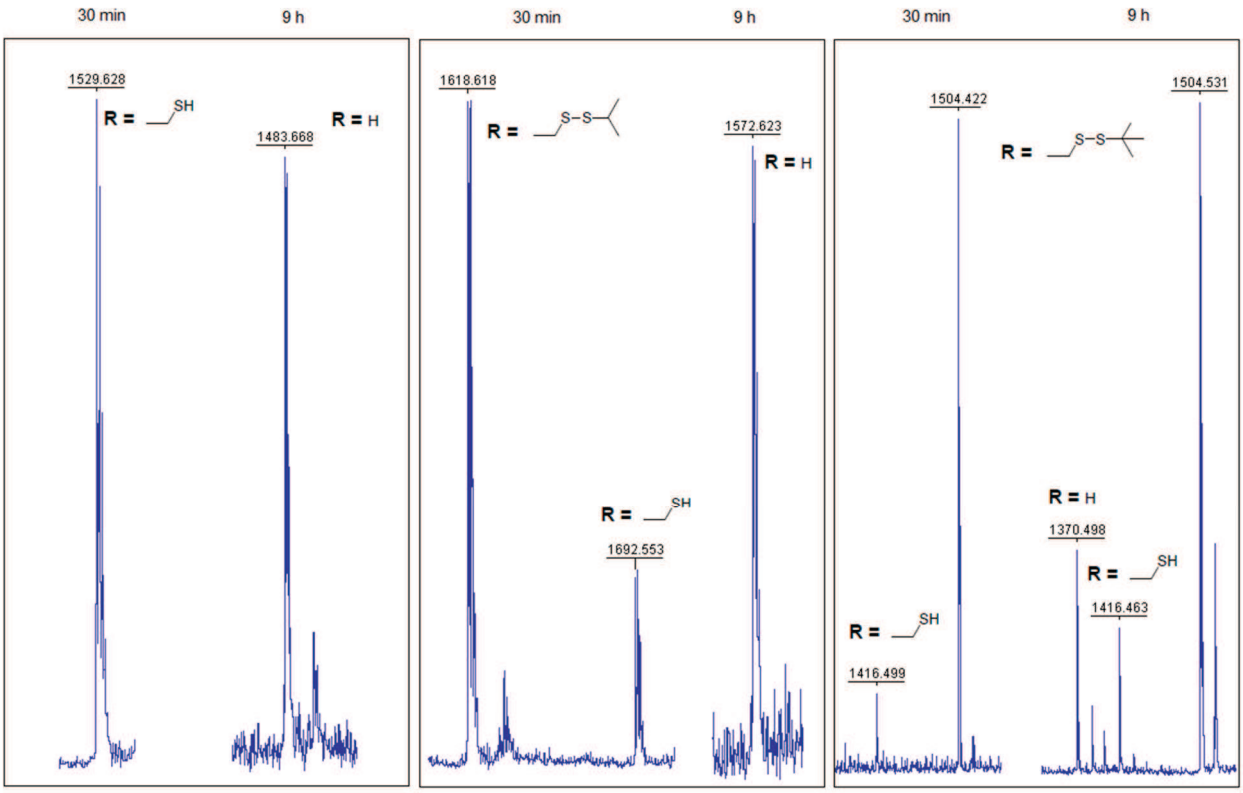
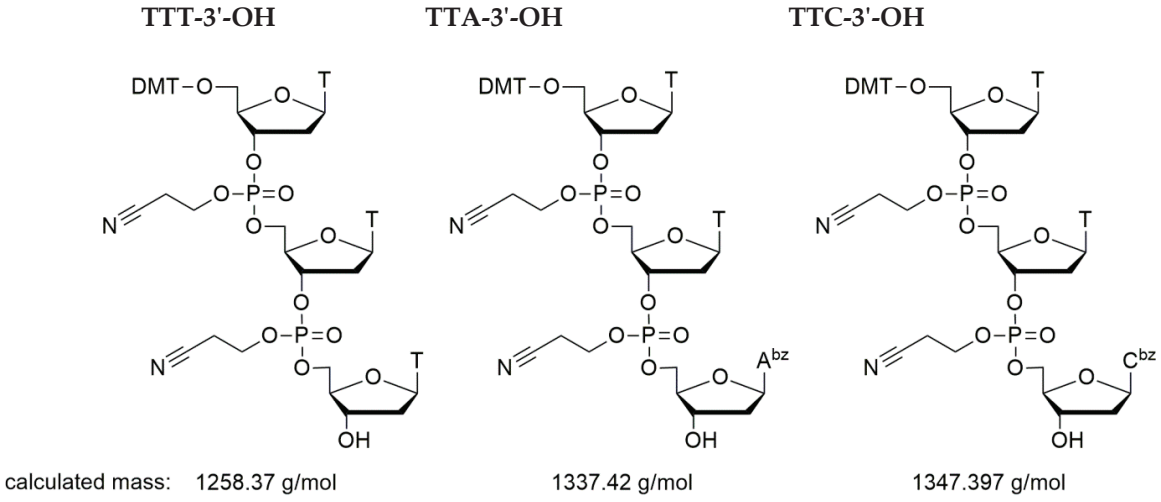


Figure S4. Reductive cleavage of the 3′-disulfide bridge of trinucleotides ATC-S_{prim}, CAC-S_{sec}, and TTC-S_{tert}. Shown are the mass spectra of trinucleotides after 30 min and 9 h of treatment with DTT.

S5. Cyanoethyl-Protected Trinucleotides Cleavage of Disulfide Bridge with DDT

cyanoethyl/protected trinucleotides TTT, TTA, and TTC cleaved from solid support with DTT were analyzed by HPLC and MALDI-TOF. Analyses showed that all protecting groups at the trinucleotide remained intact. The masses were found as sodium peaks. See below for mass spectra and HPLC diagrams.



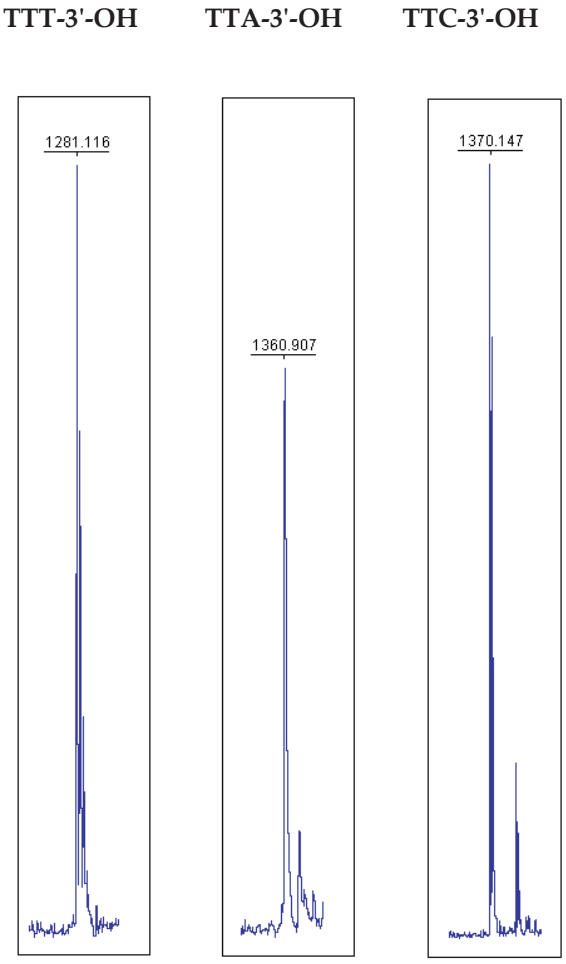


Figure S5. MS-Spectra of trinucleotides after cleavage from the support.

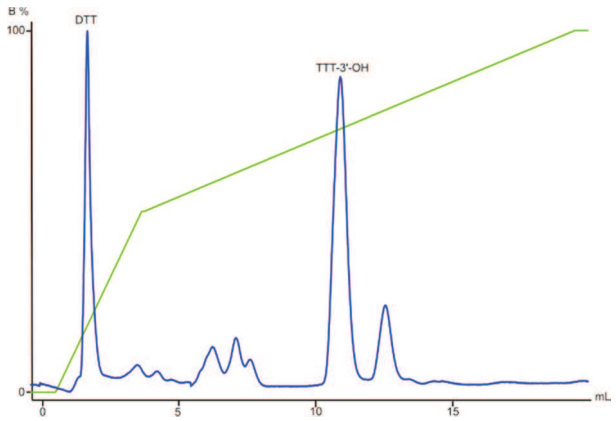


Figure S6. RP-HPLC of TTT-3′-OH after cleavage from solid support, buffer A: 5% acetonitrile, buffer B: 70% acetonitrile, VP 125/21 Nucleosil 120-10 C18.

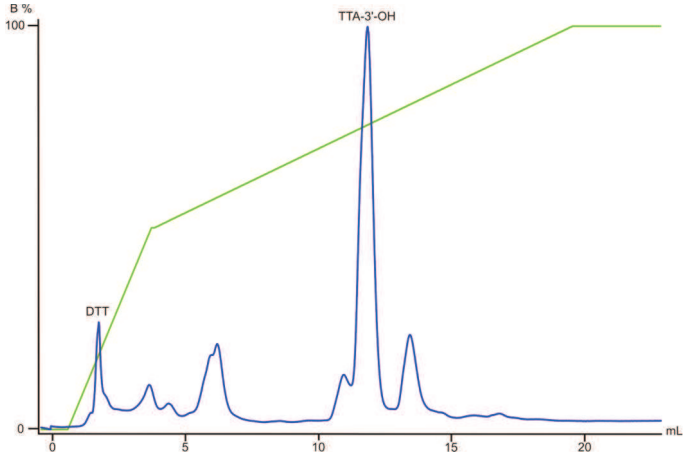


Figure S7. RP-HPLC TTA-3′-OH after cleavage from solid support, buffer A: 5% acetonitrile, buffer B: 70% acetonitrile, VP 125/21 Nucleosil 120-10 C18.

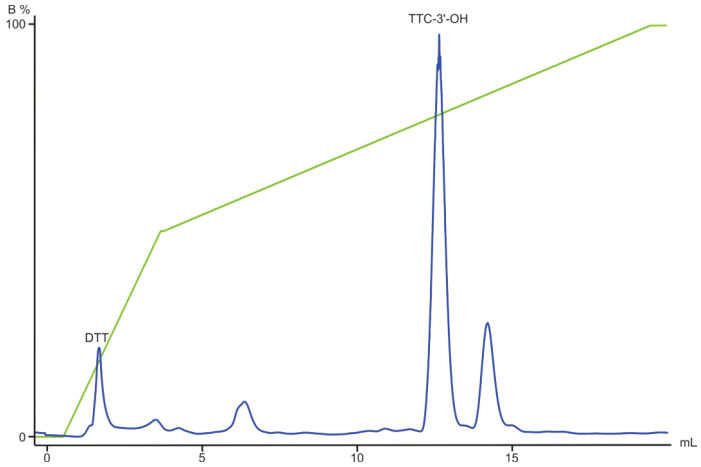


Figure S8. RP-HPLC TTC-3′-OH after cleavage from solid support, buffer A: 5% acetonitrile, buffer B: 70% acetonitrile, VP 125/21 Nucleosil 120-10 C18.

S6. Instability of Me-Protected Phosphates When Treated with DDT as Cleavage Rreagent

Methyl-protected trinucleotides GGA, GGC, and GGT cleaved from solid support with DTT were analyzed with MALDI-TOF. Analyses showed partial loss of methyl protecting groups at the phosphates. See mass spectra below.

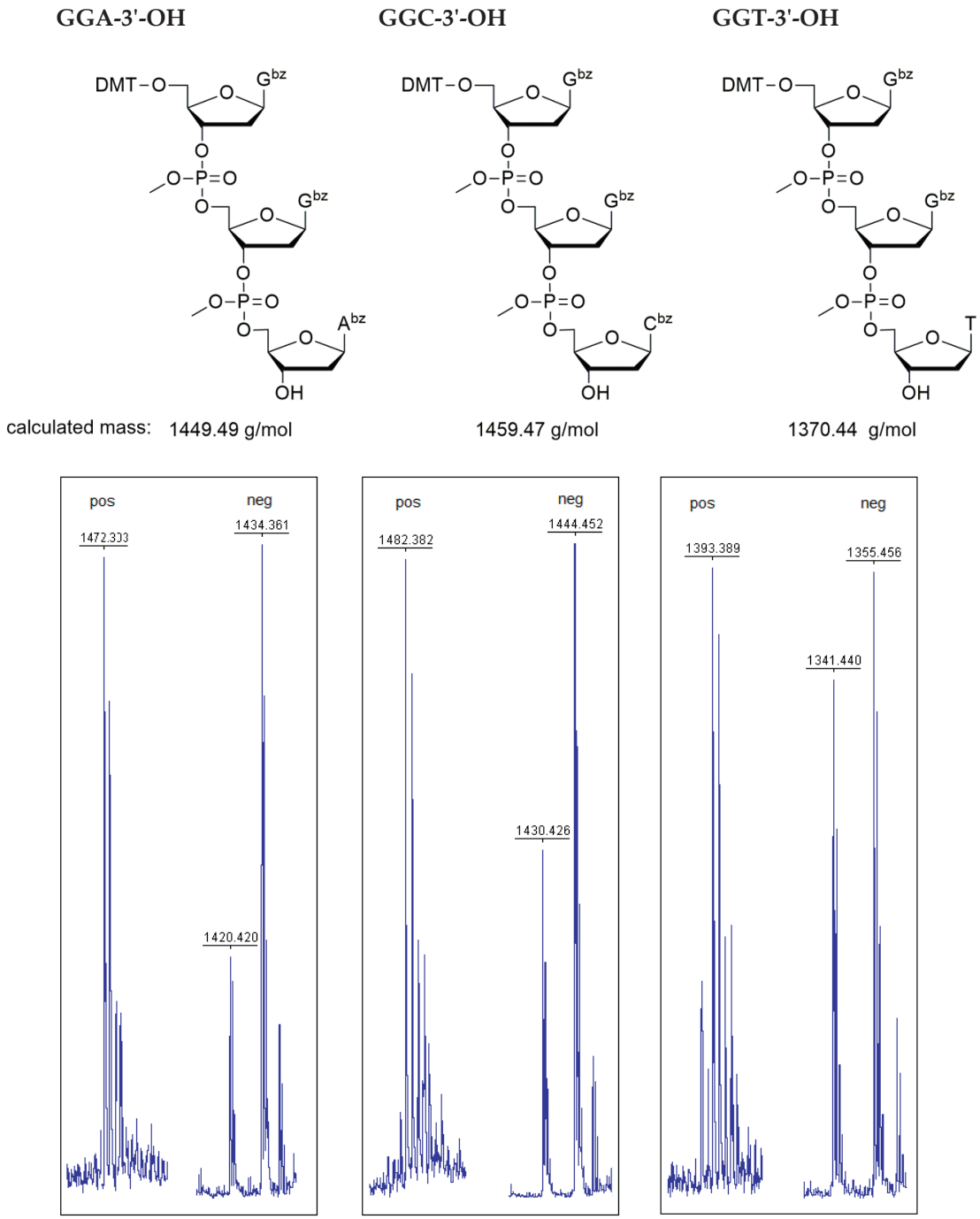
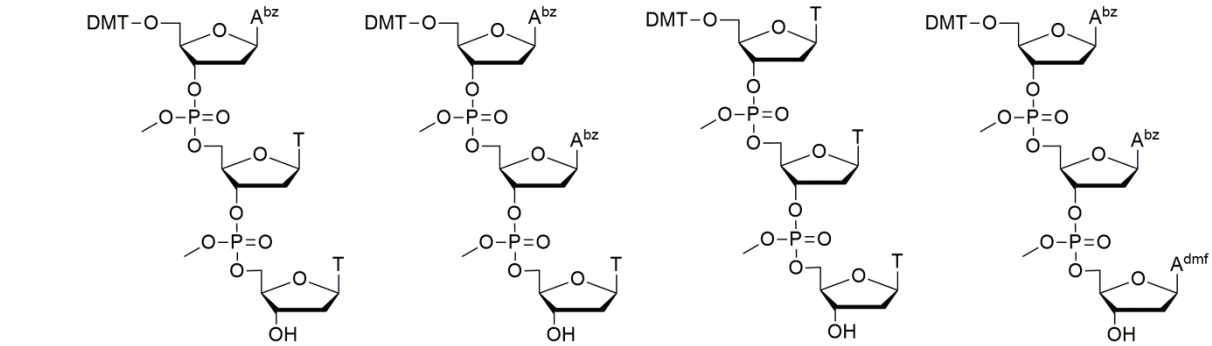


Figure S9. MS-Spectra of trinucleotides with methyl/protected phosphate after cleavage with DTT from the support. The difference of 14 indicates loss of a methyl group at the phosphate.

S7. Trinucleotides with Me-Protected Phosphates Cleavage of Disulfide Bridge with TCEP

Methyl-protected trinucleotides ATT, AAT, TTT, and AAA cleaved from solid support with TCEP were analyzed with MALDI-TOF. Analyses showed that all protecting groups at the trinucleotide remained intact. Masses were found as sodium or potassium peaks. See mass spectra below. In addition, formation of trinucleotides was confirmed by TLC with UV detection and red colouring upon acidic treatment and heat exposure, owing to the cleaved-off DMT group.



	ATT-3'-OH	AAT-3'-OH	TTT-3'-OH	AAA-3'-OH
calculated mass:	1260.14 g/mol	1339.24 g/mol	1180.34 g/mol	1402.50 g/mol
found mass:	1284.77 g/mol (+Na) 1298.43 g/mol (+K)	1384.49 g/mol (+2Na)	1180.3 g/mol	1448.29 g/mol (+2Na)
Rf (DCM:MeOH 9:1):	0.34	0.26	0.49	0.29

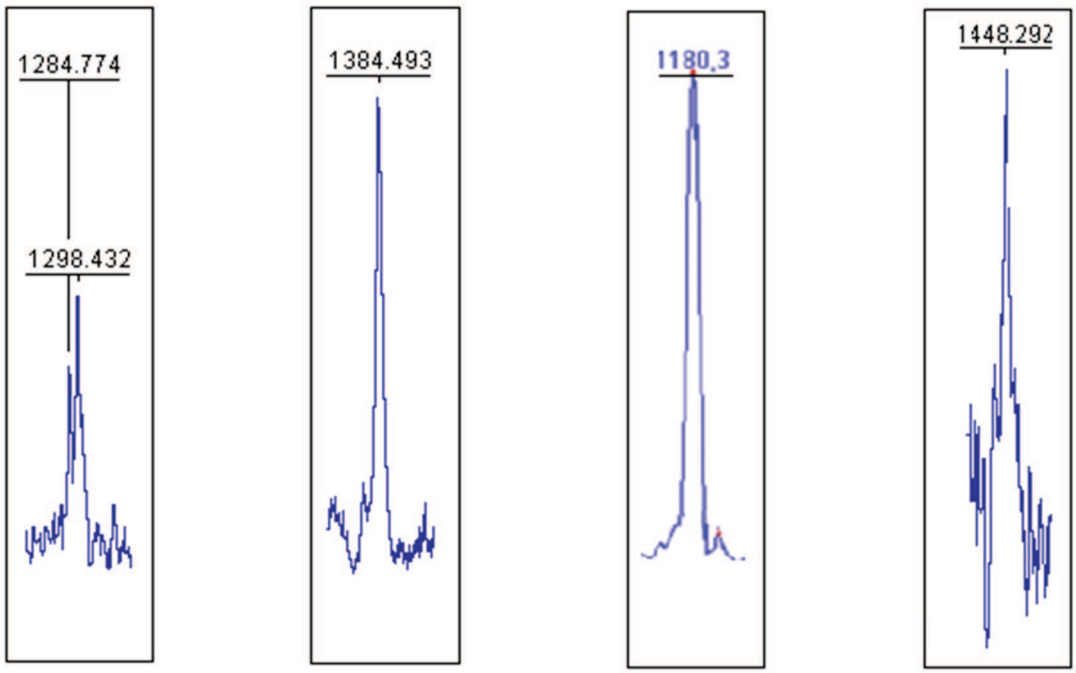


Figure S10. MS-Spectra of trinucleotides with methyl-protected phosphate after cleavage with TCEP from the support.

2.5 R. Suchsland, B. Appel, P. Virta, S. Müller, Synthesis of fully protected trinucleotide building blocks on a disulphide-linked soluble support, eingereicht am 02.12.20, Organic & Biomolecular Chemistry (OBC).

COMMUNICATION

Synthesis of fully protected trinucleotide building blocks on a disulphide-linked soluble support

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In recent years, preparation of fully protected trinucleotide phosphoramidites as synthons for the codon-based synthesis of gene libraries as well as for the assembly of oligonucleotides from blockmers has gained much attention. We here describe the preparation of such trinucleotide synthons on a soluble support using a disulphide linker.

The synthesis of fully protected trinucleotide synthons for codon-based assembly of oligonucleotides has a longer history and originally was motivated by the need for methods of combinatorial and evolutionary protein engineering, which combine combinatorial gene synthesis with functional screening or genetic selection applied at the phenotype level to an ensemble of many structural variants generated in parallel.[1-3] Among a host of related methods, the use of mixtures of pre-formed trinucleotide blocks representing codons for the 20 canonical amino acids stands out as allowing fully controlled randomization individually at any number of arbitrarily chosen codon positions of a given gene.[4-6] The chance of functional proteins in such libraries is increased, as randomization independent from the degenerated genetic code is possible and thus bias to amino acids represented by more than one codon as well as stop codons can be avoided.[6-9] The power of this method has been successfully demonstrated for randomization of immunoglobulins or at the example of a gene library of tHisF from the hyperthermophile *Thermotoga maritima*.[7,10] Apart from the preparation of gene libraries with controlled randomization, fully protected trinucleotides have potential as building blocks for oligonucleotide synthesis from blockmers (n = 1, 2, 3, 4, ...), in particular then, when the oligomer is composed of repetitive sequence patches. In an ideal case, one previously synthesized blockmer can be coupled several times to obtain the desired oligomer. Moreover, oligonucleotide assembly from blockmers is advantageous in terms of easier purification, since n-1, -2, … side products cannot be formed. A number of routes to fully protected trinucleotide building blocks have been developed, based on strategies in solution, on solid phase or on soluble supports (Fig. 1).[4,11,12] Traditionally, trinucleotide synthons have been prepared in solution, paying special attention to the pair of orthogonal protecting groups for the 5'- and 3'-OH functions, when synthesizing a dinucleotide that subsequently can be extended in 5'- or 3'-direction.[1,13-19]

Synthesis in solution requires isolation of products after each synthesis step, which can become rather tedious. Therefore, we[11,20] and others[19] have developed strategies for trinucleotide synthesis on solid support as an attractive alternative to protocols in solution.

The key issue is the attachment of the 3'-start nucleoside to the solid support via a suitable linker, allowing to cleave off the trinucleotide after synthesis without loss of the protecting groups. With regard to trinucleotide synthesis, an oxalyl anchor[19] or a disulphide linker have been described.[11,21]

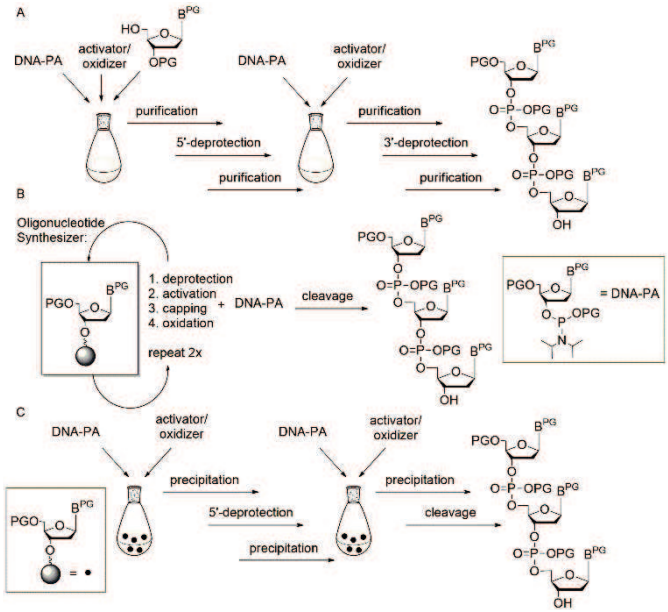

Fig. 1 Strategies for preparation of fully protected trinucleotides in solution (A), on solid support (B) and on soluble phase (C); PG = Protecting group.

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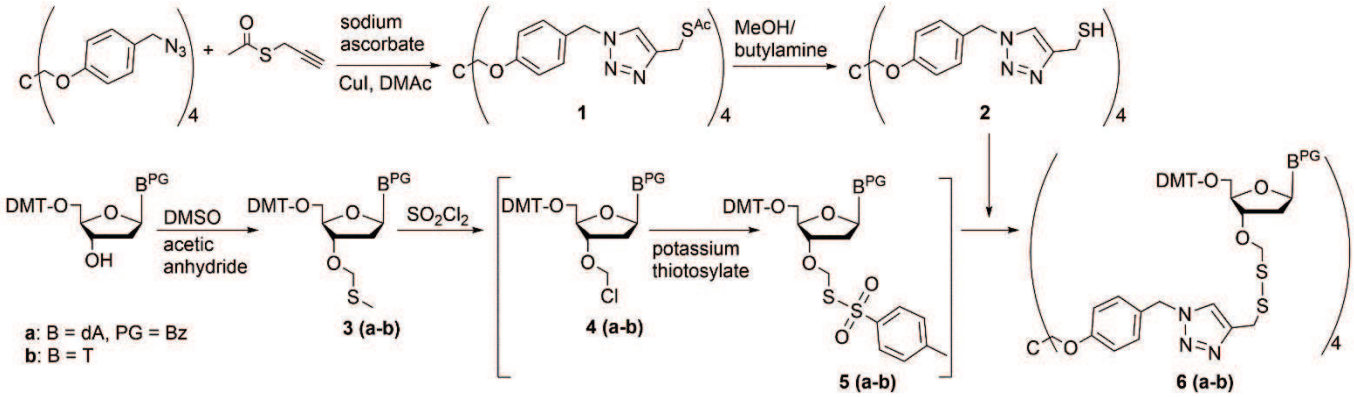
Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/x0xx00000x

In recent years, protocols for the synthesis of oligonucleotides on soluble supports have emerged,[12,21-24] and those have also been used with particular attention to the preparation of fully protected trinucleotides.[12,21] The general strategy involves iterative cycles of reaction steps in solution and precipitation for isolation/purification of reaction products. Several soluble supports have been used for oligomer synthesis (reviewed in [25]), among those, pentaerythritol-derived cores, which are easily precipitated from methanol.[24] Fully protected trinucleotides have been prepared on the pentaerythritol-derived core with the start nucleoside being tethered to the polymer via a disulphide bridge[21] or hydroquinone-*O,O*'-diacetic acid (Q-linker).[12] In both strategies, phosphotriester chemistry has been used for trimer assembly, which in the disulphide strategy resulted in a trinucleotide with 3'-terminal *ortho*-chlorophenylphosphate.[21] This 3'-remnant may be activated as a phosphotriester, but in standard automated DNA synthesis, where phosphoramidite coupling is strongly preferred, it would require to be selectively removed in order to convert the trinucleotide to the 3'-*O*-phosphoramidite building block. Moreover, the reductive cleavage of the disulphide bridge was performed in conditions that caused premature loss of the 5'-dimethoxytrityl (DMT) protecting group.[21]

Based on our previous experience,[11,14,20] we here report on the preparation of fully protected trinucleotides on a pentraerythritol-derived soluble support using phosphoramidite chemistry for nucleotide coupling. As we have reported previously, tethering the start nucleoside to a solid support (polystyrene) via a dithiomethyl linkage is a superior strategy for assembly of blockmers that upon release from the support by reductive cleavage carry protecting groups at all functionalities, but offer a free 3'-OH group for conversion to the phosphoramidite building block.[11,20] Application of this immobilization strategy to the pentaerythritol-derived core, first required appropriate functionalization of the core. This was achieved as described previously by conjugation of commercially available S-propargyl thioacetate to the tetrakis-*O*-[4-(azidomethylphenyl)pentaerythritol] support[21] by Cu(I) catalyzed 1,3-dipolar cycloaddition,[26,27] yielding tetrakis-*O*-{4-[4-[(acetylthiomethyl)]-1H-1,2,3-triazol-1-ylmethyl]-phenyl}pentaerythritol (**1**) (Scheme 1). The 3'-*O*-methylthiomethyl (MTM) modified start nucleosides (T and Bz-dA) were synthesized as described[20] following the strategy originally developed for synthesis of 2'-*O*-DTM functionalized ribonucleotide building blocks.[28] Aminolysis of the thioacetate on the support with butylamine in methanol delivered the free thiol function (**2**) required for immediate reaction with the nucleoside derivative to form the disulphide linkage. To this end, the 3'-*O*-MTM functionalized nucleoside (**3a-b**) was activated by treatment with sulfuryl chloride to give the 3'-*O*-chloromethyl ether (**4a-b**) in a Pummerer rearrangement, which immediately was converted in the presence of potassium thiotosylate to the reactive species (**5a-b**). This subsequently reacted with the support bound thiol (**2**) to form the desired loaded tetrapodal soluble support (**6a-b**, Scheme 1).

To start trinucleotide assembly, the support carrying the start nucleoside (**6a-b**) was treated with 4% dichloroacetic acid in ethylene dichloride to cleave off the 5'-*O*-DMT group (Fig. 2). The acid treatment was quenched by addition of pyridine, the solvents were evaporated, and the support linked with the deprotected start nucleoside (**7a-b**) was precipitated from methanol. The coupling reaction was carried out by taking up the precipitate in acetonitrile containing six equivalents of the *N*-acyl-5'-*O*-DMT protected nucleoside phosphoramidite in 0.1 M concentration, and addition of benzylmercapto-tetrazole[29] as activator. The resulting dimer **8a-b** was oxidized by addition of a 0.2 M solution of iodine in trimethylpyridine/ACN/H_2O (1/11/5) to give the dinucleotide product **9a-b** (Fig. 2). The support-linked dinucleotide **9a-b** was directly precipitated from the reaction mixture by addition of methanol. The solid was filtered off and used for the next coupling cycle. All steps including deprotection, coupling and oxidation were repeated to obtain the fully protected trinucleotide on the soluble support (**10a-b – 12a-b**, Fig. 2).

NMR spectroscopy was used to analyse success of the synthesis. However, as long as the start nucleoside as well as the intermediate dinucleotide and the final trinucleotide were bound to the soluble support, complexity of the products and their modest solubility in common deuterated solvents retarded the analysis. Consequently, detailed NMR characterization was applicable only for the products released from the support. After assembly of the trinucleotide was finished, it was released in fully protected form from the support by reductive cleavage of the disulphide linkage with TCEP at pH 7.5[30] (**13a-b**, Fig. 2). After treatment with TCEP, the support was thoroughly washed with acetonitrile, acetone, ethanol and ethyl acetate, in order to separate the cleaved off trinucleotide.



Scheme 1 Reaction scheme for coupling of start nucleoside to soluble support.

Traces of TCEP and of the tetrapodal support, which remained after this work up, were finally removed by chromatographic purification, which yielded sufficiently pure trinucleotide blockmers CTA and GGT in 43% and 35% overall isolated yield, respectively (both synthesized in tens of µmol scale). Extensive analysis by various NMR techniques (^{1}H, ^{13}C, DEPT, HSQC, DQF-COSY, for detailed information see SI) unambiguously confirmed the identity and purity of the desired products. For further application as synthons for DNA synthesis, the trinucleotides were converted to 3'-*O*-phosphoramidites following the standard procedure for phosphitylation (Fig. 3).[31] After aqueous work up and extensive drying, 0.1 M solutions of both trinucleotide phosphoramidites in appropriate solvents (see below) were prepared and used for coupling on the DNA synthesizer. Both trinucleotide synthons CTA (**14a**) and GGT (**14b**) were coupled in individual syntheses onto a short oligomer (CTT) on CPG. The 5'-terminal DMT group was left on, and the resulting 6mers 5'-CTACTT-3' and 5'-GGTCTT-3' were cleaved from the support by concentrated ammonia. RP-HPLC analysis was used to evaluate the coupling efficiency of the trinucleotide blockmers (Fig. 4).

It has been discussed in the past that some but not all trinucleotide synthons are soluble in acetonitrile[1,32] or various compositions of acetonitrile-dichloromethane mixtures[12,16,19] with or without addition of DMF.[18] The fully protected trinucleotide CTA showed poor solubility in acetonitrile and therefore was dissolved in dichloromethane. Solubility was

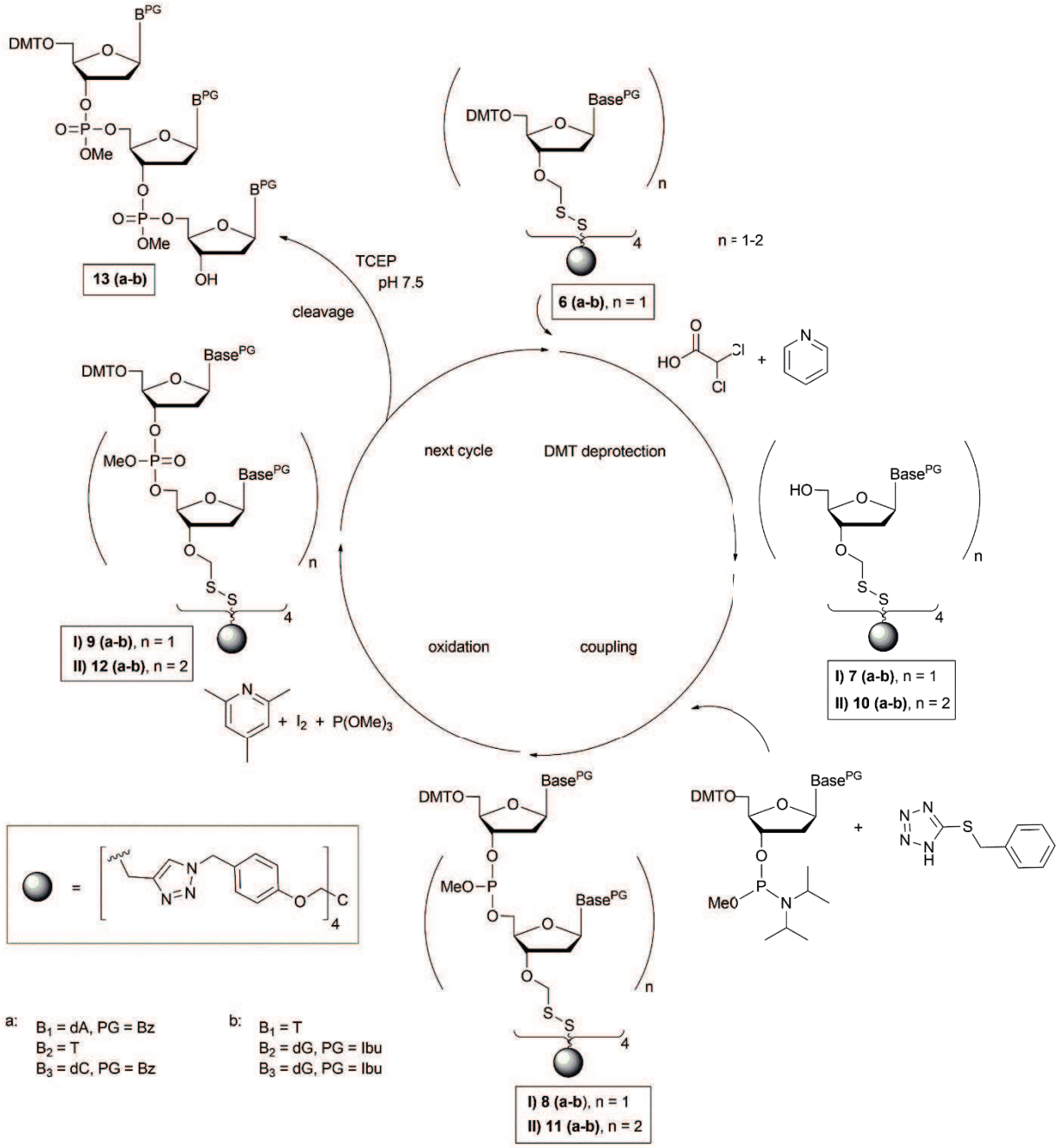


Fig. 2 Reaction scheme of trinucleotide assembly on soluble support.

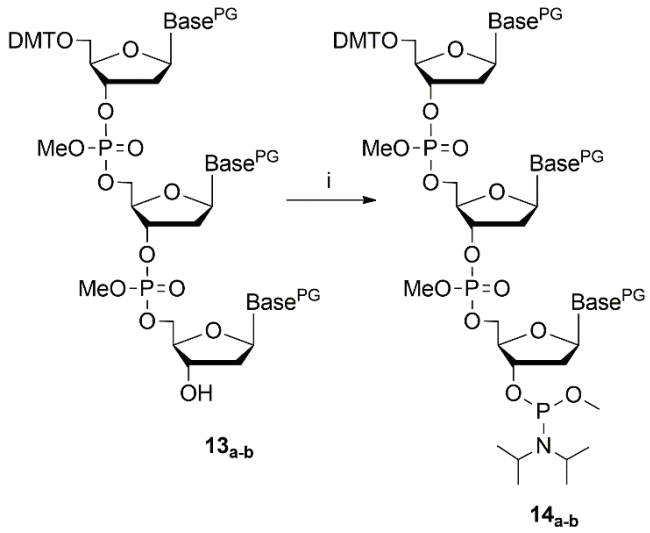


Fig. 3 Reaction scheme of trinucleotide phosphitylation, i) *N*,*N*-Diisopropyl-methyl-phosphonamidic-chloride, TEA, DCM, 3.5 h, rt; PG = Protecting group.

clearly better than in acetonitrile, but not fully satisfying. Accordingly, HPLC analysis of the 6mer after coupling and deprotection showed a rather low coupling yield (Fig. 4A). This result may be interpreted as a consequence of a low effective concentration of the CTA blockmer and of the missing acetonitrile being the ideal solvent for phosphoramidite coupling. For solving the second trinucleotide GGT, we applied a 3:1 mixture of dichloromethane and acetonitrile, which allowed to prepare a 0.1 M solution of the trinucleotide phosphoramidite ready for coupling. A double coupling cycle was used to increase the coupling efficiency. The results are shown in Fig 4B. A clearly higher coupling yield was achieved for the trinucleotide synthon GGT as compared to CTA (Fig. 4A). HPLC peak areas designated as final 6mers and abortive fragments would give rough estimation for the coupling yields, being about 17% for the CTA synthon and 72% for the GGT synthon under the described conditions (see SI for details).

For the latter, this is well in the range of coupling yields for trinucleotide synthons reported in the literature,[15,16,32] although the coupling yields given there are usually just concluded from detritylation values on the synthesizer,[1,12,18,32] or it is not at all specified how those were determined.[15,19] The identity of the assembled 6mers was confirmed by MS analysis (see SI).

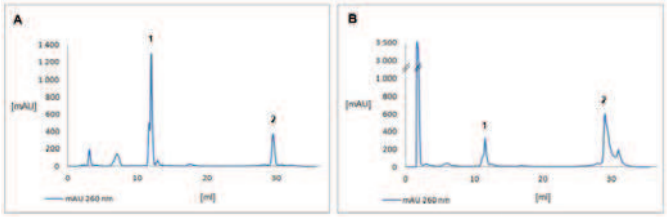


Fig. 4 HPLC analysis of CTACTT (A) and GGTCTT (B) (DMT-on). A: Peak 1: abortive fragments, Peak 2: CTACTT; B: Peak 1: abortive fragments, Peak 2: GGTCTT; AU = absorption unit. Conditions for A and B: Nucleodur 125/4, CV = 1.571 ml, 1 ml/min; buffer A: 5% ACN, 0.1 M TEAAc; buffer B: 30% ACN, 0.1 M TEAAc; Gradient: starting with 0% buffer B for 4 CV, to 40% buffer B over 3 CV, to 60% buffer B over 7 CV, to 100% buffer B over 2 CV, then 100% buffer B for another 2 CV, to 0% buffer B over 3 CV.

Conclusions

Based on our results, we conclude that the disulphide chemistry we had developed previously for the synthesis of fully protected trinucleotides on polystyrene,[11,20] is a superior strategy also for synthesis on soluble support. *S*-propargyl thioacetate was efficiently clicked onto an azido-functionalized tetrapodal support, and upon deprotection of the thiol, the 3'-*O*-dithiomethyl functionalized start nucleoside was activated and immobilized onto the support via a disulphide bridge. Assembly of the trinucleotide and release from the support by reductive treatment delivered fully protected trinucleotides, which were further converted to phosphoramidite building blocks and used in standard DNA synthesis. Moderate, however at least for GGT yet satisfying coupling yields were obtained. Most likely, the lower coupling yields result from the insufficient solubility of the two trinucleotide synthons described herein in acetonitrile. However, the encountered solubility problems are independent of the strategy for trinucleotide preparation, and need to be addressed separately for each individual sequence prior to usage in standard DNA synthesis.

Trinucleotide synthons have potential for the preparation of gene and protein libraries as well as for the assembly of functional oligonucleotides from blockmers. Their synthesis on soluble support offers a way for economic synthesis at larger scale and thus would be advantageous over synthesis on solid support, which is more expensive, or completely in solution, which includes numerous working up and product isolation steps. The disulphide chemistry presented here allows for facile immobilisation of the starting nucleoside onto the support as well as for easy release of the assembled trinucleotide with preservation of all protecting groups and with generation of a free 3'-OH group for further conversion to a phosphoramidite.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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Supporting Information

Synthesis of fully protected trinucleotide building blocks on a disulphide-linked soluble support

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Content

S1: Abbrevations

ACN	acetonitrile
Bz	benzoyl
CV	column volume
DCE	dichloroethane
DCM	dichloromethane
DEPT	distortionless enhancement by polarization transfer
DMAc	dimethylacetamide
DMF	dimethylformamide
DMT	dimethoxytrityl
DQF-Cosy	double quantum filtered correlation spectroscopy
EE	ethyl acetate
EtOH	ethanol
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	high performance liquid chromatography
HSQC	heteronuclear single quantum coherence
Ibu	isobutyryl
MeOH	methanol
MS	mass spectrometry
MTM	methylthiomethyl
NMR	nuclear magnetic resonance
PLC	preparative layer chromatography
TCEP	tris(2-carboxyethyl)phosphine
TEA	triethylamine
TEAAc	triethylammonium acetate
THF	tetrahydrofuran
TLC	thin layer chromatography

S2: General information

Mass spectra were recorded on a Bruker microflex MALDI-TOF MS. HPLC spectra were performed on an Äkta Purifier (Amersham Biosciences) with Nucleodur 125/4, CV = 1.571 ml. DNA Oligomers were synthesized using a Gene Assembler Special DNA synthesizer from Amersham Bioscience. NMR spectra were recorded on Bruker Avance 300 MHz (**1, 3**$_{a\text{-}b}$) or 600 MHz (**13**$_{a\text{-}b}$) and analyzed via TopSpin 4.0.7. Pyridine was dried overnight over KOH, heated to reflux, distilled off and stored over molecular sieve and argon. TEA was freshly distilled and stored over molecular sieve. DCM used during work-up procedure after coupling reaction was stored over $NaHCO_3$. ACN used for coupling was extra dry (10 ppm H_2O). Solid support (Thymidine 3'-lcaa CPG 500 Å) and Phosphoramidites (5'-*O*-DMT-3'-*O*-methyl phosphoramidites) were purchased from ChemGenes. All other reagents, chemicals and solvents were obtained commercially and used without further purification. All products were visualized via TLC chromatography on aluminium silica gel 60 F254 plates and UV shadowing. Synthesis of 3'-*O*-methylthiomethyl modified nucleosides (**3**$_{a\text{-}b}$) has been described previously.[1,2] Synthesis of Oligomers (5'-CTACTT-3', 5'-GGTCTT-3') was carried out according to Suchsland *et al*.[2] Synthesis of Tetrakis-*O*-{4-[4-(acetylthiomethyl)-1*H*-1,2,3-triazol-1-ylmethyl]phenyl}-pentaerythritol (**1**) was conducted according to Jabgunde *et al*. by using less amount of propargyl thioacetate and achieving equally high yields.[3] Phosphitylation was done according to McBride and Caruthers.[4]

S3: Experimental procedures

S3.1: Tetrakis-*O*-{4-[4-(acetylthiomethyl)-1*H*-1,2,3-triazol-1-ylmethyl]phenyl}-pentaerythritol (**1**)

0.29 g Tetrakis-{[4-(acidomethyl)phenoxy-]methyl}methane (0.44 mmol), 8.6 mg sodium ascorbate (44 µmol) und 33 mg CuI (0.18 mmol) were dissolved in 1 ml DMAc under dry conditions. Then 250 mg propargylthioacetate (2.2 mmol) were added. After four Pump-Freeze-Thaw cycles, the solution was stirred at 50 °C for 16 h. 2.5 ml H_2O were added and the crude product was extracted three times with ethyl acetate. The organic layer was washed with saturated aqueous $NaHCO_3$ solution and dried over Na_2SO_4. After evaporation to dryness the crude product was purified via column chromatography (DCM:MeOH / 97:3). Upon lyophilisation the product **1** was obtained with 70% yield (0.36 g, 0.32 mmol).

^{1}H NMR (300 MHz, DMSO): δ [ppm] = 7.92 (s, 4H, H5 of triazole), 7.22 (d, *J* = 8.71 Hz, 8H, H2/H6 of Ph), 6.94 (d, *J* = 8.71 Hz, 8H, H3/H5 of Ph), 5.43 (s, 8H, N-C*H*$_2$-Ph), 4.23 (s, 8H, AcS-C*H*$_2$-), 4.11 (s, 8H, C*H*$_2$-pentaerythritol), 2.33 (s, 12H, -SAc).

^{13}C NMR (300 MHz, DMSO): δ [ppm] = 194.47, 158.32, 143.29, 129.53, 128.34, 122.93, 114.80, 66.00, 52.22, 44.25, 30.20, 23.21.

MS: calculated: 1117.33 m/z, found: 1117.19 m/z.

S3.2: Coupling of start nucleoside to soluble support (**6$_{a-b}$**)

0.22 g Tetrakis-*O*-{4-[4-(acetylthiomethyl)-1*H*-1,2,3-triazol-1-ylmethyl]phenyl}pentaerythritol (**1**, 0.19 mmol) was solved in 1.4 ml degassed ACN and 0.7 ml MeOH. Then 1 ml degassed 5 M butylamine solution (in MeOH) was added. The reaction mixture was stirred over night at room temperature. 2.8 ml of a 1:1 solution DCM:ACN were added. After 10 min 50 mg Amberlyst 15 were added. The ion exchange resin was filtered off and the solvents were removed in vacuo to get the deprotected soluble support **2**.

0.58 g (0.80 mmol) 5'-*O*-DMT-3'-*O*-MTM-*N*-Bz-dA (**3$_a$**) were dissolved in 3.2 ml DCM under dry conditions. Then 0.35 ml TEA (3 eq) were added. The solution was stirred in an ice bath. 70 µl (1 eq) freshly distilled SO_2Cl_2 in 0.65 ml dry DCM were added dropwise. The reaction mixture was stirred for another 10 min in an ice bath. 0.23 g (1.5 eq) potassium thiotosylate (in 0.45 ml dry DMF) were added at room temperature and the mixture was stirred for 10 min. The deprotected soluble support with the free thiol (**2**) was completely solved in 1.4 ml DCM:ACN / 1:1 and added to the reaction mixture. The solution was stirred for 3.5 h at room temperature. The solution was divided over three 50 ml plastic tubes and each was refilled with MeOH. The tubes were stored in the freezer at -20 °C overnight. Then the tubes were centrifuged at 6000 rpm for 30 min, the solid was isolated and lyophilized. MS: calculated: 3756.37 m/z, 940.09 ($4H^+$/4), found: 940.9 m/z.

Accordingly, 5'-*O*-DMT-3'-*O*-MTM-T (**3$_b$**) was coupled to soluble support using 0.19 mmol soluble support and 1.54 mmol 5'-*O*-DMT-3'-*O*-MTM-T.

Yield over two steps: **6$_a$**= 54% (0.10 mmol, 0.38 g), **6$_b$**= 49% (0.09 mmol, 0.32 g)

S3.3: General procedure for detritylation, coupling, oxidation and cleavage:

Detritylation ($7_{a\text{-}b}$, $10_{a\text{-}b}$)

7.5 ml Dichloroacetic acid solution (4% in DCE) were added to 0.24 g 5'-*O*-DMT-3'-*O*-MTM-*N*-Bz-dA bound on soluble support (63 µmol, $\mathbf{6_a}$) and stirred for 5 min, whereby the solution turned red. 7.5 ml pyridine were added, thereby the solution turned colourless again. Pyridine was evaporated, and the residue was divided over two 50 ml plastic tubes, each of which was filled up with about 40 ml MeOH. After 15 min at -20 °C, the tubes were centrifuged at 6000 rpm for 30 min, the solid was filtered and lyophilized to yield in 5'-OH-3'-*O*-MTM-*N*-Bz-dA bound on soluble support $\mathbf{7_a}$ to be used for the following phosphoramidite coupling.

Detritylation was carried out in the same manner to get the other 5'-OH-derivatives:

34 µmol $\mathbf{9_a}$ (5'-*O*-DMT-TdA^{Bz} on soluble support) and 4 ml acid solution to give $\mathbf{10_a}$
89 µmol $\mathbf{6_b}$ (5'-*O*-DMT-T on soluble support) and 11 ml acid solution to give $\mathbf{7_b}$
46 µmol $\mathbf{9_b}$ (5'-*O*-DMT-dG^{Ibu}T on soluble support) and 6 ml acid solution to give $\mathbf{10_b}$

Coupling ($8_{a\text{-}b}$, $11_{a\text{-}b}$)

5 ml of a 0.1 M solution of 5'-*O*-DMT protected 3'-*O*-methylphosphoramidite of T in ACN (6 eq) was added under dry conditions to the detritylated nucleoside bound on the soluble support ($\mathbf{7_a}$, 63 µmol). Then 2.1 ml 5-(benzylmercapto)-1*H*-tetrazole (0.3 M in ACN) were added, and the reaction mixture was stirred for 3 h at room temperature under argon to form the protected dimer $\mathbf{8_a}$ which was used immediately for oxidation.

Coupling was carried out in the same manner with the following compounds:

$\mathbf{10_a}$ (TdA^{Bz} on soluble support, 34 µmol), 2.2 ml of a 0.1 M solution of the 5'-*O*-DMT-dC^{Bz}-3'-*O*-methylphosphoramidite in ACN and 1.2 ml BMT-solution to give $\mathbf{11_a}$
$\mathbf{7_b}$ (T on soluble support, 89 µmol), 5.4 ml of a 0.1 M solution of the 5'-*O*-DMT-dG^{Ibu}-3'-*O*-methylphosphoramidite in ACN and 3 ml BMT-solution to give $\mathbf{8_b}$
$\mathbf{10_b}$ (dG^{Ibu}T on soluble support, 46 µmol), 2.8 ml of a 0.1 M solution of the 5'-*O*-DMT-dG^{Ibu}-3'-*O*-methylphosphoramidite in ACN and 1.6 ml BMT-solution to give $\mathbf{11_b}$

Oxidation ($9_{a\text{-}b}$, $12_{a\text{-}b}$)

0.2 M iodine solution (Trimethylpyridine/ACN/H_2O (1:11:5)) were added until a yellow colour remained (about 70 ml). A few drops of a 1 M trimethyl phosphite solution in DMF were added until the solution was colourless again. The solution was divided over four 50 ml plastic tubes, refilled with MeOH and stored in the freezer at -20 °C overnight. The solid was filtered and the product $\mathbf{9_a}$ was lyophilized (34 µmol, 0.17 g, 53%).

Oxidation was carried out in the same manner for the other oxidation reactions:

$\mathbf{11_a}$ (5'-*O*-DMT-dC^{Bz}TdA^{Bz} on soluble support) and about 35 ml iodine solution to give $\mathbf{12_a}$ (70%, 24 µmol, 0.16 g)
$\mathbf{8_b}$ (5'-*O*-DMT-dG^{Ibu}T on soluble support) and about 95 ml iodine solution to give $\mathbf{9_b}$ (55%, 46 µmol, 0.23 g)
$\mathbf{11_b}$ (5'-*O*-DMT-dG^{Ibu}-dG^{Ibu}T on soluble support) and about 40 ml iodine solution to give $\mathbf{12_b}$ (70%, 33 µmol, 0.22 g)

All yields for oxidized compounds were calculated in relation to DMT-protected starting materials $6_{a\text{-}b}$ and $9_{a\text{-}b}$.

Cleavage from support (**13$_{a-b}$**)

0.30 g TCEP were solved in a mixture of 2 ml aqueous HEPES buffer (pH 7.5) and 4 ml ACN. This TCEP solution was added to trinucleotide linked soluble support (0.16 g, 27 µmol, **12$_a$**,) and stirred overnight at room temperature under argon. The reaction mixture was evaporated and lyophilized. The soluble support was washed extensively with ACN, acetone, EtOH, THF and EE, until no colour appeared when the soluble support was treated with acid-solution (dichloroacetic acid, 4% in DCE). The combined organic layers were evaporated and lyophilized. Purification was carried out by PLC (DCM:MeOH / 95:5 + 1% TEA). The product was eluted from silica gel first with DCM + 1% TEA and afterwards with ACN/acetone + 1% TEA. The combined organic layers were evaporated and lyophilized to give **13$_a$** (44%, 48 µmol, 66 mg).

Cleavage from support was achieved in the same manner for trinucleotide **12$_b$** (31 µmol) with 0.39 g TCEP to give **13$_b$** (39%, 48 µmol, 0.07 g).

13$_a$ (5'-*O*-DMT-dC^{Bz}TdA^{Bz}):

^{1}H NMR (600 MHz, DMSO): δ [ppm] = 11.21 (s, 1H, NH), 11.17 (s, 1H, NH), 10.44 (s, 1H, NH), 7.81 (m, 4H, Bz-arom), 7.68-7.65 (m, 2H, A-H6, H8), 7.38 (m, 1H, T-H6), 7.21 (m, 1H, C-H6), 7.20-7.19 (m, 13 H, DMT-arom), 6.88-6.87 (m, 6 H, Bz-arom), 6.48 (m, 1H, H1'), 6.11 (m, 1H, H1'), 5.79 (m, 1H, H1'), 5.39 (m, 1H, C H5), 5.32 (m, 1H, H4'), 4.82 (m, 1H, H4'), 4.68 (m, 1H, H4'), 4.27 (m, 1H, H3'), 3.84 (m, 1H, H3'), 3.76-3.71 (m, 12H, CH_3 (DMT/$POCH_3$)), 3.69 (m, 1H, H3'), 3.59 (m, 2H, H5'/H5''), 3.46 (m, 2H, H5'/H5''), 3.25 (m, 2H, H5'/H5''), 2.85 (m, 2H, H2'/H2''), 2.58 (m, 2H, H2'/H2''), 2.35 (m, 2H, H2'/H2''), 1.34 (s, 3H, T-CH_3).

^{13}C NMR (600 MHz, DMSO): δ [ppm] = 174.25, 166.98, 165.79, 164.76, 158.35, 158.33, 157.71, 156.66, 147.02, 146.98, 144.05, 137.97, 136.23, 131.60, 130.23-128.44, 129.79, 127.18, 127.75-114.88, 118.45, 109.69, 89.54, 89.40, 84.48, 83.74, 78.25, 74.32, 72.90, 69.79, 69.30, 67.80, 65.95, 65.17, 62.62, 55.05, 43.4, 35.11, 31.93, 26.53, 29.8. For more information see table S1 in section NMR spectra (**13a**).

MS: calculated: 1382.41 m/z, found: 1405.63 (**13a** +Na) m/z.

13$_b$ (5'-*O*-DMTdG^{Ibu}dG^{Ibu}T):

^{1}H NMR (600 MHz, DMSO): δ [ppm] = 11.31 (s, 2H, NH), 10.46 (s, 1H, NH), 9.07 (s, 2H, NH Ibu), 7.91 (s, 2H, G-C8), 7.80 (m, 1H, T-H6), 7.16-6.86 (m, 13 H, DMT-arom), 6.65 (m, 2H, H1'), 5.97 (m, 1H, H1'), 5.31 (m, 1H, H3'-OH), 4.46 (m, 1H, H4'), 4.31 (m, 2H, H4'), 4.01 (m, 1H, H3'), 3.87 (m, 2H, H3'), 3.76-3.71 (m, 12H, CH_3 (DMT/$POCH_3$)), 3.51 (m, 2H, H5'/H5''), 3.36 (m, 2H, 2H''), 3.01 (m, 4H, 2H' + 2x Ibu-H), 2.87 (m, 4H, 2x H5'/H5''), 1.97 (m, 2H, H2'/H2''), 1.23 (s, 3H, T- CH_3).

^{13}C NMR (600 MHz, DMSO): δ [ppm] = 180.90, 163.70, 158.41, 158.39, 158.35, 157.71, 143.08, 136.23, 130.23-128.33, 122.95, 122.25, 120.88, 119.24, 113.31, 87.16, 86.37, 84.45, 83.60, 81.42, 77.19, 74.66, 69.78, 65.75, 55.05, 45.69, 39.47, 32.14, 29.96, 18.04. For more information see table S2 in section NMR spectra (**13b**).

MS: calculated: 1370.44 m/z, found: 1370.80 m/z.

S3.4: Synthesis of fully protected phosphoramidite trinucleotides (**14a-b**)

Trinucleotide (**13$_a$**, 27 µmol, 37 mg) was dried under high vacuum and dissolved in 250 µl DCM under dry conditions. 20 µl TEA were added. 10 µl *N*,*N*-Diisopropylmethyl-phosphonamidic-chloride were

added dropwise. The reaction mixture was stirred for 3.5 h at room temperature under argon. Synthesis was controlled via TLC (EE:DCM:TEA / 45:45:10). Samples were visualized with ethanolic ninhydrin solution. Reaction was stopped by addition of 40 µl MeOH. The organic reaction mixture was washed with saturated $NaHCO_3$-solution containing 2% TEA. The separated organic layer was evaporated at 35 °C, and the remaining oil (32 mg, 21 µmol, **14a**) was lyophilized and stored under argon at -20 °C.

Phosphitylation was carried out in the same manner with trinucleotide **13_b** to give **14b** (66 mg, 48 µmol).

S3.5: Coupling of trinucleotides on DNA synthesizer – 6mer synthesis

The trinucleotide phosphoramidite (5'-CTA-3', **14_a**) was solved in DCM (0.1 M) under dry conditions and used for coupling to the sequence 5'-CTT-3' on CPG, assembled directly prior to trinucleotide coupling from standard monomer 3-*O*-methyl-phosphoramidites. Synthesis scale was 1 µmol. Coupling time was 2 x 300 s (double-coupling) for the trinucleotide building block. Synthesis was performed via DMT-on method.

The trinucleotide phosphoramidite (5'-GGT-3', **14_b**) was solved in DCM:ACN/3:1 (0.1 M) under dry conditions. Coupling to the sequence 5'-CTT-3' on CPG was carried out as described above for **14_a**.

S3.6: Deprotection of synthesized 6mers

The solid support of each individual synthesis was divided over two screw cap plastic tubes. 1 ml 32% aqueous ammonia solution was added to each vial, and the vials were shaken at room temperature overnight. The oligonucleotide solution was transferred to a new screw cap plastic tube, the support was washed with another 1 ml of ammonia solution, which was then added to the solution in the tube. The tube was sealed and treated at 55°C overnight. The supernatant was removed and the solid support was washed four times with each time 100 µl EtOH:H_2O/1:1. The combined supernatant and washing solutions were evaporated to dryness and the remaining solid was dissolved in buffer A (5% ACN, 0.1 M TEAAc) and analyzed by HPLC.

S3.7: HPLC analysis of 6mers

HPLC conditions: Nucleodur 125/4, CV = 1.571 ml; 1 ml/min; room temperature; buffer A: 5% ACN, 0.1 M TEAAc; buffer B: 30% ACN, 0.1 M TEAAc; Gradient: starting with 0% buffer B for 4 CV, to 40% buffer B over 3 CV, to 60% buffer B over 7 CV, to 100% buffer B over 2 CV, then 100% buffer B for another 2 CV and to 0% buffer B over 3 CV. Detection was by UV-light absorption at 260 nm. Collected fractions corresponding to Peak 2 (see Fig. 4 HPLC Purification of CTACTT (A) and GGTCTT (B) (DMT-on) in main text) were analyzed by MALDI mass spectroscopy.

MS: full length 5'-CTACTT-3' (Peak 2, Fig. 4A) calculated: 1742.18 m/z, found: 1742.15 m/z.
MS: full length 5'-GGTCTT-3' (Peak 2, Fig. 4B) calculated: 1798.21 m/z, found: 1799.21 m/z ($+H^+$).

S3.8: Coupling yields for GGT and CTA building blocks

Coupling yields for trinucleotide phosphoramidites were determined from peak areas of the HPL chromatogram (Figure 4, main text), corrected by the extinction coefficients.

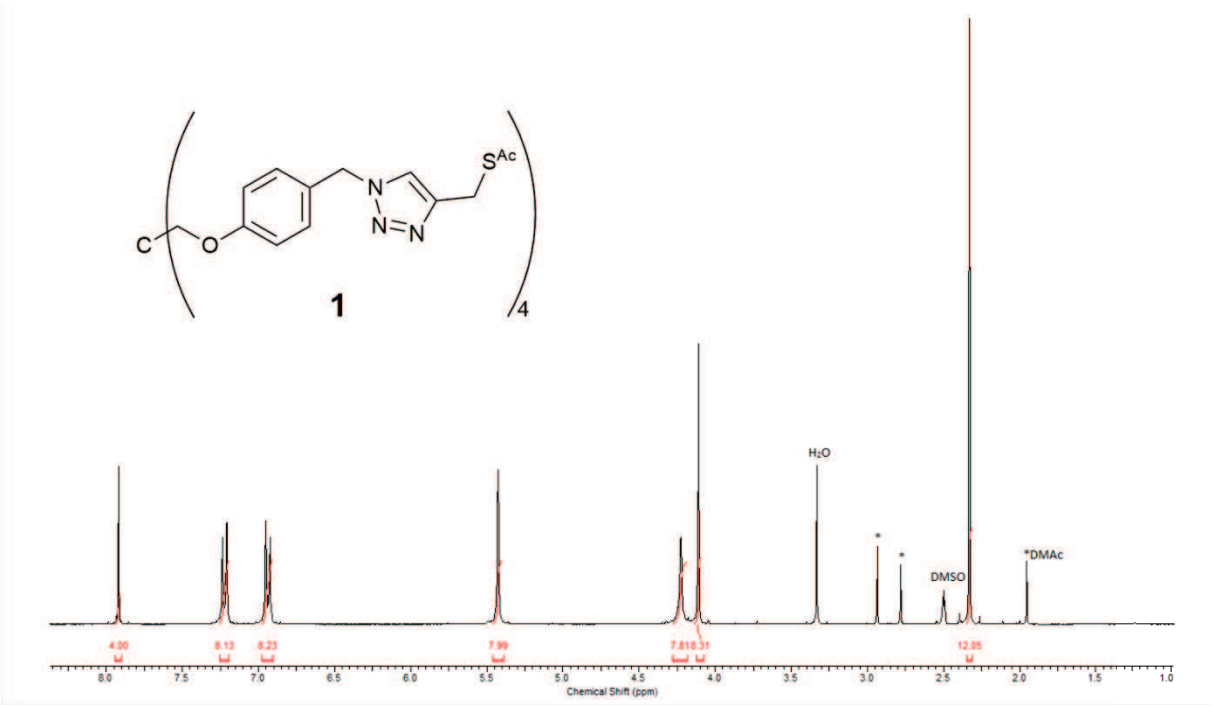
Coupling yield for CTA: 17%.

Coupling yield for GGT: 72%.

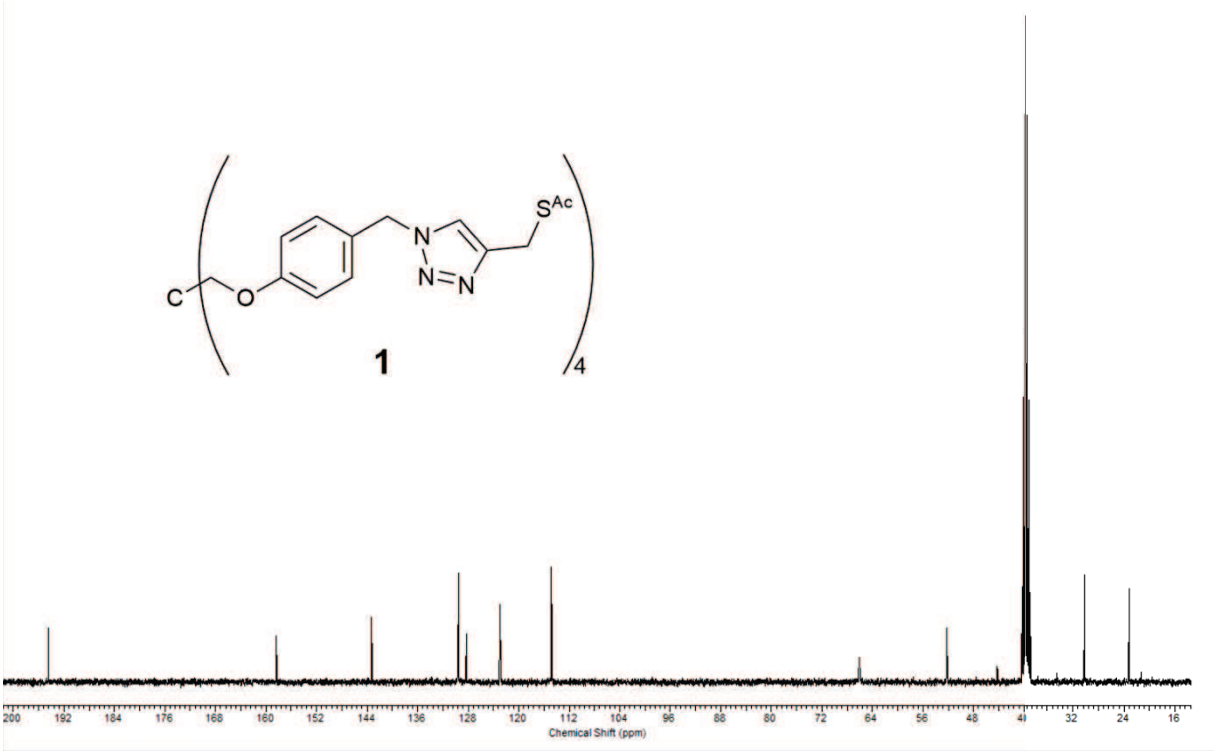
S4: NMR Spectra

Tetrakis-*O*-{4-[4-(acetylthiomethyl)-1*H*-1,2,3-triazol-1-ylmethyl]-phenyl}-pentaerythritol (**1**), ^{1}H,^{13}C spectra

^{1}H spectrum (1):

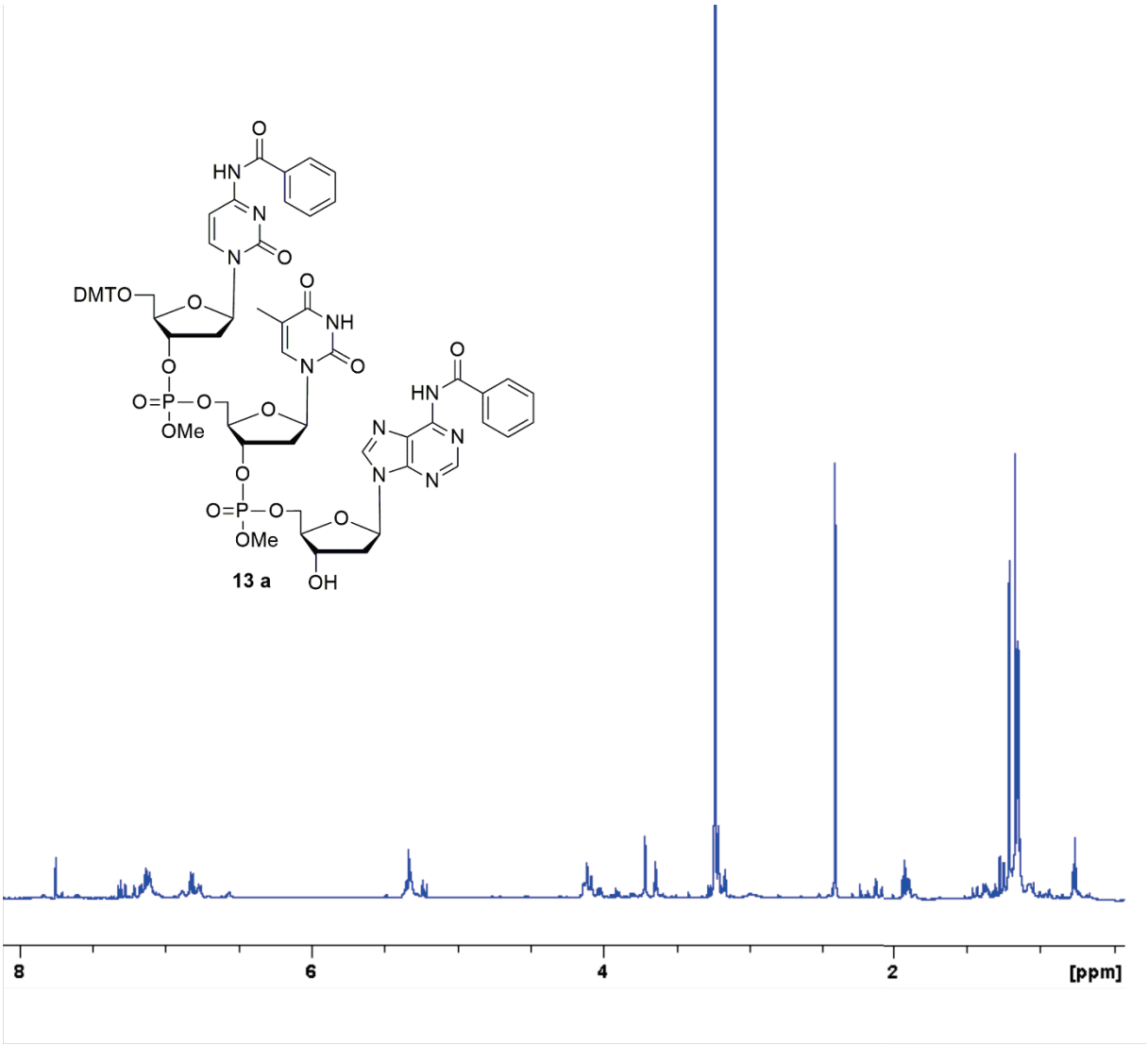


^{13}C spectrum (1):

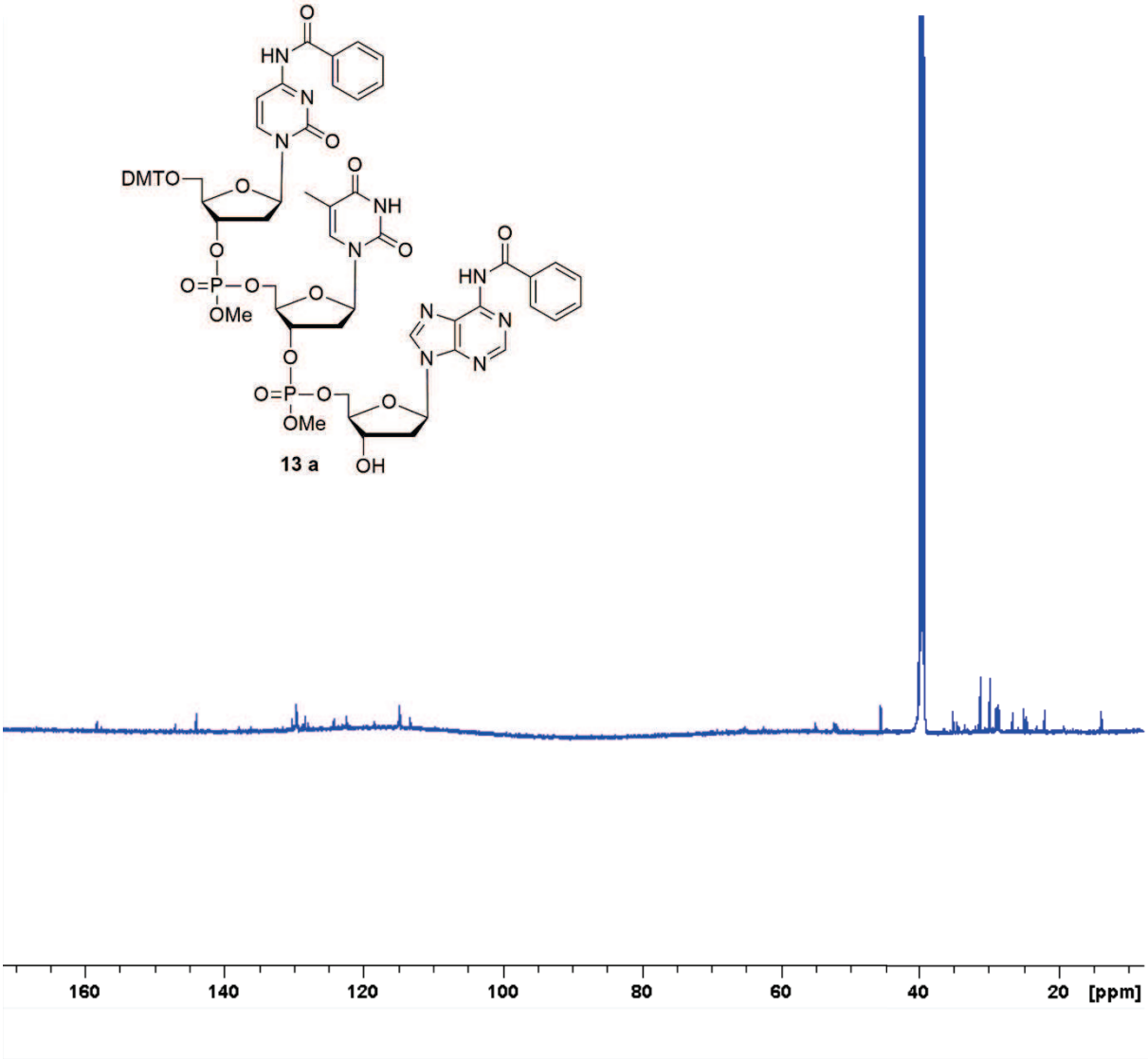


Trinucleotide $C^{Bz}TA^{Bz}$ (**13$_a$**), ^1H, ^{13}C, DEPT, DQF-COSY, HSQC spectra

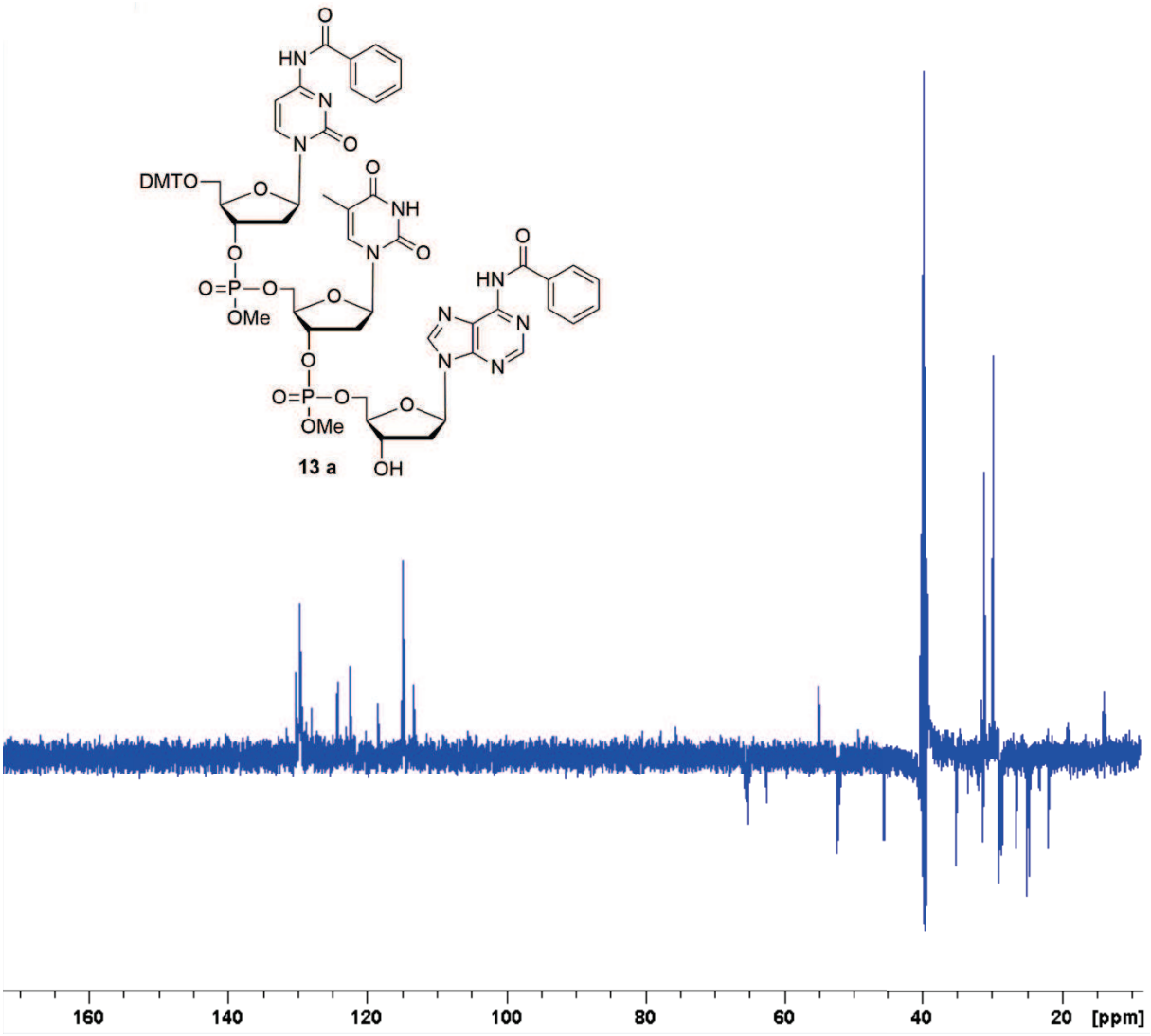
^{1}H spectrum (13a):



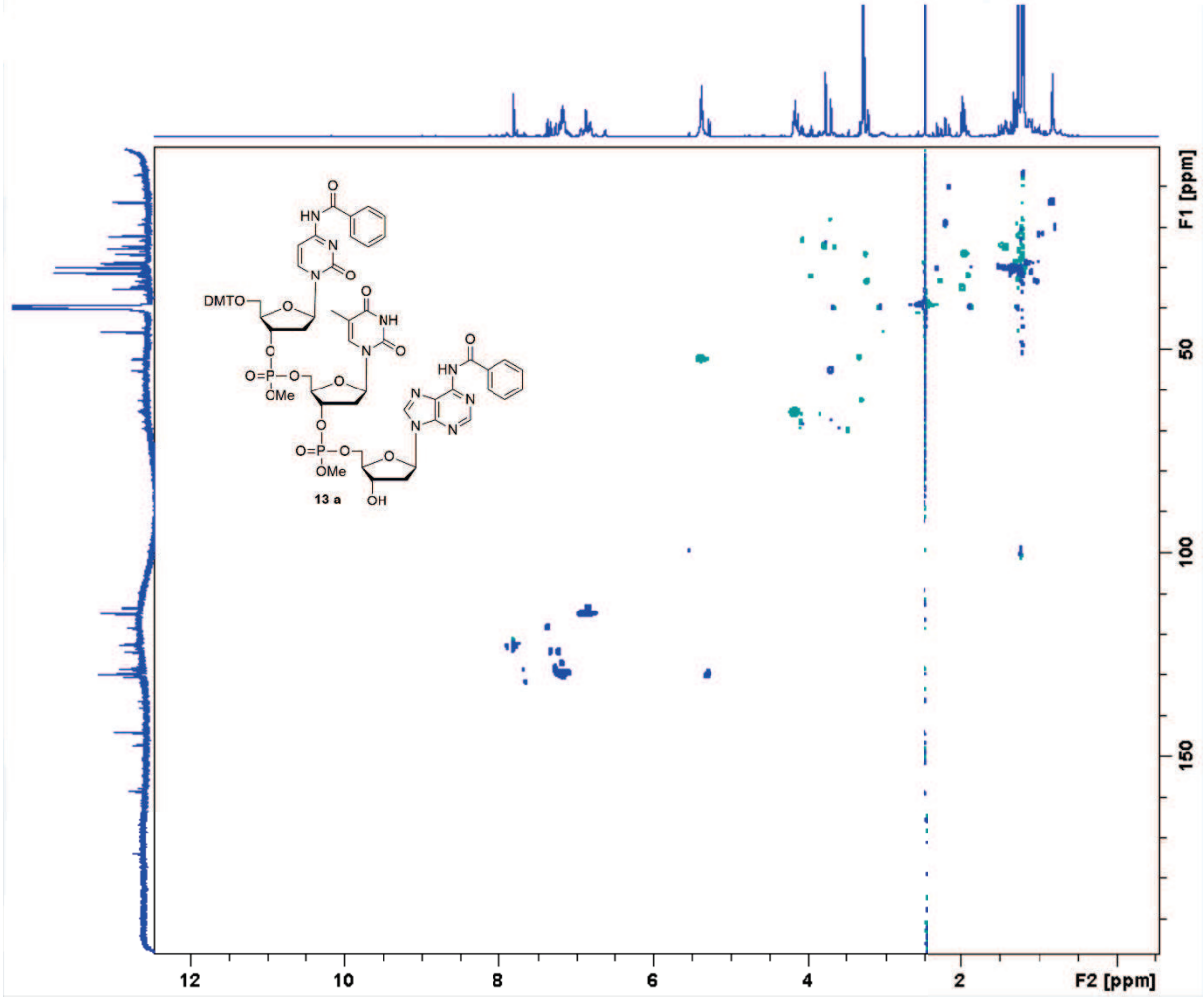
^{13}C spectrum (13a):



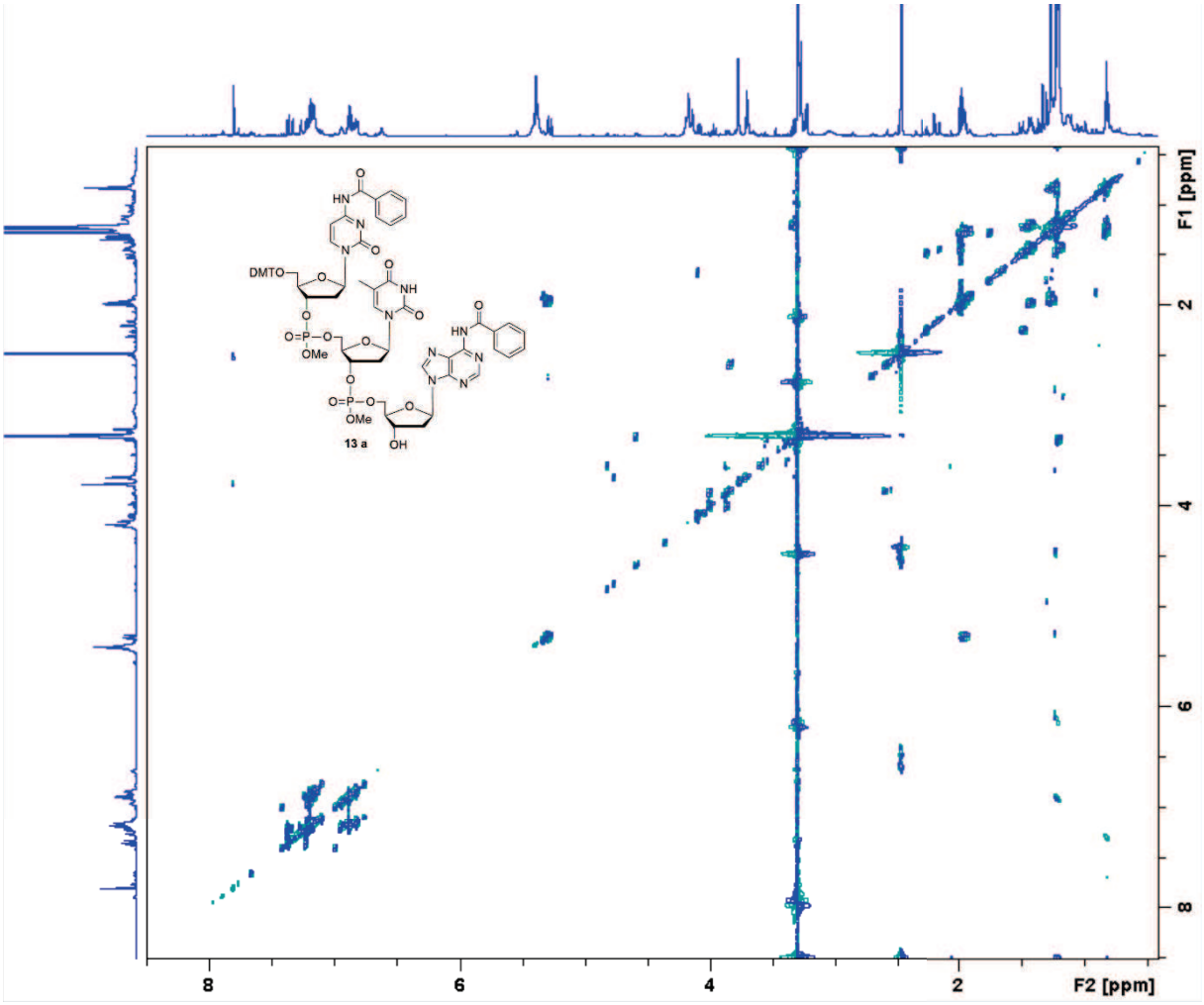
DEPT spectrum (13a):



HSQC spectrum (13a):



DQF-COSY spectrum (13a):



Details of ^{1}H and Cosy spectra (13a):

Object	Integral [abs]	Integral [rel]	Peaks	v(F1) [ppm]	
Integral 1	146872918016.00	1.0874	0	11.2141	NH
Integral 2	128184184832.00	0.9491	0	11.1748	NH
Integral 3	122552209408.00	0.9074	0	10.4424	NH
Integral 4	629390876672.00	4.6600	0	7.8099	BZ arom
Integral 5	187295752192.00	1.3867	0	7.6808	
Integral 6	178513494016.00	1.3217	0	7.6532	Ade H6/H8
Integral 7	189182054400.00	1.4007	0	7.3814	Thy H6
Integral 8	134741446656.00	0.9976	0	7.2162	Cyt H6
Integral 9	692917497856.00	5.1303	0	7.2041	
Integral 10	713390256128.00	5.2819	0	7.1907	DMT arom
Integral 11	421545852928.00	3.1211	0	6.8867	
Integral 12	459441123328.00	3.4017	0	6.8740	Bz arom
Integral 13	106472710144.00	0.7883	0	6.4854	H1'
Integral 14	135063437312.00	1.0000	0	6.1115	H1'
Integral 15	146421608448.00	1.0841	0	5.7955	H1'
Integral 16	199132606464.00	1.4744	0	5.3942	Cyt H5
Integral 17	224739733504.00	1.6640	0	5.3280	H4'
Integral 18	131820859392.00	0.9760	0	4.8246	H4'
Integral 19	122597601280.00	0.9077	0	4.6878	H4'
Integral 20	173989359616.00	1.2882	0	4.2754	H3'
Integral 21	143954042880.00	1.0658	0	3.8494	H3'
Integral 22	959010983936.00	7.1004	0	3.7699	methyl (DMT/POCH3)
Integral 23	798403350528.00	5.9113	0	3.7116	
Integral 24	174303420416.00	1.2905	0	3.6916	H3'
Integral 25	289091543040.00	2.1404	0	3.5970	H5'/"
Integral 26	291325337600.00	2.1570	0	3.4684	H5'/"
Integral 27	328343789568.00	2.4310	0	3.2524	H5'/"
Integral 28	313418035200.00	2.3205	0	2.8574	H2'/"
Integral 29	271217479680.00	2.0081	0	2.5878	H2'/"
Integral 30	268220178432.00	1.9859	0	2.3557	H2'/"
Integral 31	440311259136.00	3.2600	0	1.3349	Thy methyl

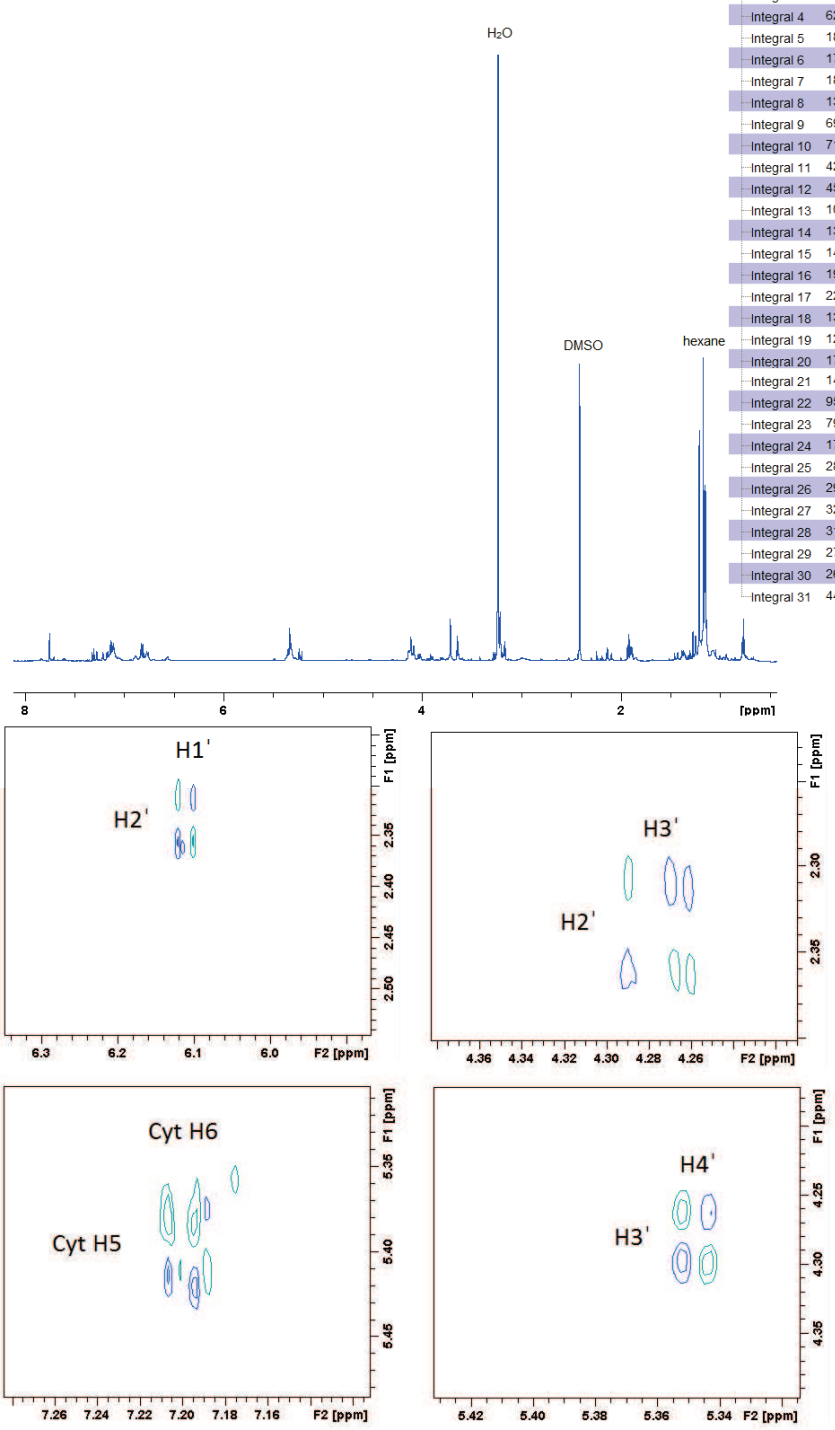
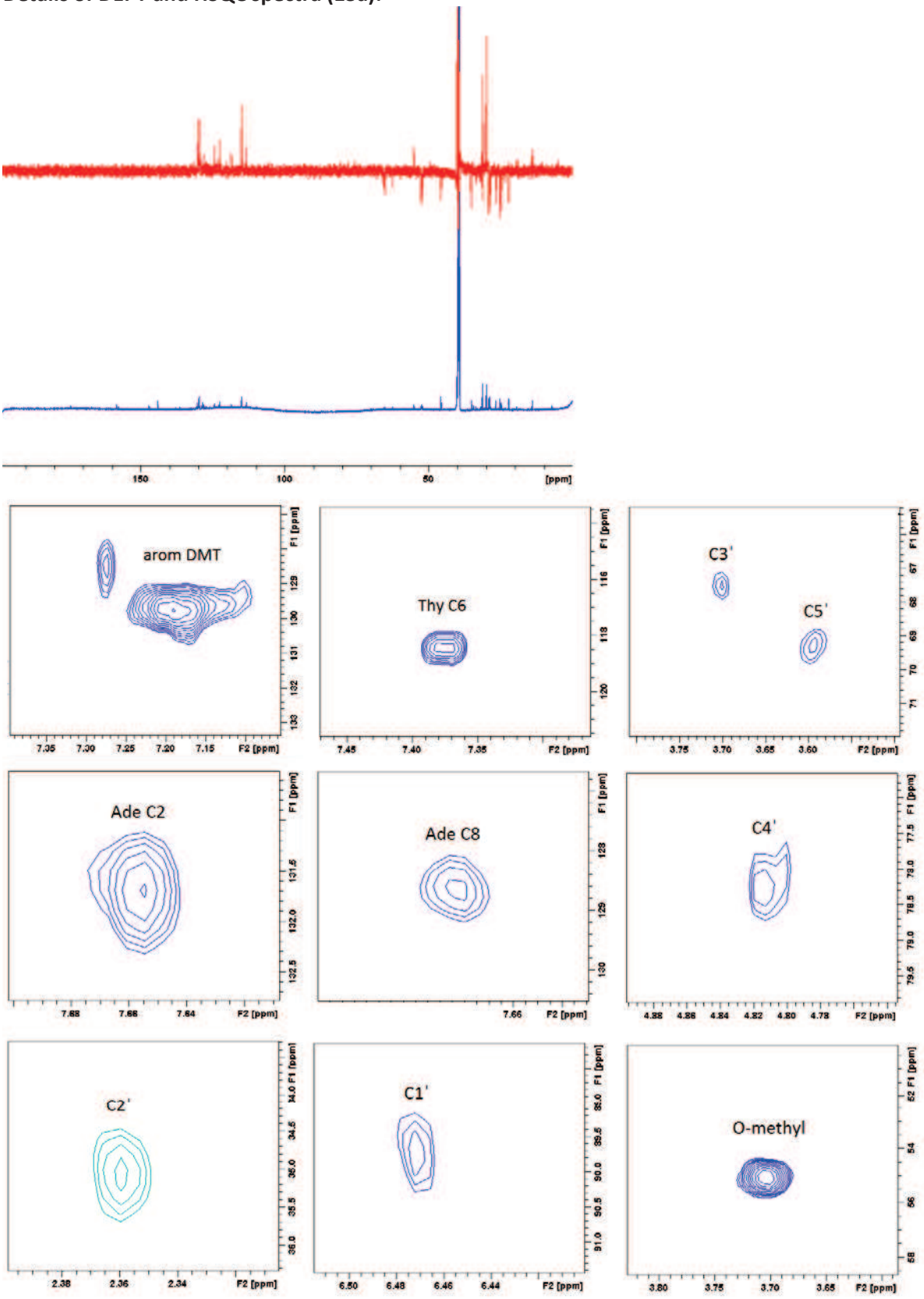


Table S1: Details of ^{13}C and DEPT spectra of **13_a**, DEPT: + ≙ positive signal, - ≙ negative signal, / ≙ no signal.

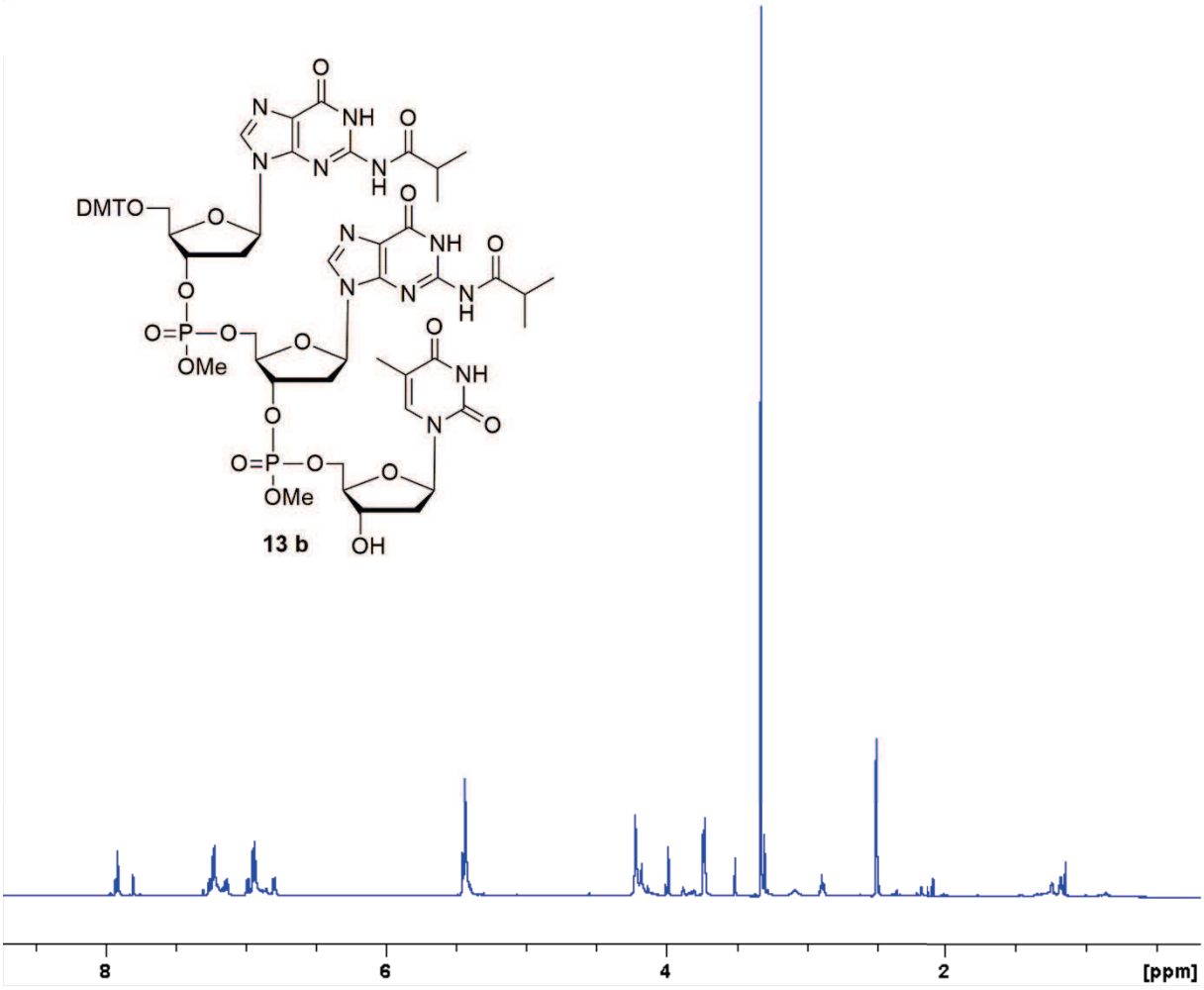
ppm	DEPT	Signal
174.25	/	Cyt C1
166.98, 165.79	/	BZ C=O
164.76	/	Thy C3
158.35, 158.33	/	DMT arom
157.71	/	Ade C5
156.66	/	Cyt C3
147.02, 146.98	/	Ade C3/4
144.05	/	DMT arom
137.97	/	Bz arom
136.23	/	DMT arom
131.60	+	Ade C2
130.23-128.44	+	DMT arom
129.79	+	Cyt C5
127.18	+	Cyt C6
127.75-114.88	+	Bz arom
118.45	+	Thy C6
109.69	/	Thy C5
89.54	+	C1'
89.40	/	DMTq
84.48	+	C1'
83.74	+	C1'
78.25	+	C4'
74.32	+	C4'
72.90	+	C4'
69.79	-	C5'/''
69.30	-	C5'/''
67.80	+	C3'
65.95	+	C3'
65.17	+	C3'
62.62	-	C5'/''
55.05, 43.40	+	O-CH_3 (DMT/$POCH_3$)
35.11	-	C2'/''
31.93	-	C2'/''
26.53	-	2'/''
10.50	-	Thy methyl

Details of DEPT and HSQC spectra (13a):

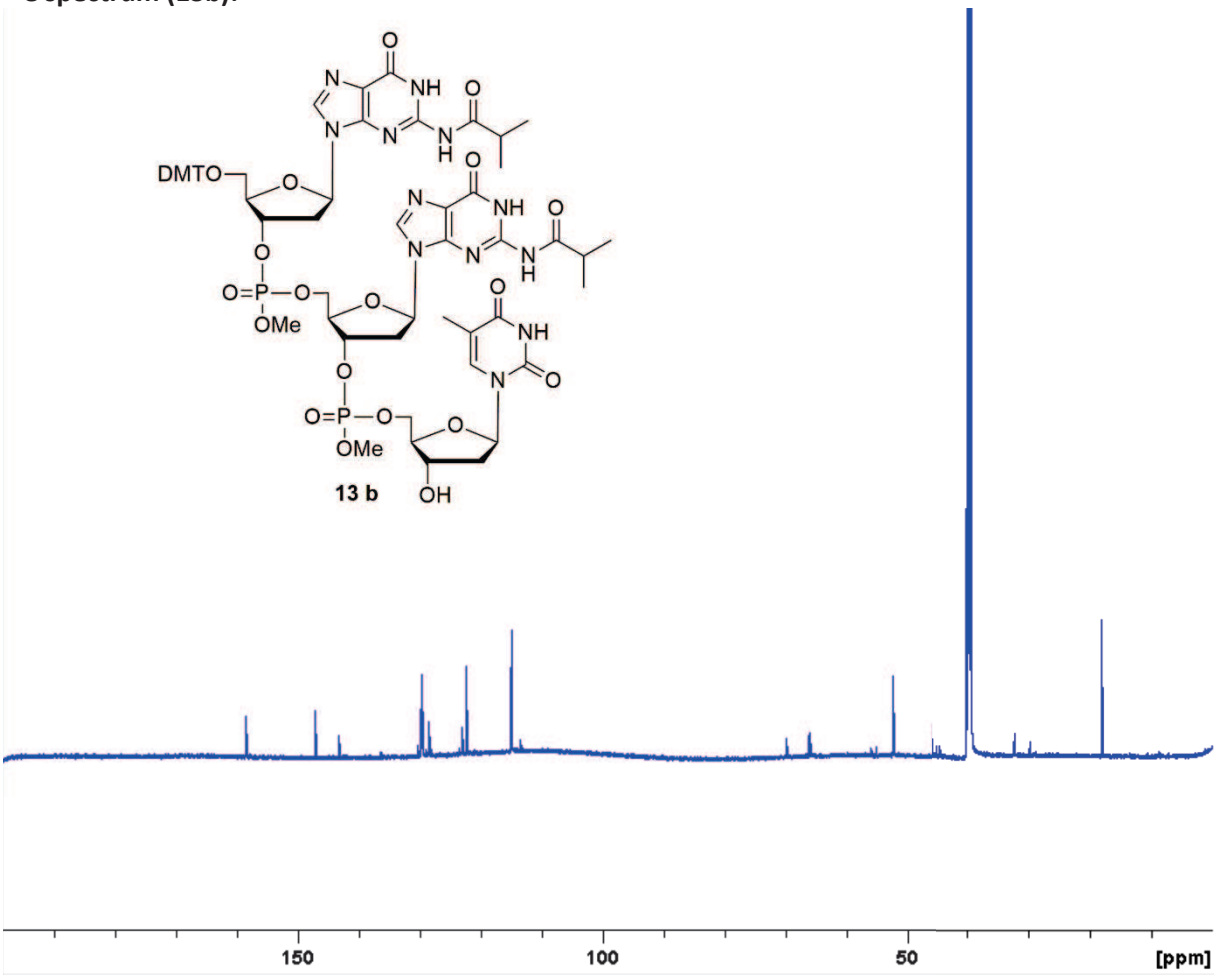


Trinucleotide $G^{Ibu}G^{Ibu}T$, (**13$_b$**), ^{1}H, ^{13}C, DEPT, DQF-COSY, HSQC spectra

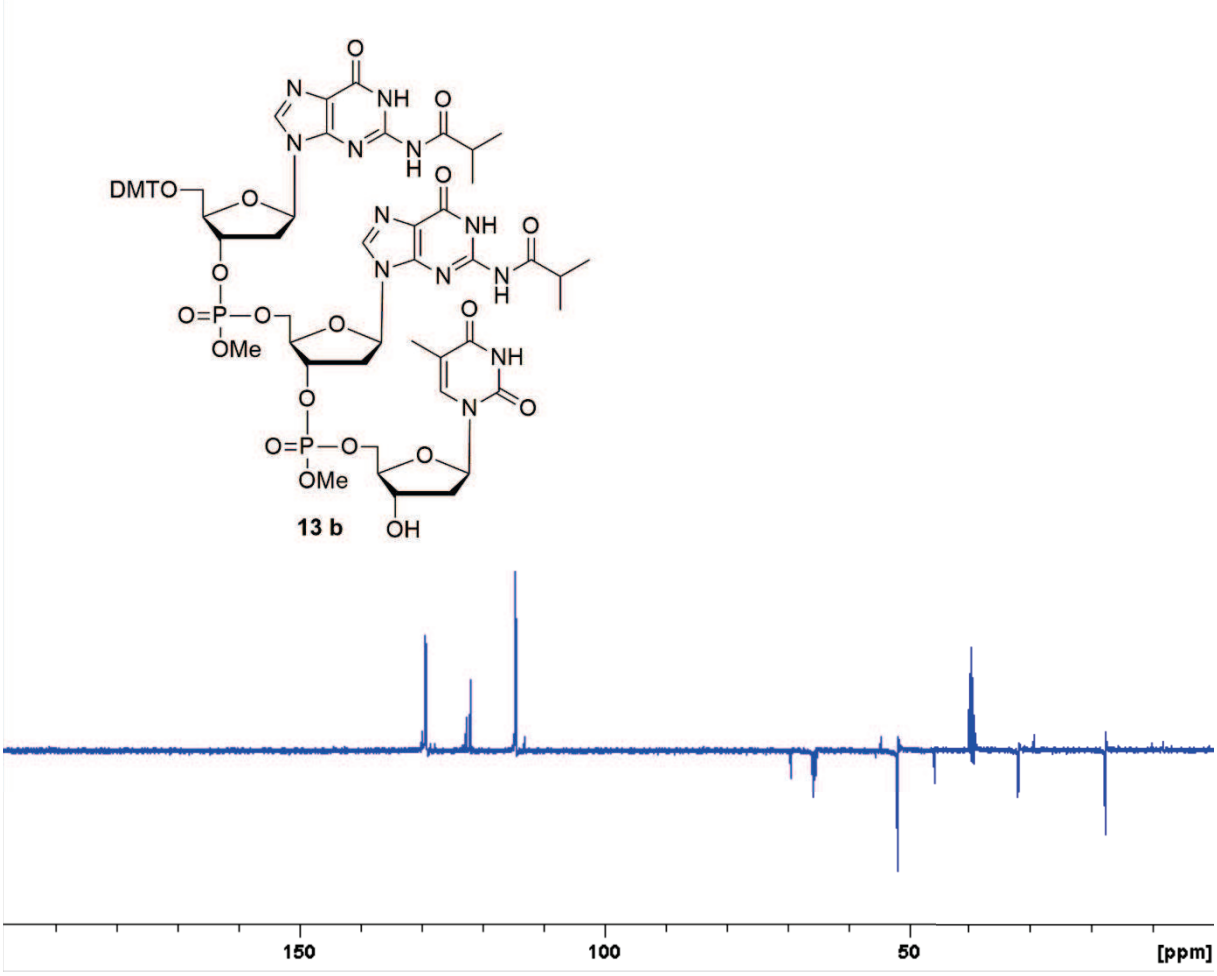
^{1}H spectrum (13b):



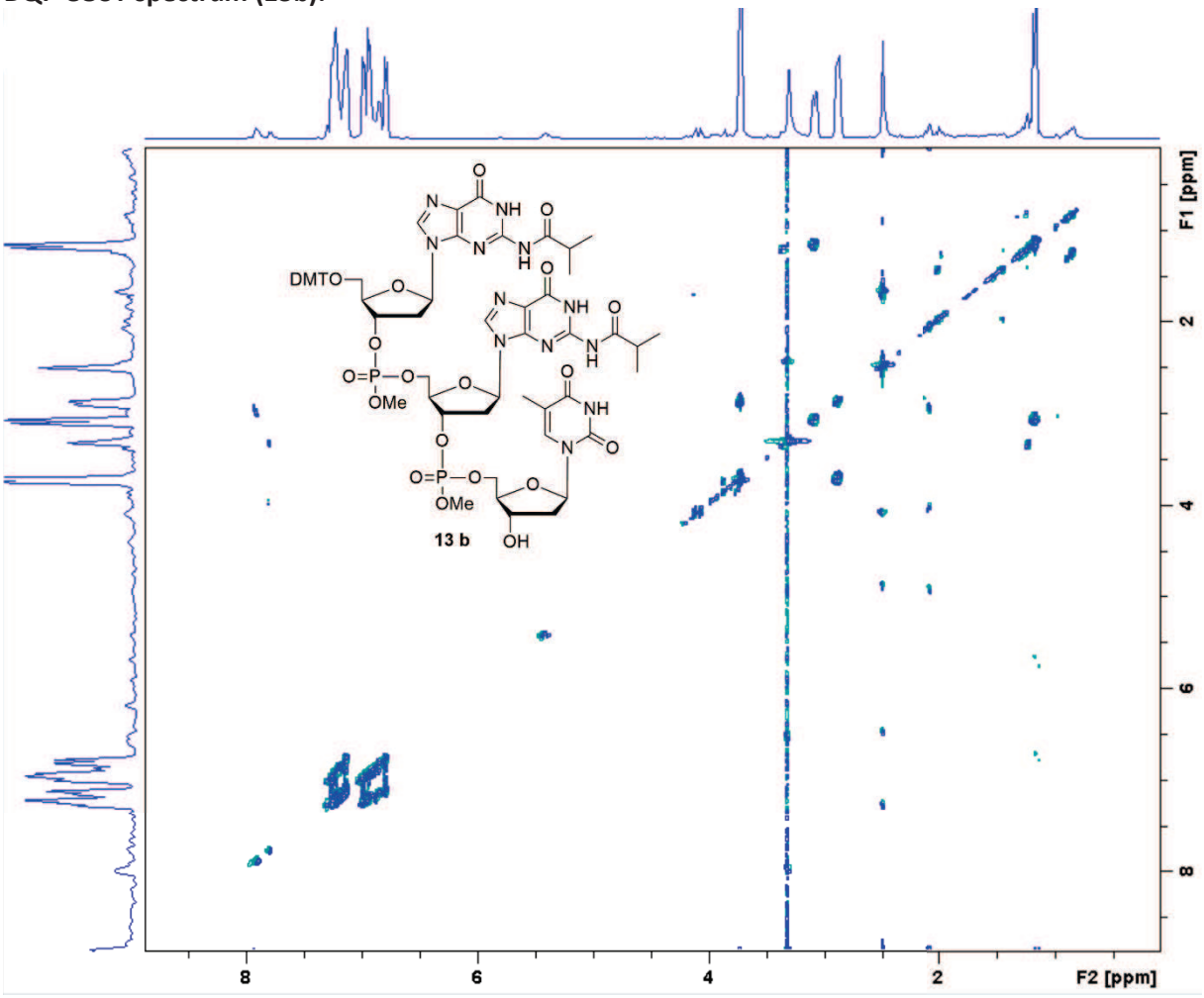
^{13}C spectrum (13b):



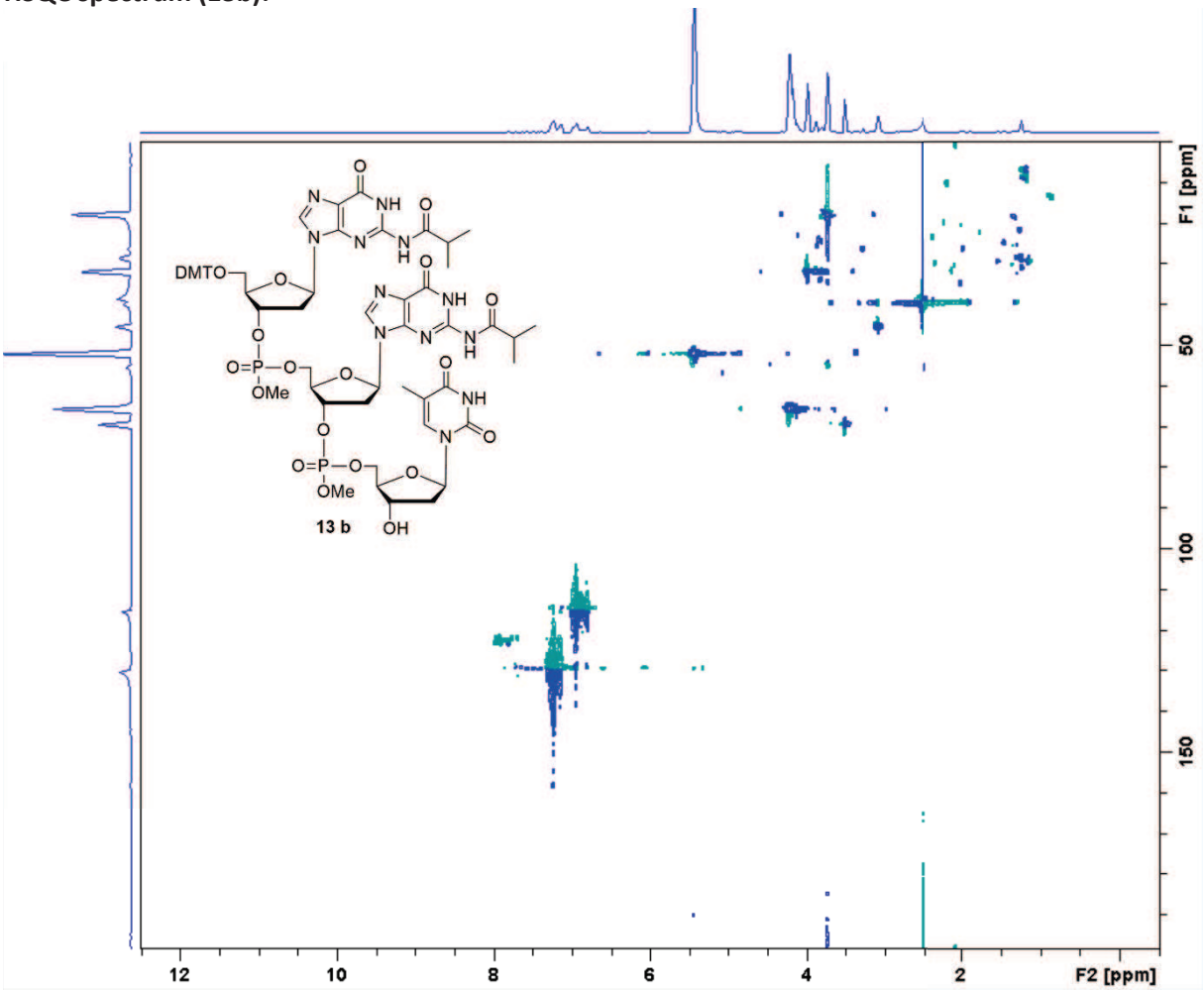
DEPT spectrum (13b):



DQF-COSY spectrum (13b):

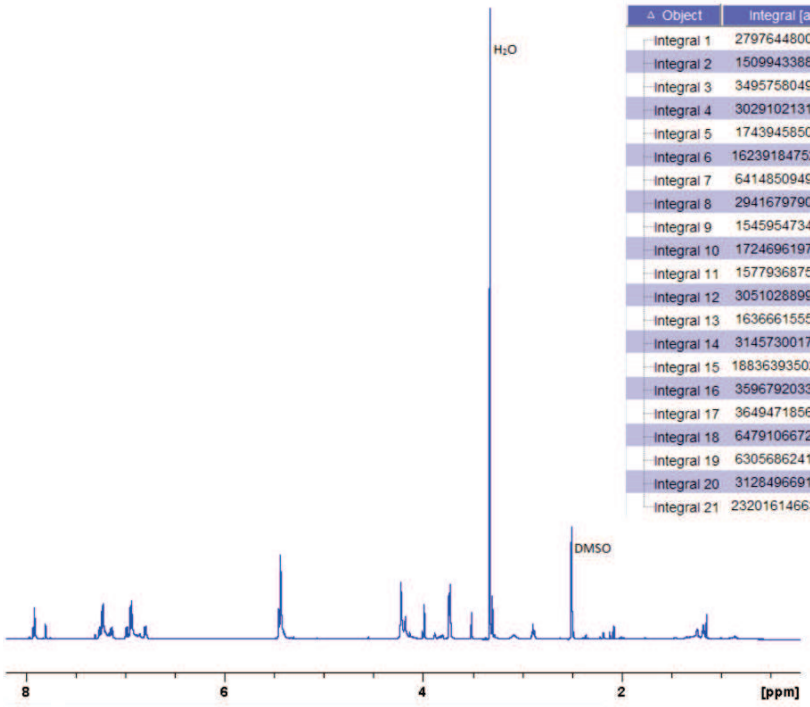


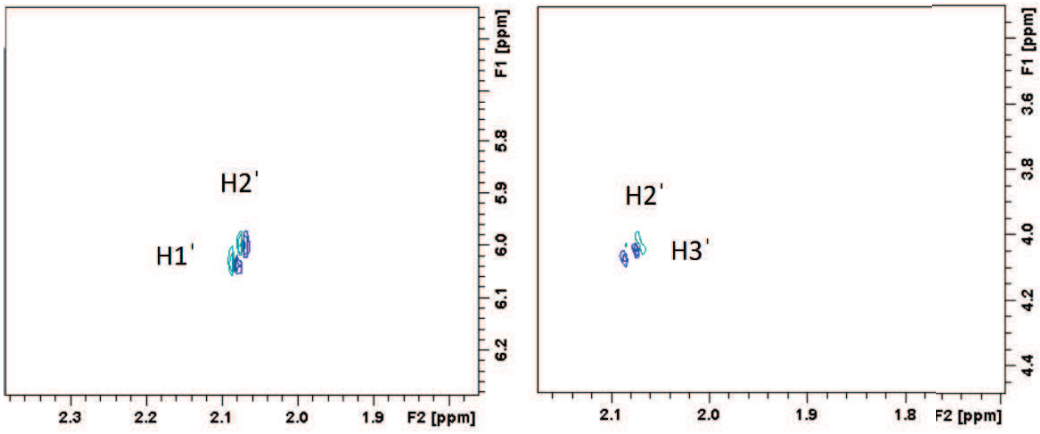
HSQC spectrum (13b):



Details of ^{1}H and COSY spectra (13b):

Object	Integral [abs]	Integral [rel]	Peaks	v(F1) [ppm]	
Integral 1	279764480000.00	1.3097	0	11.3102	2xNH
Integral 2	150994338816.00	0.9767	0	10.4567	NH
Integral 3	349575804928.00	2.2612	0	9.0698	2x NH (Ibu)
Integral 4	302910213120.00	1.9594	0	7.9154	2x Gua C8
Integral 5	174394585088.00	1.1281	0	7.8006	Thy C6
Integral 6	1623918475264....	10.5043	0	7.1645	DMT arom.
Integral 7	641485094912.00	4.1494	0	6.8609	
Integral 8	294167979008.00	1.9028	0	6.6526	2x H1'
Integral 9	154595473408.00	1.0000	0	5.9742	H1'
Integral 10	172469619712.00	1.1156	0	5.3157	H3'-OH
Integral 11	157793687552.00	1.0207	0	4.4658	H4'
Integral 12	305102889984.00	1.9736	0	4.3190	2xH4'
Integral 13	163666155520.00	1.0587	0	4.0044	H3'
Integral 14	314573001728.00	2.0348	0	3.8704	2x H3'
Integral 15	1883639350272....	12.1843	0	3.7262	methyl (DMT/POCH$_3$)
Integral 16	359679203328.00	2.3266	0	3.5068	H5'/''
Integral 17	364947185664.00	2.3607	0	3.3581	2x H''
Integral 18	647910667264.00	4.1910	0	3.0895	2x Ibu-H, 2`
Integral 19	630568624128.00	4.0788	0	2.8754	2x 5'/5''
Integral 20	312849669120.00	2.0237	0	1.9713	H2'/''
Integral 21	2320161466368....	15.0080	0	1.2472	methyl Ibu/Thy





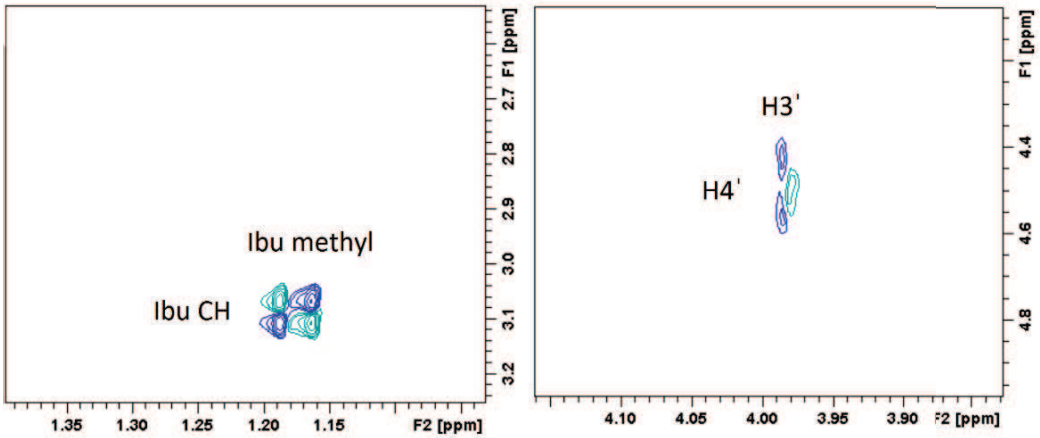
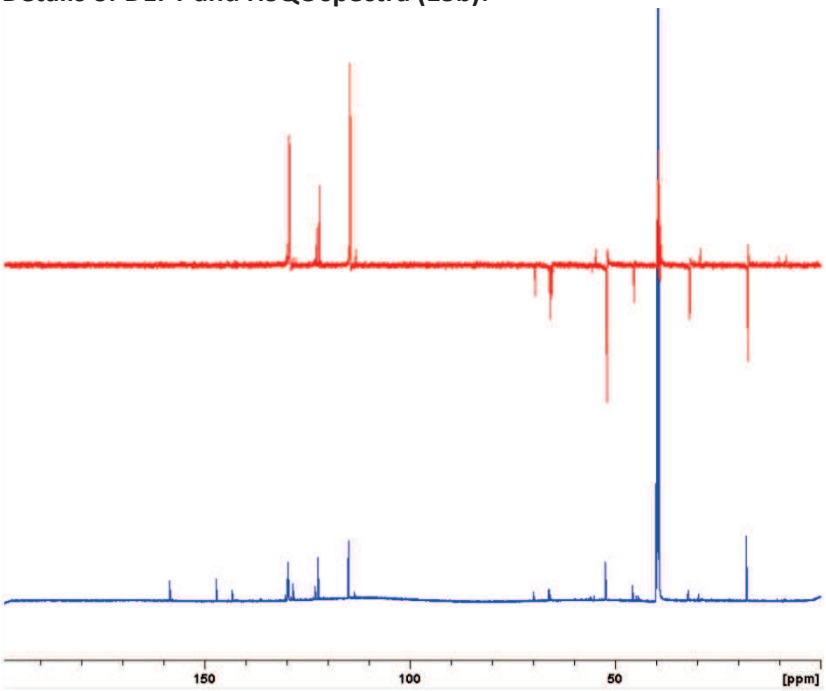
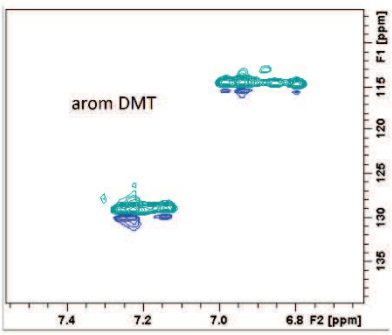


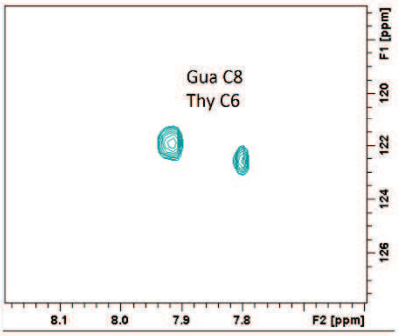
Table S2: Details of ^{13}C and DEPT spectra of **13_b**, DEPT: + ≙ positive signal, - ≙ negative signal / ≙ no signal.

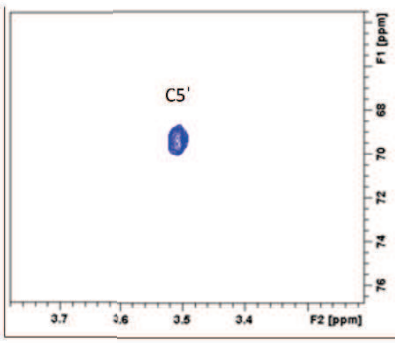
ppm	Dept	Signal
180.90	/	Ibu C=O
163.70	/	Thy C4
158.41, 158.39	/	DMT arom
158.35	/	Gua C6
157.71, 147.02	/	Thy C2; Gua C4
143.08	/	Gua C2
136.23	/	DMT arom
130.22-128.33	+	DMT arom
122.95, 122.25	+	Thy C6, Gua C8
120.88, 119.24	/	Gua C5; Thy C5
113.31	+	DMT arom
87.16	/	DMTq
86.37	+	C4'
84.45	+	C4'
83.60	+	C1'
81.42	+	C1'
77.19	+	C3'
74.66	+	C3'
69.78	-	C5'
65.75	-	C5'
55.05, 39.47	+	O-CH_3 (DMT/$POCH_3$)
45.69	-	C2'
32.14	-	C2'
29.60, 18.04	+	Methyl Thy/Ibu

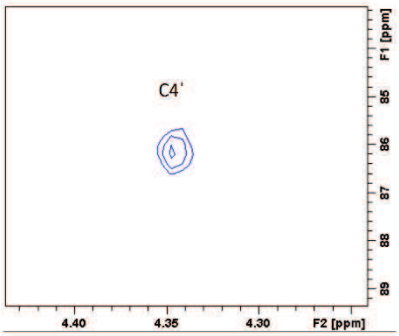
Details of DEPT and HSQC spectra (13b):

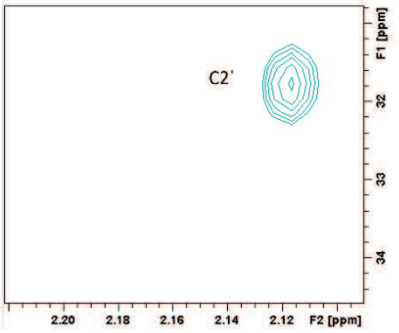


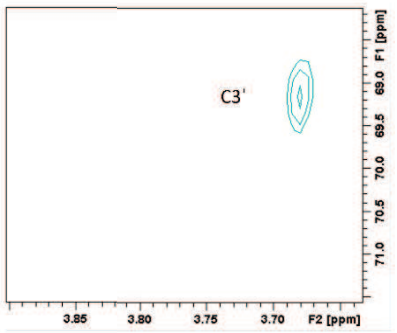


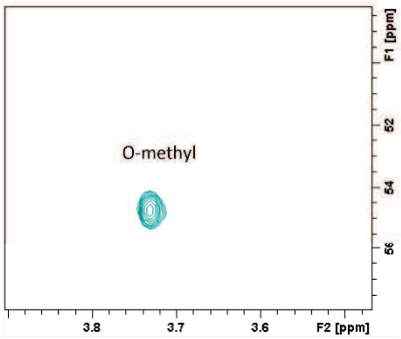


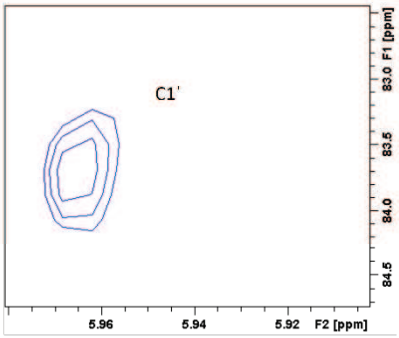












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3. Anhang
3.1 Eigenständigkeitserklärung

Hiermit erkläre ich, dass diese Arbeit bisher von mir weder an der Mathematisch-Naturwissenschaftlichen Fakultät der Universität Greifswald noch einer anderen wissenschaftlichen Einrichtung zum Zwecke der Promotion eingereicht wurde.
Ferner erkläre ich, dass ich diese Arbeit selbstständig verfasst und keine anderen als die darin angegebenen Hilfsmittel und Hilfen benutzt und keine Textabschnitte eines Dritten ohne Kennzeichnung übernommen habe.

_____

Ruth Suchsland

3.2 Anteil der Autoren an den Publikationen

1.) R. Raetz, B. Appel, S. Müller, Preparation of trinucleotide synthons for the synthesis of gene libraries. Chim. Oggi 2016, 34, 14-17.

R. Raetz: Literaturrecherche, Abfassen von Textteilen, Erstellen von Abbildungen, Überarbeitung des Manuskripts
B. Appel: Literaturrecherche, Abfassen von Textteilen, Korrektur von Abbildungen, Überarbeitung des Manuskripts
S. Müller: Abfassung und Korrektur des Manuskripts

2.) R. Suchsland, B. Appel, S. Müller, Preparation of trinucleotide phosphoramidites as synthons for gene library synthesis. Beilstein J. Org. Chem. 2018, 14, 397-406.

R. Suchsland: Literaturrecherche, Abfassen von Textteilen, Erstellen von Abbildungen, Überarbeitung des Manuskripts
B. Appel: Literaturrecherche, Abfassen von Textteilen, Korrektur von Abbildungen, Überarbeitung des Manuskripts
S. Müller: Abfassung und Korrektur des Manuskripts

3.) R. Suchsland, B. Appel, S. Müller, Synthesis of trinucleotide building blocks in solution and on solid phase. Curr. Protoc. Nucleic Acid Chem. 2018, 75, 1-26.

R. Suchsland: Durchführung Synthesen/Experimente, Methodik, Validierung, Erstellung erster Manuskriptentwurf
B. Appel: Konzepterstellung, Methodik, Überarbeitung des Manuskripts
S. Müller: Konzepterstellung, Methodik, Validierung, Überarbeitung und Korrektur des Manuskripts, Leitung

4.) R. Suchsland, B. Appel, M. Janczyk, S. Müller, Solid phase assembly of fully protected trinucleotide building blocks for codon based gene synthesis. Appl. Sci. 2019, 9, 2199-2210.

R. Suchsland: Durchführung Synthesen/Experimente (Anbindungen an verschiedene Festphasen, Verwendung verschiedener Linker, Trinukleotidsynthesen), Methodik, Validierung, Erstellung erster Manuskriptentwurf
B. Appel: Konzepterstellung, Methodik, Überarbeitung des Manuskripts
M. Janczyk: Durchführung Synthesen/Experimente (Trinukleotidsynthesen), Methodik, Validierung
S. Müller: Konzepterstellung, Methodik, Validierung, Überarbeitung und Korrektur des Manuskripts, Leitung

5.) R. Suchsland, B. Appel, P. Virta, S. Müller, Synthesis of fully protected trinucleotide building blocks on a disulphide-linked soluble support, eingereicht am 02.12.20, Organic & Biomolecular Chemistry (OBC).

R. Suchsland: Durchführung Synthesen/Experimente, Methodik, Validierung, Erstellung erster Manuskriptentwurf
B. Appel: Methodik, Überarbeitung des Manuskripts/Abbildungen
P. Virta: Bereitstellung Tetrakis-*O*-[4-(azidomethyl)phenyl]pentaerythritol, Korrektur des Manuskripts
S. Müller: Konzepterstellung, Methodik, Validierung, Überarbeitung und Korrektur des Manuskripts, Leitung

Ruth Suchsland	Prof. Dr. Sabine Müller

Publikationen

R. Raetz, B. Appel, S. Müller, Preparation of trinucleotide synthons for the synthesis of gene libraries. *Chim. Oggi* **2016**, 34, 14-17.

R. Suchsland, B. Appel, S. Müller, Preparation of trinucleotide phosphoramidites as synthons for gene library synthesis. *Beilstein J. Org. Chem.* **2018**, 14, 397-406.

R. Suchsland, B. Appel, S. Müller, Synthesis of trinucleotide building blocks in solution and on solid phase. *Curr. Protoc. Nucleic Acid Chem.***2018**, 75, 1-26.

R. Suchsland, B. Appel, M. Janczyk, S. Müller, Solid phase assembly of fully protected trinucleotide building blocks for codon based gene synthesis. *Appl. Sci.* **2019**, 9, 2199-2210.

R. Suchsland, B. Appel, P. Virta, S. Müller, Synthesis of fully protected trinucleotide building blocks on a disulphide-linked soluble support, eingereicht am 02.12.20, Organic & Biomolecular Chemistry (OBC).

Posterpräsentationen

September 2015 – VII. Nukleinsäurechemietreffen (Berlin): "Preperation of trinucleotide phosphoramidites on solid phase"

Juli 2016 – XXII. International Round Table on Nucleosides, Nucleotides and Nucleic Acids (Paris): "Preparation of trinucleotide phosphoramidites"

September 2017 – 7th Cambridge Symposium Nucleid Acids Chemistry and Biology (Cambridge): "Preparation of trinucleotide phosphoramidites"

September 2017 – VIII. Nukleinsäurechemietreffen (Mainz): "Preparation of trinucleotide phosphoramidites"

Vorträge

September 2016 – III. Doktorandenseminar Nukleinsäurechemie (Bad Herrenalb): "Synthesis of Trinucleotide Synthons"

November 2017 – Stipendiatentreffen 2017/2018 des Fonds der Chemischen Industrie (Berlin): "Synthese von Trinukleotidsynthons"

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