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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
DIVISION DE NUEVAS CREACIONES
E. S. D.

Re: Expediente No. 06.027.688
Solicitud de patente a nombre de **NOVARTIS AG**

Yo, DILIA MARIA RODRIGUEZ D'ALEMAN, actuando como apoderada sustituta de acuerdo con la sustitución presentada el 21 de septiembre de 2010, identificada como aparece al pie de mi firma, domiciliada en la carrera 11 A No. 86-53, piso 6, obrando como apoderada de la sociedad **NOVARTIS AG.**, domiciliada en Basilea Suiza, solicitante de la patente de la referencia, en respuesta al oficio No. 08910 notificado por fijación en lista el 3 de junio de 2011, relacionado con el concepto técnico de fondo No. 1619, me permito manifestar lo siguiente:

1. Con fundamento en la facultad que confiere el artículo 34 de la Decisión 486 al solicitante para modificar la solicitud de patente en cualquier momento del trámite, presentamos junto con este memorial nuevo juego de reivindicaciones, que ahora consta de 6 reivindicaciones dirigidas al ARN de estructura de cadena doble con por lo menos un extremo como con una modificación química hidroxipropilfosfodiéster, la composición farmacéutica y el método in vitro para identificar y/o caracterizar los agentes farmacológicos en la proteína objetivo.

El sustento para el nuevo capítulo reivindicatorio se encuentra entre las páginaS 5 a 21 de la memoria descriptiva. Por lo tanto, nos permitimos manifestar que, el nuevo capítulo reivindicatorio no involucra ampliación alguna de la materia originalmente presentada en esta solicitud y se brinda como respuesta a las objeciones de claridad expuestas por el señor examinador en su concepto técnico No. 1619.

Teniendo en cuenta el concepto técnico emitido por el Señor Superintendente Delegado para la propiedad intelectual GianCarlo Marcenaro Jiménez a la doctora Ana María Castro Wey, que da alcance a las comunicaciones No. 09-001524 y 09-012566, no se presenta una tasa adicional, toda vez que la solicitud fue presentada con anterioridad al 1 de enero de 2009 y tiene menos de 10 reivindicaciones.

2. Claridad de la solicitud

En relación con las objeciones expuestas en el acápite 3 del concepto técnico No. 1619, nos permitimos decir que las mismas quedan cabalmente superadas gracias al nuevo capítulo reivindicatorio.

En relación con el término “RNA de cadena doble con al menos un extremo romo”, nos permitimos decir que, el extremo romo es independiente de las modificaciones, y resulta de ambas cadenas, que tienen la misma secuencia y punto final sobre dicho al menos un extremo. En un ejemplo de RNA de cadena doble con dos extremos romos, ambas cadenas pueden tener la misma secuencia y longitud, sin tener en cuenta las modificaciones. Por lo tanto, el solicitante respetuosamente se permite decir que considera que las reivindicaciones son claras y no requieren de clarificación adicional.

Respecto a que la antigua reivindicación 8 no es clara, respetuosamente nos permitimos decir que la misma (ahora nueva reivindicación 4) establece que la estructura de cadena doble de ARN comprende una cadena de 19 pares de bases de ribonucleótidos contiguos. Por lo tanto, cada cadena del ARN de cadena doble es por lo menos 19 nucleótidos de longitud y existe una región de pares de bases entre las dos cadenas que es al menos 19 pares de bases en longitud. Por lo que se considera que no es necesario realizar clarificaciones adicionales.

3. Excepciones a la patentabilidad

En cuanto a las antiguas reivindicaciones 8, 9 y 12, nos permitimos decir que únicamente se dejó la reivindicación 12 relacionada a un método de identificación o caracterización, pero se especificó que es un método *IN VITRO*, motivo por el cual se entiende que no se realiza al interior de un organismo, humano o animal y no es tampoco un método de tratamiento.

4. Reivindicaciones de uso

Atentamente nos permitimos manifestar que la antigua reivindicación 13 que hacía mención a un uso, ya no hace parte del pliego reivindicatorio.

5. Novedad

El señor examinador ha indicado que tanto el documento D1 (WO02/096927) como la anterioridad D2 (WO03/070918) contienen todas las características técnicas esenciales de la presente invención y que por ello,

la novedad se encuentra afectada.

En relación con los argumentos presentados en el concepto técnico No. 1619, respetuosamente nos permitimos decir que el artículo 16 de la Decisión 486 de la Comisión de la Comunidad Andina establece que la invención se considera como nueva cuando no está comprendida en el estado de la técnica.

Si de acuerdo con el espíritu de la norma legal indicada, la invención no se considera como nueva si está "comprendida" en el estado de la técnica, dicha norma se está refiriendo, naturalmente, a la totalidad del invento y no a alguno o a algunos de los componentes. Para que la invención pierda su calidad de novedosa, es claro que ella, TODA ELLA, ha de estar contenida en el "*conjunto de conocimientos existentes que son aplicables al proceso industrial*" como define el estado de la técnica el Tribunal de justicia del Acuerdo de Cartagena (Proceso No. 6-IP-89, sentencia de 31 de octubre de 1989, publicada en Jurisprudencia del Tribunal de Justicia del Acuerdo de Cartagena, Tomo 11, BID-INTAL, Buenos Aires, 1994, página 60).

Esto se conoce como el concepto de NOVEDAD ABSOLUTA, de conformidad con lo cual, para desvirtuar el requisito de novedad de una invención será necesario que lo reivindicado por la invención bajo estudio corresponda de forma total con lo revelado en **un solo** documento o anterioridad señalada, conclusión a la que se llega después de un análisis exhaustivo mediante una comparación entre el capítulo reivindicatorio de la invención en estudio y la divulgación por separado de cada una de las referencia D1 y D2.

Ahora bien, si un determinado elemento, condición o circunstancia de la nueva creación ya hace parte del estado de la técnica no quiere ello decir, desde luego, que la invención deba ser rechazada por carecer del requisito de novedad. No sin razón ha dicho el mencionado Tribunal: "*hay que advertir que toda creación es en cierta forma, producto del estado de la técnica existente, en el sentido de que el inventor ha de tener en cuenta un conjunto de conocimientos técnicos que le permitan crear nuevos procedimientos aplicables a la industria*" (Ibidem, páginas 53 y 54) (El subrayado es nuestro).

Por lo tanto, como mínimo la novedad debe contemplar los siguientes aspectos:

-Que la invención en estudio esté contenida de forma TOTAL y ABSOLUTA en el documento que se menciona como anterioridad o de forma independiente en los documentos que se mencionan, si son varios; y

-Que la invención en estudio puede partir de conocimientos conocidos, caso en el cual habrá que hacer la comparación no de lo conocido sino de lo inédito y novedoso, teniendo que analizarse el valor agregado que aporta la invención con sus nuevas características al campo técnico al cual pertenece la invención en estudio.

Aplicando lo anterior al caso concreto, para que las razones que aduce ese despacho para desvirtuar la novedad fuesen ciertas, sería forzoso que se demostrara que la invención de mi mandante, como ahora se reivindica en el nuevo pliego de reivindicaciones, esté comprendida en el estado de la técnica de forma TOTAL, lo cual no sucede como ahora se demostrará.

5.1 Novedad de la solicitud en estudio sobre lo divulgado en el documento WO 02/096927 (D1).

El documento en cuestión se encuentra dirigido a moléculas de ácido nucleico que incluyen dsARN, siARN, antisense, 2,5-A-quiméricos y aptámeros entre otros y moléculas enzimáticas de ácido nucleico tal como ribozimas, DNA-cimas y otros, las cuales modulan la expresión del receptor del factor de crecimiento endotelial vascular.

Sin embargo, la anterioridad D1 no reporta un dsARN con extremos romos y una cap 3' como se reivindica en la presente invención. De hecho, D1 se encuentra enfocado en un ARN de cadena sencilla, es decir, ribozimas, pero no menciona un ARN de cadena doble. Si bien, D1 menciona modificaciones químicas de los nucleótidos, es pertinente señalar que dicho documento no menciona la modificación en el cap 3' como se reivindica en la nueva reivindicación 1.

Sumado a lo anterior, atentamente nos permitimos hacer énfasis sobre el hecho que, D1 reporta una lista extensa de posibles modificaciones 3' en la página 45 de su descripción. Dicha lista se deriva de una publicación previa de Beaucage y colaboradores publicada en la revista *Tetrahedron* 49:1925 del año 1993 (Véase por favor anexo 1).

En dicho artículo se puede corroborar que incluso el mismo autor Beaucage no hace referencia siquiera a un dsARN útil para interferencia de ARN. Aun más, el autor únicamente reporta el uso de dichas modificaciones sobre un DNA de cadena sencilla pero no sobre un siARN. De hecho, Beaucage y colaboradores nunca hacen mención a una molécula ARN basada en un dúplex.

Además, el documento D1 establece sin ningún sustento o evidencia que la lista de “modificaciones terminales protegen la molécula de ácido nucleico de la degradación de exonucleasa, y que puede ayudar en la liberación y/o localización dentro de una célula” (por favor véase página 44, línea 26 a 28 de D1).

Así las cosas, el documento D1 citado por el señor examinador no puede ser relevante para afectar la novedad de la presente solicitud, aún más, a la luz del nuevo capítulo reivindicatorio.

5.2 Novedad de la solicitud en estudio sobre lo divulgado en el documento WO 03/070918 (D2).

Por su parte, la anterioridad D2 se encuentra dirigida a métodos y reactivos útiles en la expresión del gen modulador en una variedad de aplicaciones terapéuticas. Principalmente, D2 se relaciona con moléculas pequeñas de ácido nucleico sintetizadas y modificadas químicamente tal como siARN, ARN de cadena doble (dsARN) microARN (miARN) y shARN entre otros. Dichas moléculas son capaces de mediar la interferencia de ARN contra secuencias de ácidos nucleicos objetivo.

No obstante y como ese Despacho podrá corroborar, la anterioridad D2 tampoco hace referencia a caps 3' asociados con dsARNs de extremos romos, como se reivindica en la cláusula 1 modificada. De hecho, el documento D2 no reporta las caps 3' ni el sorprendente hallazgo en relación con que las caps 3' son efectivas para alcanzar actividad ARNi.

En consecuencia, el documento D2 tampoco resulta relevante para afectar la novedad de la solicitud en estudio.

En este sentido, atentamente nos permitimos señalar que la intención de la presente invención es reportar un extremo cap particular, que comprende hidroxipropilfosfodiéster (C3), el cual puede ser empleado sobre cualquier ARN de cadena doble de cualquier secuencia. El extremo cap: (1) estabiliza el siARN contra la degradación, esto es, de nucleasas y (2) permite la inhibición de un gen objetivo por interferencias de ARN (ARNi). Estas cualidades son descritas en la memoria descriptiva (página 12 línea 14 hasta la página 13, línea 15):

“En otro aspecto, la presente invención provee métodos para la inhibición de un gen objetivo que comprenden introducir dentro de una célula de ARNds de acuerdo con la presente invención, la cual es capaz de inhibir por lo menos un gen objetivo a través de ARNi. También, más de una especie de ARNds, la cual cada una es específica para

otra región objetivo, se puede introducir dentro de una célula al mismo tiempo o secuencialmente. El ARNds de acuerdo con la presente invención, se puede introducir dentro de una célula a través de varios métodos estándares en ingeniería genética, incluyendo métodos físicos, por ejemplo, difusión simple, a través de inyección de una solución conteniendo el ácido nucleico, bombardeo con partículas cubiertas con el ácido nucleico, empapando la célula u organismo en una solución del ácido nucleico, lipofección o electroporación de las membranas de célula en la presencia de ácido nucleico. Un método particularmente preferido para entregar ácidos nucleicos es el uso de lipofección. Las células subsecuentemente se controlan bajo condiciones bajo las cuales el ARNi ocurre. Será fácilmente aparente para el técnico experto bajo que condiciones una línea de célula dada se controla con el fin de que ocurra ese ARNi. Este método para la inhibición de un gen objetivo se puede utilizar terapéuticamente (por ejemplo, para destruir un gen sobre expresado en una enfermedad particular) o para investigación (por ejemplo, examinar la función de un gen o validar objetivos para el descubrimiento de fármacos). En una modalidad preferida, la función del gen se elimina completamente a través de este método, es decir, la destrucción de las células para un gen particular se puede generar a través de este método.”

De otra parte, el señor examinador indica en el folio 116 de su concepto técnico que *“Además de los documentos citados aquí, en el informe de búsqueda internacional se citaron dos documentos adicionales que no están disponibles para esta oficina, por lo que se le realiza requerimiento por artículo 46 de estos documentos para que sean anexados al expediente. Se solicita presentar sus descargos frente a la afectación de todos estos documentos con el fin de agilizar el trámite.”*

En relación con el documento de Czauderna y colaboradores, el solicitante desea mencionar que dicha anterioridad reporta experimentos que emplean dsRNA de extremo romo para actividad de interferencia de RNA efectiva (ó RNAi). No obstante, estos compuestos de extremos romos NO tienen el cap 3' como se describe en la presente invención. De hecho, la anterioridad de Czauderna y colaboradores reporta dsRNA con 3' caps, los dúplex reportados allí no son de extremos romos, por ejemplo, véase por favor la Figura 4A. Por lo tanto, debe resultar claro que esta referencia no divulga la presente invención de un dsRNA de extremo romo que tenga un cap 3' como se reivindica.

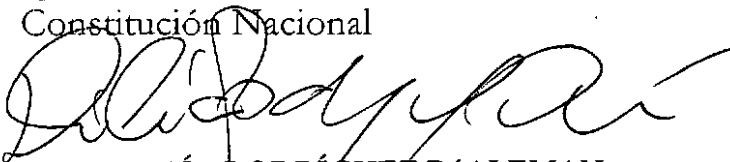
Por su parte, el documento de Hamada y colaboradores, reporta dos tipos de caps. Uno de ellos es el 2'-O-4'-C-etilentimidina (eT), como se puede observar en la figura 3A, el cual se encuentra ubicado en el dúplex de extremo romo, pero este cap no es similar al cap con la estructura química que se reivindica en la solicitud en estudio. El otro cap es hidroxietilfosfato (hp), véase también en la Figura 3A. Este última cap es empleado sobre dsRNA con dos “overhangs” o “voladizos” en la molécula (#4, #5 y #6 en

la Figura 3B), por lo que este cap también es diferente del cap reclamado en la presente invención.

Así las cosas, es evidente que las referencias que se allegaron con nuestra respuesta del 2 de agosto de 2011 al oficio No. 09762 (concepto técnico No. 1618), al igual que los documentos D1 y D2 no afectan ni la novedad ni el nivel inventivo de la presente invención, más aún, a la luz del nuevo juego de reivindicaciones.

Habiendo atendido cabalmente las objeciones presentadas por ese despacho, y habiendo el mismo aceptado los argumentos presentados por el solicitante que demuestran el cumplimiento de los requerimientos de patentabilidad de los compuestos de la solicitud en estudio, atentamente solicitamos se conceda el privilegio de patente de invención a la presente invención o si el examinador considera que subsisten razones para objetar la presente invención ruego expedir otro examen de fondo con los debidos motivos y razones que sustenten su objeción con el fin de que mi mandante tenga la posibilidad de exponer sus argumentos con base en la interpretación de los artículos 45 de la Decisión 486 -el cual otorga la facultad al examinador para emitir los exámenes de fondo-, 35 y 59 del Código Contencioso Administrativo que señala que: *"habiéndose dado oportunidad a los interesados para expresar sus opiniones, y con base en las pruebas e informes disponibles, se tomará la decisión que será motivada al menos en forma sumaria si afecta a particulares"* y *"(..) deberá proferirse la decisión definitiva. Esta se motivará en sus aspectos de hecho y de derecho, y en los de conveniencia si es el caso"*, respectivamente (El subrayado es nuestro).

En consecuencia, solicitamos a ese despacho pronunciarse en relación con todos los argumentos presentados en este memorial dando estricta aplicación al principio del debido proceso consagrado en el artículo 29 de la Constitución Nacional



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COLOMBIA

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ANEXO 1

Publicación previa de Beaucage y colaboradores publicada en la revista
Tetrahedron 49:1925 del año 1993

TETRAHEDRON REPORT NUMBER 329

The Functionalization of Oligonucleotides Via Phosphoramidite Derivatives

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(Received 27 October 1992)

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INTRODUCTION

The rapid growth of biotechnology has triggered an increasing demand for modified oligonucleotides dedicated mostly to diagnostic and therapeutic applications. The plethora of such applications has spurred the development of nucleoside and non-nucleoside phosphoramidite derivatives for the functionalization of specific oligonucleotides. While the major contribution of the phosphoramidite approach to the evolution of oligonucleotide synthesis has been addressed earlier,¹ the

1925

scope of the present Report encompasses the synthetic applications of a wide spectrum of phosphoramidite derivatives allowing the incorporation of fluorescent, lipophilic, intercalating, cross-linking, alkylating and DNA cleaving entities into oligonucleotides. Such derivatizations have been targeted to the 5'/3'-termini, to the internucleotide phosphodiester linkages, or to the nucleobases and will be discussed at length.

1. FUNCTIONALIZATION OF OLIGONUCLEOTIDES AT THE 5'-TERMINUS

1.1. Phosphorylation

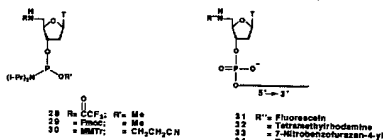
The automated phosphorylation of the 5'-OH function of oligonucleotides synthesized by the current solid-phase methods represents an attractive feature, as most biological applications require oligonucleotides carrying a terminal 5'-phosphate group. Engel² and others³⁻⁵ have addressed the issue by reporting the preparation of the phosphoramidites 1-3 (Table 1) for this specific application.

Table 1. Phosphoramidite Derivatives for the Functionalization of Oligonucleotides at the 5'-Terminus

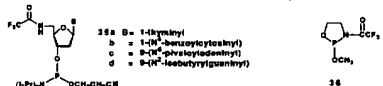
Compound	R	R'	R''	References
1a	HC(CH ₃)CH ₂ O-	-N(Pri)-H ₂	-CH ₂ CH ₂ CH ₂ N	2a,3a,4,5
b	CH ₃ O-	-N(Pri)-H ₂	-CH ₂ CH ₂ CH ₂ N	
2	CH ₂ O-	-N(Pri)-H ₂	-CH ₂ CH ₂ -C ₆ H ₄ -NO ₂	2a,b,5
3		-N(Pri)-H ₂	-CH ₂ CH ₂ -C ₆ H ₄ -NO ₂	2a
4		-N(Pri)-H ₂	-CH ₂ CH ₂ -C ₆ H ₄ -NO ₂	7
5		-N(Pri)-H ₂	-CH ₂ CH ₂ -C ₆ H ₄ -NO ₂	7
6	HC(CH ₃)CH ₂ O-	-N(Pri)-H ₂	-CH ₂ -C ₆ H ₄ -Cl	3a,b
7	H ₂ C=CHCH ₂ O-	-N(Pri)-H ₂	-CH ₂ CH=CH ₂	3b
8	Ph ₃ SiCH ₂ CH ₂ O-	-N(Pri)-H ₂	-CH ₂ CH ₂ CH ₂ N	1
9	(CH ₃) ₃ CO-	-N(Pri)-H ₂	-C(CH ₃) ₃	9,10
10	DMTrO(CH ₂) ₂ SO ₂ CH ₂ CH ₂ O-	-N(Pri)-H ₂	-CH ₂ CH ₂ CH ₂ N	11
11	CH ₃ O-	-N(Pri)-H ₂	-[(CH ₂) ₆ BTf] see 2,3,6	21,62

1.2. Activation, Aminoalkylation, and Ligand Incorporation.

The synthetic challenge involved with the derivatization of oligonucleotides with non-radioactive markers has led to the development of methods for the generation of suitable amino groups at the 5'-terminus of these oligonucleotides. One strategy in achieving this goal has been published by Smith *et al.*²¹ The approach involved the preparation of 5'-amino-5'-deoxythymine and its conversion into the deoxyribonucleoside phosphoramidites 28-30. These were incorporated at the 5'-end of oligonucleotides by standard solid-phase synthesis. Once deprotected, the 5'-aminated oligomers were reacted with either fluorescein isothiocyanate, tetramethyl rhodamine isothiocyanate, 4-fluoro-7-nitrobenzofuran or Texas Red sulfonyl chloride yielding the conjugates 31-34. These oligomers have been used as primers in DNA sequence analysis according to the Sanger approach.²² The hybridization of these primers to their respective templates was unaltered by the presence of the fluorophores. Furthermore, the facile detection of fluorescent DNA bands on polyacrylamide gels stressed the potential application of oligomers such as 31-34 in automated DNA sequence analysis.^{23a,b}



Synthetic routes for the preparation of the protected 5'-amino-2',5'-dideoxyribonucleoside-3'-O-phosphoramidites 35a-d have been developed.²⁴ Specifically, the phosphoramidite 35b has been applied to the synthesis of 5'-amino-4-(CCCATATGCG) which was, subsequently, reacted with an excess of the pentachlorophenyl ester of a tetraethylammonium salt. Co-crystals of the complex formed between the metallated self-complementary dodecamer and EcoRV, were obtained. These crystals were suitable for electron microscopy studies and X-ray crystallography.²⁴ Alternatively, the incorporation of the phosphoramidite 30 into oligonucleotides generated 5'-aminated oligomers which were condensed with the *N*-hydroxysuccinimide ester of biotinophenanthroline-nickel(II) complexes. These metallated oligomers can be detected by time-resolved fluorescence techniques.²⁵

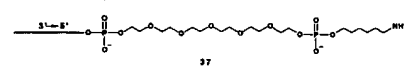


To avoid the preparation of 5'-amino-2',5'-dideoxynucleosides, the phosphoramidites 12,23,37 13a-b, 25-33 14,34,35 15a-b, 25,36 16,37 17a-b, 38-41 18,39 19,40 and 36,42 have been synthesized and applied to the aminoalkylation of synthetic oligodeoxynucleotides at the 5'-terminus. The reaction of deprotected 5'-aminoalkylated oligonucleotides with excess biotin *N*-hydroxysuccinimide ester^{36,37} or biotin *p*-nitrophenyl ester³⁸ produced the corresponding 5'-biotinylated oligomers in yields exceeding 85% with no detectable side reaction.³⁶ The purified 5'-biotinylated oligomers were used as hybridization probes for the detection of specific gene sequences.^{36,39} The strong and noncovalent interaction between biotin and avidin or streptavidin leading to the formation of a high affinity complex

($K_d \approx 10^{-15}$ M) that is easily detectable by enzymatic methods, has offered an alternative to conventional ³²P radiolabeling. In addition to hybridization probes, 5'-biotinylated oligonucleotides have found application as primers in experiments involving the Polymerase Chain Reaction (PCR).^{31,32,43,45} to facilitate the sequencing of amplified DNA.^{46,47}

The versatility of the phosphoramidite 20 has been exemplified by the solid-phase synthesis of an oligonucleotide carrying five primary amine functions attached to the 5'-terminus. The multiple incorporation of the phosphoramidite 20 occurred with a coupling efficiency of 95%.⁴⁸ Upon removal of the protecting groups with ammonium hydroxide, the aminoalkylated oligonucleotide was biotinylated and then hybridized to complementary M13mp18 single-stranded DNA immobilized on nitrocellulose filters. Colorimetric detection showed specific hybridization with a sensitivity of 0.5 ng.⁴⁸ In an attempt to facilitate the detection of PCR-amplified DNA sequences through an improved immobilization of sequence-specific oligonucleotide probes to nylon membranes, Zhang *et al.*⁴⁹ described the reaction of a hexamethylene glycol-based 8-cyanoethyl-*N,N*-diisopropylphosphoramidite with the 5'-OH of an oligonucleotide linked to a solid support. The resulting "spacer-armed" oligonucleotide was aminoalkylated with the trifluoroacetylated phosphoramidite 33b yielding, after deprotection, the oligomer 37. The purified aminoalkylated oligonucleotide was then efficiently coupled to nylon membranes activated with 1-cybi-3-(*N,N*-dimethylaminopropyl)carbodiimide hydrochloride.⁴⁹

Alternatively, 5'-aminoalkylated oligomers generated from the incorporation of the phosphoramidite 17b were activated with cyanuric chloride and then coupled to a nylon support covalently coated with poly(ethyleneimine). This support may also find application in the capture of specific RNA and DNA targets.⁵⁰



The conjugation of 5'-aminoalkylated oligomers to fluorophores has allowed the classification of *Fluorescein* strains by fluorescence microscopy on the basis of complementarity to partial 16S rRNA sequences.⁴⁴ In addition to enhancing the detection of specific DNA sequences by fluorescence amplification,⁵¹ fluorescently labelled oligodeoxynucleotides have served in fluorescence energy transfer experiments⁵² and as primers in automated DNA sequence analysis.^{45,53}

The phosphoramidite 17b has been applied to the attachment of a 5'-amino linker to a ribavirin phosphate homopolymer. The amino linker could permit the potential conjugation of the ribavirin oligomer to a monoclonal antibody.³⁸

5'-Aminoalkylated oligodeoxynucleotides resulting from the incorporation of the phosphoramidite 17b have also found application in the preparation of oligonucleotide-quinone conjugates.⁴¹ When hybridized to their complementary sequences, these conjugates were activated by cytochrome *c* reductase in the presence of NADH and triggered the formation of cross-linked strands by reductive alkylation.^{41b} These oligonucleotide conjugates may find interesting applications in antisense experiments. Similar applications are anticipated for those 5'-aminoalkylated oligomers which have been reacted with *N*-succinimidyl-4-(*N*-maleimidomethyl)pyridoxane-1-*o*-carboxylate or *N*-succinimidyl-4-(*p*-maleimidophenyl)butyrate and conjugated to interleukin-1B as a means to deliver such conjugates to cells harboring the interleukin-1 receptor.²⁹

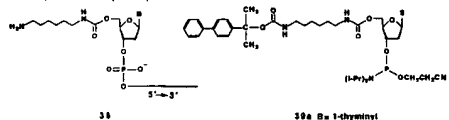
While the synthesis of oligodeoxynucleoside methylphosphonates from deoxyribonucleoside methylphosphonates has been reported in the mid-eighties, only recently has the 5'-aminoalkylation of a methylphosphonate oligomer been effected by the methylphosphoramidite 21 (Table 1).⁵¹ The deprotected oligomer was biotinylated and, after purification, was immobilized on streptavidin-agarose. Given the stability of the internucleotide methylphosphonate linkages to the nucleolytic activity of nucleases, the biotinyl-oligonucleoside methylphosphonate was employed in the capture of small U2

RNA/ribonucleoprotein complexes from nuclear extracts of cultured human cells.⁵¹

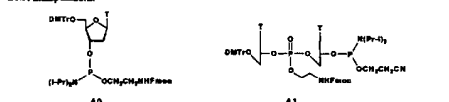
It is to be noted that solid-phase syntheses of 5'-aminoalkylated oligonucleotides and oligodeoxyribonucleotides containing a 5'-aminoalkylphosphate function have also been achieved by the phosphotriester approach.^{52,53}

Wachter *et al.*⁵⁴ further investigated the 5'-aminoalkylation of oligodeoxyribonucleotides. Their approach involved the reaction of the 5'-OH function of solid-phase linked oligomers with carbonyldiimidazole, followed by benzamylchloride/amine. The deprotected 5'-aminoalkylated deoxyribonucleotide 38 was biotinylated with the *N*-hydroxysuccinimide ester of biotin at pH 7.7. Interestingly, the carbamate linkage in 38 survived the usual basic conditions required for the removal of the base protecting groups.^{54,55} This methodology was also employed by Beck *et al.*⁵⁶ toward the development of a chemiluminescent detection of DNA and by others^{56,57} to evaluate the cellular uptake and trace the intracellular localization of fluorescently-labelled oligonucleotide-antibody/protein conjugates.

Along similar lines, De Vos *et al.*⁵⁵ described the synthesis of the deoxyribonucleotide phosphoramidite 39a-h to facilitate the incorporation of an aminoalkyl function at the 5'-end of oligonucleotides. The (*p*-biphenyl)isopropylcarbamoyl group was conveniently removed under the acidic conditions required for the cleavage of a DMT group. The resulting 5'-aminoalkylated oligomer was biotinylated while the oligonucleotide was still attached to the solid support or, alternatively, in solution phase after complete deprotection.⁵⁵



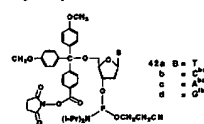
Seliger *et al.*⁵⁷ reported the synthesis of the deoxyribonucleotide phosphoramidite 40 and the dinucleotide phosphoramidite 41 as a means of inserting aminoalkyl functions in oligonucleotides. The monomeric phosphoramidite 40 led to coupling yields of ca. 99% whereas the coupling efficiency of the dimeric phosphoramidite 41 was ca. 87%. The purified aminoalkylated oligomers were biotinylated in yields up to 90% with the *N*-hydroxysuccinimide ester of biotin at pH 7.5.⁵⁷ A biotinylated hairpin loop was immobilized on an avidin-coated solid support to demonstrate the accessibility to double-stranded DNA manipulations.⁵⁷



A versatile linker for the modification of synthetic biomolecules has been reported by Glides *et al.*⁵⁸ The linker was incorporated into oligonucleotides via the phosphoramidites 42a-d. The solid-phase synthesis of a pentadecamer was performed and the oligomer was reacted with 1,6-diaminohexane followed by the *N*-hydroxysuccinimide ester of biotin. The crude biotinylated oligonucleotide was purified by binding to streptavidin-agarose and washing away non-biotinylated

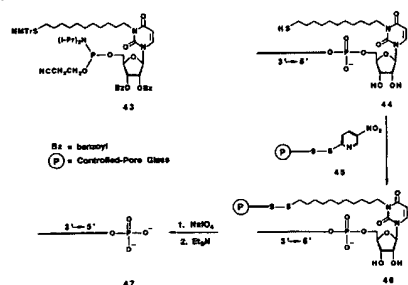
oligonucleotide sequences. Treatment of the agarose with aqueous acetic acid released the captured oligomer in 85% yield.⁵⁸

In addition to this specific application, the phosphoramidites 42a-d could potentially lead to the attachment of a variety of ligands at the 5'-end of oligonucleotides with the unique possibility of recovering the unmodified oligomers upon mild acidic conditions.

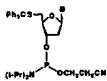


1.3. Thiolation, Mercaptoalkylation, and Ligand Incorporation

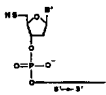
A strategy for solid-phase purification of oligonucleotides has also been proposed by Bammwirth and Wippler.⁵⁹ The approach relied on the incorporation of the ribonucleotide phosphoramidite 43 at the 5'-end of an oligonucleotide linked to a solid support. The derivatized oligonucleotide was then deprotected with concentrated ammonium hydroxide and treated with aqueous silver nitrate. The thiol-containing oligonucleotide (44) along with the underivatized oligonucleotide sequences were reacted with the activated disulfide support 45 at pH 7.0. The resulting support 46 was freed from underivatized sequences and the pure 5'-phosphorylated oligonucleotide 47 was released from 46 upon treatment with aqueous sodium metaperiodate and 5% aqueous triethylamine, respectively.⁵⁹ The yield of pure 47 has not, however, been reported.



The synthesis of protected 5'-mercapto-2',5'-dideoxycytidyl-3'-O-phosphoramidites (48a-d) and their application toward the preparation of 5'-mercapto-oligonucleotides have been reported.⁵⁹ Like 5'-amino-oligonucleotides, 5'-mercapto-oligonucleotides, represented by the structure 49, can react with a variety of electrophiles, heavy metals and fluorescent markers.⁶¹



48a: **Ba** 1-(4-phenyl)
b 1-(4-(2,4-dinitrophenyl))
c 1-(4-(2,4,6-trinitrophenyl))
d 1-(4-(2,4,6-trinitrophenyl))



49: **B'** = T, C, A or G

The mercaptoalkylated phosphoramidites (17a,c) and 22²⁰ALC₆/AA⁶¹ provided an alternate route to the synthesis of 5'-thiolated oligonucleotides. It has been shown that 5'-mercapto-oligonucleotides can be derivatized with tetramethylrhodamine iodoacetamide or fluorescein iodoacetamide to yield fluorescent M13 primers suitable for automated DNA sequencing.⁶²

Furthermore, Mori *et al.*⁶³ described the synthesis of the phosphoramidite 33 and its incorporation at the 5'-end of synthetic oligonucleotides. The 5'-mercaptoalkylated oligonucleotides were then coupled with *N*-6-acetylthymine affording fluorescent oligonucleotides which were used in cellular uptake experiments.⁶⁴

5'-Mercaptoalkylated oligodeoxynucleotides have also been applied to the synthesis of sequence-defined polypeptide-oligonucleotide conjugates.⁶⁵ Specific polypeptides were prepared by standard solid-phase methodology and were reacted with *N*-hydroxysuccinimide-5-maleimido benzotriazole. Purified maleimide-containing polypeptides were reacted with purified 5'-mercaptoalkylated oligonucleotides at pH 6-7 to yield the desired polypeptide-oligonucleotide conjugates. It is noteworthy that the conjugation of 5'-thiolated oligonucleotides with a maleimido-poly-L-histidine derivative⁶⁶ led to the formation of conjugates that were particularly effective in the selective inhibition of gene expression in intact cells.⁶⁷

Of interest, 5'-thiolated oligomers have been synthesized from the incorporation of a tritylated mercaptoacrylate glycol phosphoramidite derivative by solid-phase techniques.⁶⁸ These thiolated oligomers were converted with horseradish peroxidase derivatized with maleimide functions to generate valuable conjugates for the sequence-specific detection of PCR-amplified DNA.⁶⁹

The reaction of 5'-mercaptoalkylated oligodeoxynucleotides with Trestyl-activated Sepharose 4B or Eporo-activated Sepharose 6B resulted in the covalent attachment of specific oligomers to Sepharose. The addition of complementary unmodified oligodeoxynucleotides yielded DNA duplexes as affinity ligands for the isolation of specific DNA binding proteins.⁶³

It is important to mention that 5'-aminoalkylated oligonucleotides can easily be converted into oligonucleotides carrying a 5'-mercapto function upon reaction with dithiothreitol (*N*-succinimidylpropionate at pH 7.7 followed by treatment with dithiothreitol at pH 8.5).⁷ 5'-Mercaptoalkylated oligonucleotides were obtained in 70-80% yields. Similarly, Ganz *et al.*⁶⁹ reported the reaction of a 5'-aminoalkylated oligonucleotide with *N*-succinimidyl-3-(2-pyridyldithio)propionate⁷¹ instead of dithiothreitol (*N*-succinimidylpropionate). The activated disulfide-containing oligonucleotide produced the 5'-mercaptoalkylated oligonucleotide upon treatment with dithiothreitol (DTT) at pH 9.0.^{72,69} Furthermore, the reaction of a 5'-aminoalkylated oligomer with *N*-acetyl-DL-homocysteine thiolactone at pH 8.0⁷³ or with *N*-succinimidyl-5-acetyl thioacetate followed by hydrazylamine

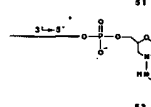
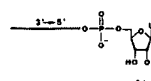
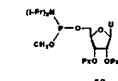
hydrochloride,⁷¹ generated the corresponding oligonucleotide carrying a free thiol group in yields greater than 95%. The thiolated oligomer was then derivatized with *N*-(4-dimethylaminobenzene-4'-iodobenzoyl)-5-*N*-methylmaleimide.^{70,71}

In this context, the reaction of the 5'-hydroxy function of an oligonucleotide linked to a solid support with, successively, carbonyl diimidazole and 5-DMTT-cytosine yielded, after deprotection and purification, an oligomer derivatized with both mercapto and carboxylic acid functionalities.⁷⁴ Thang and Chasagis⁷⁵ described the synthesis of oligonucleotides carrying a 5'-terminal thiophosphate monoester function. Their approach was based on the phosphorylation of the 5'-OH function of oligomers anchored to a solid support with the phosphoramidite 1a followed by sulfuration of the phosphite triester intermediate with elemental sulfur. The purified 5'-thiophosphorylated oligonucleotides were reacted with various electrophiles in aqueous solution, affording the efficient incorporation of photoactivatable cross-linkers,⁷⁶ intercalators and chelators^{77,78} in addition to fluorescent and radioactive markers.^{79,80} This strategy has been particularly useful for the introduction of prodrugs at the 5'-end of α- or β-oligodeoxynucleotides as a means to direct photoinduced reactions to the complementary DNA sequences.^{71,78} In a similar manner, the phosphoramidite 24 (Table I) enabled the incorporation of a 5'-benzylthiothiophosphate group into oligonucleotides and the subsequent functionalization of the thioester oligomers with a phenanthroline-copper chelate.⁷⁸

Recently, the coupling of a specific 5'-thiophosphorylated oligomer with *p*-azidophenacyl bromide was performed to demonstrate that the inhibition of SV40 DNA replication in CV-1 cells resulted from the formation of a triple helix between the viral DNA and the oligonucleotide carrying the cross-linking agent.⁷⁹ Pararibon-oligonucleotide conjugates have also been similarly prepared by reacting 5-(4-iodobenzyl)cytosine with oligodeoxynucleotides carrying a 5'-thiophosphate group.⁸⁰ The sequence-specific photoinduced cross-linking of the two DNA strands by the triple helix-forming pararibon-oligonucleotide conjugates occurred with efficiencies greater than 80%.^{80,81} Thus, pararibon-oligonucleotide conjugates can be designed to recognize and irreversibly modify specific DNA sequences.⁸⁰ Such modifications are expected to block replication and transcription by preventing the opening of the double helix.

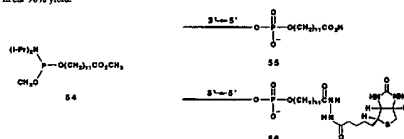
1.4. Oxidation, Esterification, and Ligand Incorporation

Alternate methods for linking non-radioactive labels to the 5'-end of synthetic oligodeoxynucleotides employing phosphoramidite intermediates have been reported. Specifically, the ribonucleoside phosphoramidite 50 was used to generate a (5'-5')-internucleotide link with an oligomer covalently bound to a solid support.⁸² The deprotected oligomer 51 was oxidized to 52 with sodium metaperiodate. The reaction of 52 with biotin hydrazide followed by reduction with sodium



borohydride afforded the biotinylated oligonucleotide 53 which has been applied to the detection of M13 DNA immobilized on nitrocellulose filters.³⁶

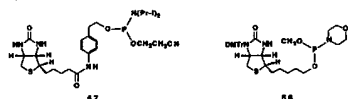
The phosphoramidite 54 has been recommended by Krcinsky *et al.*³⁷ for the phosphorylation of the 5'-OH function of an oligonucleotide anchored to a solid support. Iodine oxidation followed by complete deprotection yielded the oligomer 55 which upon condensation with biotin hydrazide in the presence of 1-(3-dimethylamino-propyl)-3-ethylcarbodiimide (DEC) afforded the biotinylated oligomer 56 in ca. 90% yield.



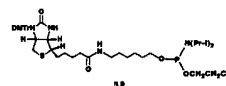
Noteworthy procedures for the 5'-biotinylation of synthetic oligonucleotides have also been reported by Kempe *et al.*,³⁸ Chollet *et al.*,³⁹ and Chu *et al.*³⁴

1.5. Direct Ligand Incorporation

1.5.1. Biotinyl Phosphoramidites. 5'-Biotinylated oligonucleotides were easily prepared by reacting the phosphoramidite 57 with the 5'-OH group of solid-phase bound oligomers under standard conditions.⁴⁰ The synthesis of 57 was accomplished by coupling biotin *N*-hydroxysuccinimide with *p*-(2-hydroxyethyl)aniline. The resulting ethanalamide was then phosphorylated with bis-(*N,N*-diisopropylamino)-6-cyanoethylphosphine and *N,N*-diisopropylammonium tetrazolide.⁴¹ Alternatively, the phosphorylation of a biotin derivative with chloro-(methoxy)-*N*-morpholinophosphine led to the biotinylated phosphoramidite 58.⁴² The coupling efficiency of 58 with the 5'-OH function of solid-phase bound oligonucleotides can potentially be measured during the removal of the *N*⁴-DMTr group of the biotin moiety.

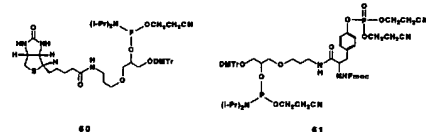


In a recent report, Pon described the preparation of [1-*N*-(4,4'-dimethoxybiphenyl)-biotinyl-6-aminoethyl]-6-cyanoethyl-*N,N*-diisopropylphosphoramidite (59) for the automated synthesis of 5'-biotinylated oligonucleotides.⁴³ The *N*⁴-DMTr group provided solubility and purification advantages while the 6-aminoethyl linker facilitated the interaction of biotin with large proteins. The biotinylated phosphoramidite 59 was incorporated into oligonucleotides with an efficiency of ca. 85-95%. The biotinylated oligonucleotides could be heated in concentrated ammonium hydroxide for 24 h at 30 °C without significant loss of biotin.⁴³

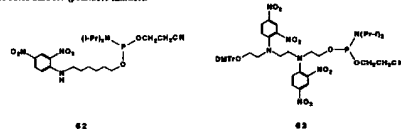


Phosphoramidite linkers have been designed for the direct insertion of biotin at multiple sites in synthetic oligonucleotides.⁴⁴ For example, the biotinyl phosphoramidite 60 was prepared in six steps from sulfolink and was repeatedly incorporated at the 5'-end of a universal M13 sequencing primer with a coupling efficiency of ca. 99%. Oligonucleotides having up to eight biotin residues were synthesized. Depending on the assay used, it was found that an oligomer having eight biotin residues was detected at a concentration 10 fold lower than that of a singly biotinylated oligomer.⁴⁵

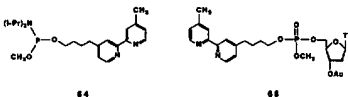
1.5.2. Phosphorylated Phosphoramidites. The phosphorylated phosphoramidite 61 was synthesized in nine steps from the *p*-toluenesulfonate salt of L-tyrosine benzyl ester and was also repeatedly inserted in the sequence of the universal M13 sequencing primer (vide supra) with a coupling efficiency of ca. 96%. Phosphorylated oligonucleotides enabled the detection of M13 DNA with a signal strength increasing with the number of phosphorylated residues.⁴⁶



1.5.3. 2,4-Dinitrophenyl Phosphoramidites. Will *et al.*⁴⁷ reported the synthesis of the phosphoramidites 62 and 63 for the single or multiple incorporation of dinitrophenyls (DNP) at the 5'-terminus of oligonucleotides. The insertion of 63 in oligonucleotides by solid-phase techniques occurred with a coupling efficiency of ca. 97% and afforded oligonucleotides having up to ten DNP groups.⁴⁸ Due to the sensitivity of the multiple DNP labels to concentrated ammonium hydroxide under the usual deprotection conditions, FOD-amidites⁴⁹ carrying base protecting groups that can be removed under milder conditions have been used in the synthesis of DNP-labelled oligonucleotides. The facile detection of such oligonucleotides was effected with anti-DNP monoclonal or polyclonal antibodies harboring suitable markers.⁵⁰



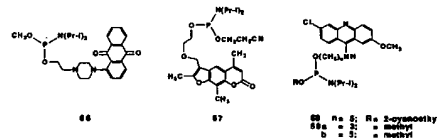
1.5.4. Bipyridinyl Phosphoramidite. The phosphoramidite **64** has recently been prepared by Modak *et al.*⁹¹ and employed in the phosphorylation of 5'-O-acetyl-2'-deoxythymidine. After oxidation and purification, the bipyridinyl phosphorimier **65** was complexed with Cu(II) and incubated with poly (A)₁₂₋₁₈ at 37 °C, pH 7.1, for 24 or 48 h. The RNA substrate was degraded to the extent of 21% within 48 h. Adenosine (2'-3') cyclic monophosphate was identified as the major hydrolysis product. No degradation of the RNA homopolymer was detected in the absence of Cu(II) under similar conditions.⁹¹ The phosphoramidite **64** may, therefore, be useful toward the incorporation of a bipyridinyl group at the 5'-end of oligonucleotides and the development of sequence-specific RNA cleaving reagents.



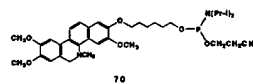
1.5.5. Anthraquinonyl Phosphoramidite. Mori *et al.*⁹² developed the phosphoramidite **66** for the derivatization of oligonucleotides at the 5'-end with an anthraquinone function. Anthraquinones are potential radical producing entities which do not necessarily require photoactivation or the presence of metal ions. Consequently, anthraquinone-linked oligonucleotides may find application in the delivery of radical-producing anti-cancer drugs to cellular nucleic acid targets.⁹²

1.5.6. Psoralenyl Phosphoramidite. The direct incorporation of psoralen derivatives at the 5'-terminus of oligodeoxynucleotides was effected by the phosphoramidite **67** via solid-phase methods.^{93,94} Psoralen derivatives can intercalate between the base pairs of double-stranded nucleic acids and may become covalently attached to thymine residues upon exposure to long-wavelength UV irradiation. Under these conditions, each psoralen-DNA adduct is generated through the formation of a cyclobutane ring. These adducts have been applied to the elucidation of nucleic acid structures and functions.^{95,96}

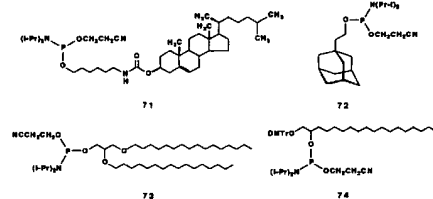
1.5.7. Acridinyl and Fagaroninyl Phosphoramidites. The synthesis of the acridinyl phosphoramidites **68**⁹⁷ and **69**^{98,99} and their introduction at the 5'-terminus of modified and unmodified oligodeoxynucleotides has been documented.^{74,97b} In addition to providing fluorescence to these oligonucleotides, the acridinyl group stabilized the formation of double- and triple-helical structures with complementary nucleic acid targets by virtue of its intercalating properties.^{74,75,97b,99,100} Such fluorescent oligonucleotides have been particularly useful in kinetic experiments pertaining to the cellular uptake of these biomolecules.⁹⁸



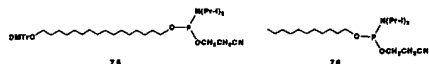
It has been demonstrated that the hexaphenanthridine alkaloid, fagarine chloride, isolated from *Fagopyrum esculentum* inhibited the reverse transcriptase activity of several viruses.¹⁰¹ These findings provided the impetus to determine whether antisense oligonucleotide-fagarine conjugates would enhance the inhibition of retroviral reverse transcriptases. Fagarine chloride was converted to the phosphoramidite **70** which was then reacted with the 5'-hydroxy function of oligonucleotides.¹⁰¹ Relative to unmodified oligomers, oligonucleotide-fagarine conjugates hybridized better to complementary RNA sequences presumably because of the intercalating abilities of the fagarine entity. The conjugates were ca. 3 times more potent than unmodified oligomers at inhibiting HIV reverse transcriptase but were ca. 3 times less inhibitory than fagarine chloride itself.¹⁰¹



1.5.8. Cholesteryl and Other Lipophilic Phosphoramidites. The application of oligonucleotides as potential therapeutics has been hampered by the low efficiency with which these polyanionic molecules permeated intact cells. It has been rationalized that the attachment of lipophilic groups known to interact with cell membranes, to oligonucleotides, may increase the therapeutic potency of these biopolymers.¹⁰² To test this rationale, the cholesteryl phosphoramidite **71** along with the adamantanylethyl phosphoramidite **72** and the polyalkyl phosphoramidites **73-75** were synthesized and incorporated at the 5'-terminus of an oligonucleotide phosphorothioate complementary to a region of the HIV-1 rev gene.^{102a} It has been shown, so far, that the denaturation temperatures of duplexes composed of the lipophilic oligonucleotide phosphorothioates and complementary DNA strands were identical to that of the duplex resulting from an equivalent underivatized phosphorothioate oligomer and its complementary DNA sequence.^{102a}

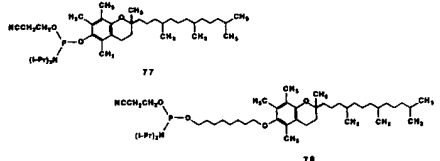


Along similar lines, Kabanov *et al.*^{102b} reported the synthesis of the phosphoramidite **76** as a means to introduce a hydrophobic group at the 5'-end of oligonucleotides. Targeting an underacylated oligonucleotide against a loop forming-site of the influenza A/PR8/34 viral RNA, considerably suppressed the development of the virus in permissive MDCK cells relative to that observed with unmodified oligonucleotides under identical conditions.^{102b} The attachment of hydrophobic groups to

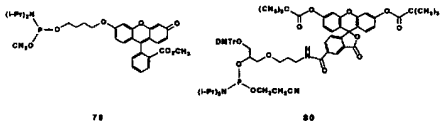


oligonucleotides has therefore demonstrated its efficacy at improving the biological activity of these biomolecules.

In an additional effort to impart lipophilicity to oligonucleotides and improve their cellular uptake and cellular distribution, the synthesis of the DL- α -tocopherol (Vitamin E) phosphoramidites 77 and 78 has been described.^{102d} Similar to the phosphoramidite 76, the incorporation of 77 and 78 into oligonucleotides occurred in yields greater than 95% according to HPLC analysis of the conjugates.^{102d} The anti-HIV activity of these modified oligonucleotides are currently being evaluated in cell cultures.



1.5.9. Fluoresceinyl Phosphoramidites. The preparation of fluorescently labeled oligonucleotides for automated dideoxy-DNA sequencing has been further simplified by Schubert *et al.*¹⁰³ who reported the facile integration of the fluoresceinyl phosphoramidite 79 into synthetic oligonucleotides. The phosphoramidite was prepared from the alkylation of fluorescein methyl ester with 4-chloro-1-(4,4'-dimethoxytrityloxy)butane followed by detritylation and phosphorylation with bis-(*N,N*-diisopropylamino)methoxyphosphine. The phosphoramidite 79 has been used in the solid-phase synthesis of various M13mp18 sequencing primers. Coupling yields were equivalent as those recorded with the standard deoxyribonucleoside phosphoramidites.^{103a}



The unlit step synthesis of the 5-carboxyfluoresceinyl phosphoramidite 80 from 5-carboxyfluorescein and sulfonal has recently been reported by Thelen *et al.*^{103b} It was argued that because of the steric hindrance imparted by the trityl group, the coupling of 80 at the 5'-OH function of

a solid-phase bound oligonucleotide necessitated a coupling time of 120 sec and proceeded with an efficiency of only 70%. The fluorophore and its linkage to the oligomer were stable during chain assembly and to the conditions required for complete oligonucleotide deprotection.^{103b} Like the bioinyl- and phosphoroyl phosphoramidites (80 and 81), the fluoresceinyl phosphoramidite 80 can be incorporated at any site within a DNA sequence or can serve in the derivatization of solid supports to yield oligonucleotides labelled at the 3'-terminus.

Aside from the 5'-OH group of oligonucleotides, the internucleotide phosphodiester function of these biomolecules could also be functionalized. This subject will be dealt with in the forthcoming section.

2. FUNCTIONALIZATION OF OLIGONUCLEOTIDES AT THE INTERNUCLEOTIDIC LINKAGES

Jäger *et al.*¹⁰⁴ demonstrated that aminoalkylated phosphoramidate oligonucleotides can be prepared by routine solid-phase synthesis using deoxyribonucleoside 5'-O-(*N,N*-diisopropylamino)methoxyphosphine monomers. For example, the oxidation of the dinucleoside phosphite triester 81 with iodine and 1,5-diaminopentane afforded the aminoalkylated phosphoramidate dimer 82 which was subsequently coupled to 6-chloro-9-(*p*-chlorophenyl)-2-methoxyacridine.¹⁰⁴



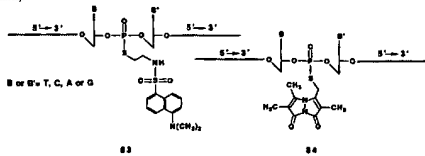
Due to the stability of the phosphoramidate linkage to the conditions used in solid-phase oligonucleotide synthesis by the phosphoramidite method, the combination of deoxyribonucleoside phosphoramidites and *H*-phosphonate monomers has allowed the insertion of cholesterol,¹⁰⁵ tetraazabipiperidine-*N*-oxyl^{106a} or phenaziny di-*N*-oxide^{106b} phosphoramidite links at predetermined sites. The validity of this approach has been previously demonstrated by Letsinger *et al.*¹⁰⁷ in a report delineating the preparation of cationic oligonucleotides.

In a similar manner, Agrawal *et al.*¹⁰⁸ and others¹⁰⁹ introduced either (*N*-1-trifluoroacetyl)hexanediamine arms at specific sites along the oligonucleotide chains¹⁰⁸ or ethylenediamine and hexamethylenediamine (ethers) at the 5'-end of oligonucleotides.¹⁰⁹ After complete deprotection, the aminoalkylated phosphoramidate oligomers were coupled to ligands such as fluorescein isothiocyanate, rhodamine isothiocyanate or activated esters of biotin. This approach provided multiple labelling capabilities and thereby increased sensitivity for diagnostic purposes.¹⁰⁹

The specific functionalization of oligonucleotides with two different reporter groups has been described by Agrawal and Zantek.¹¹⁰ Oligonucleotide assembly began with the incorporation of a nucleoside *H*-phosphonate monomer followed by oxidation with carbon tetrachloride and (*N*-1-trifluoroacetyl)hexanediamine to generate a phosphoramidate link. Standard deoxyribonucleoside phosphoramidite monomers were then employed until completion of the synthesis. The last oxidation step was effected with a sulfurylating reagent^{111,112} to generate a phosphorothioate link which, after oligonucleotide deprotection, was reacted with monobromobimane at pH 7.2. The aminoalkyl function of the purified bimane-labelled oligomer was then reacted with fluorescein isothiocyanate and the resulting bifunctionalized oligomer was isolated in 60% yield. Such bifunctionalized oligomers may find application in cellular uptake experiments or toward the targeting of specific messenger RNAs.

Numerous reporter groups have been introduced at specific sites in oligonucleotides carrying phosphorothioate dimer links.¹¹³ For example, the reaction of an oligonucleoside phosphorothioate with *N*-dansylaziridine afforded the dansylated phosphorothioate oligomer 83 in yields greater than 85%.¹¹⁴ Sulfurylating monobromobimane for *N*-dansylaziridine at pH 7 led to the complete formation

of the biamine-labelled phosphorothioate oligomer **84**.¹¹⁵ Interestingly, biamine labels have been incorporated at multiple sites into oligonucleotide phosphorothioates while the oligomers were still embedded in the polyacrylamide gel matrix after electrophoresis. Oligonucleotides containing two hundred to four hundred labels can be detected with the naked eye at the low femtomole level (10–20 fmol).^{115,116}

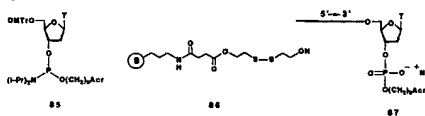


The nucleophilicity of phosphorothioate diesters has been exploited in the sequence determination of DNA and RNA.^{117,118} Specifically, the reaction of oligodeoxy- or oligoribonucleotides having randomly distributed phosphorothioate links with iodoethane or 2,3-epoxy-1-propanol generated hydrolytically labile phosphorothioate triesters which promoted the subsequent cleavage of the oligonucleotide chains.¹¹⁷ This approach represents an alternative to the current DNA sequencing procedures.

The following section will assess the recent developments toward the derivatization of oligonucleotides at the 3'-end.

3. FUNCTIONALIZATION OF OLIGONUCLEOTIDES AT THE 3'-TERMINUS

The incorporation of a fluorescent marker at the 3'-terminus of oligonucleotides was reported by Asseline and Thoenig.¹¹⁹ Their approach involved the phosphorylation of the solid support **85** with the fluorescent deoxyribonucleoside phosphoramidite **86** in the presence of 1H-tetrazole. The labelled oligomers (**87**) were subsequently released from the support by treatment with DTT under alkaline conditions. This methodology also permitted the multiple insertion of fluorescent labels within an oligonucleotide chain.



Ac(CH₂)₆OH 3-methoxy-5-chloro-8-*α*-hydroxypropylbenzylamine

(**8**) = Fraction **808**

In recent years, solid supports have been engineered to facilitate the derivatization of synthetic oligonucleotides with functional groups including masked 5'-phosphate,^{120,121} or masked 3'-hydroxyl.^{120,122}

terminal 3'-phosphate,^{120,121,122,123} 3'-thiophosphate,^{120,121,124} and 3'-aminoalkyl.^{120,125-129} These functional groups can then be further derivatized with various ligands to serve as tools in molecular biology experiments. Solid supports functionalized with acridine,¹³⁰ cholesterol,^{130a} DL- α -tocopherol¹³¹ or fluorescein¹³² have also been described for the synthesis of oligonucleotides bearing these ligands at the 3'-terminus.

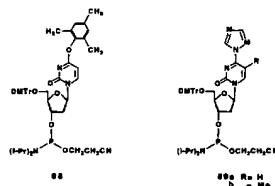
Aside from the internucleotide linkage and both terminal, the functionalization of oligonucleotides at the nucleobases has been thoroughly investigated. The results of these investigations will be summarized in the next section.

4. FUNCTIONALIZATION OF OLIGONUCLEOTIDES AT THE NUCLEOBASES

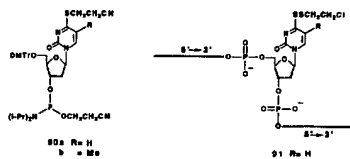
4.1. Derivatization of Activated Purines and Pyrimidines

It is well-known that the triazole function of 4-triazolopyrimidinone nucleosides are susceptible to nucleophilic displacement by amines.^{133,134} This feature has been exploited by Le Brun *et al.*¹³⁵ who inserted 4-triazolopyrimidinones at predetermined sites in oligonucleotides according to the phosphoramidite methodology. The triazole groups were then displaced with 1,10-diaminodecane and the corresponding aminoalkylated oligomers were subsequently biotinylated. The specificity of these biotinylated oligomers was tested in hybridization assays with plasmid DNA immobilized on nitrocellulose filters.¹³⁵ It was found that the stability of the resulting hybrids was not affected by biotinylated arms located at the 5'-end of the oligomers. Conversely, the stability of the hybrids was strongly impaired by the location of the biotinylated linkers within the oligonucleotide chains. The denaturation temperature (*T_m*) of these hybrids was at least 10 °C lower than that measured with unmodified oligomers.¹³⁵ Consequently, the 5'-terminus appears to be the ideal position for the incorporation of these modified nucleobases into oligonucleotides.

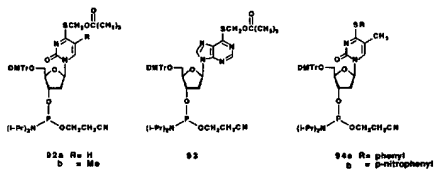
MacMillan and Verdine^{136a} reported the synthesis of functionally tethered oligodeoxynucleotides by incorporating the deoxyribonucleoside phosphoramidite **88** at a preselected site during oligonucleotide assembly. The use of deoxyribonucleoside phosphoramidites having base-labile protecting groups (*N*⁴-isobutyryl cytosine, *N*⁶-phenylacetyl adenine and *N*²-phenylacetyl guanine)¹³⁶ permitted rapid oligonucleotide deprotection with concentrated ammonium hydroxide without affecting the *O*⁴-arylated uracil residue. Treatment of the modified oligonucleotides with amines such as *N*-methylamine, 1,2-diaminoethane, 1,4-diaminobutane, glycine or bis-(aminoethyl)amine at 65 °C resulted in functionally tethered oligonucleotides with a conversion efficiency of 85–100%.^{136a} A similar postsynthetic generation of oligomers containing *O*⁴-alkylthymine, 5-methylcytosine, *N*⁶-(dimethylamino)-5-methylcytosine and 4-thiothymine residues stemmed from the insertion of the triazole phosphoramidite derivative **89b** in oligonucleotides.^{136c}



It has been recently shown that 4-thio-2'-deoxyuridine-containing oligonucleotides led to better attachment by postsynthetic S-alkylation or mixed disulfide formation.^{13a} The 4-thio-2'-deoxyuridine phosphoramidite 90a has been synthesized and incorporated into oligonucleotides by solid-phase synthesis.

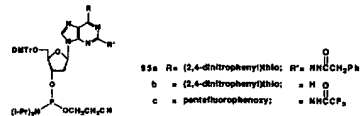


Quantitative 5'-deprotection of each oligonucleotide was effected by treatment of the solid support with 1 M DBU in acetonitrile. The modified oligodehydriplate was then released from the stationary phase and reacted with *N*-(2-chloroethylthio)phthalimide in phosphate buffer (pH 8) to yield the oligonucleotide 91 which can be further derivatized with selected reporter groups.^{13a} The phosphoramidites 90b and 92-94 have analogously been applied to the synthesis of oligonucleotides containing thioated nucleobases.^{13a-b}



To investigate the synthesis of oligonucleotides containing purine modified at C-6 with S-, N-, and O-derivatives, the 6-(2,4-dinitrophenyl)thiopurine phosphoramidites 95a-b were prepared in several steps from 3',5'-di-O-acetyl-N⁷-phenylacetyl-2'-deoxyguanosine and 3',5'-di-O-acetyl-2'-deoxyinosine, respectively.^{13a-b} The insertion of 95a into oligonucleotides occurred within 3 min with an efficiency of 98%. A dodecamer was synthesized and fractions of the solid support carrying the fully protected oligonucleotide were each treated with different reagents. For example, the reaction of one of these fractions with 10% mercaptoethanol in concentrated ammonium hydroxide at ambient temperature produced a deprotected dodecamer having a 6-thioguanine residue. Alternatively, the treatment of similar oligonucleotide fractions with either concentrated ammonium hydroxide containing *N*¹,*N*¹,*N*¹,*N*¹-tetramethylguanidine (65 mM) and *o*-nitrobenzaldehyde (75 mM), 40% aqueous

methylamine, methanol/DBU (9:1) or 0.5 M aqueous sodium hydroxide at 25 °C, afforded deprotected dodecamers having either 2,6-diaminopurine, 2-amino-6-methylaminopurine, 6-methylguanine or guanine residues.^{13a} This general procedure also allowed the introduction of NMR sensitive groups such as ¹⁹F, ¹³C, and ¹⁷O derived from [¹⁹F]-ammonia, [¹³C]-methanol and (¹⁷O)-water, at defined locations into synthetic oligonucleotides.^{13a} Like 95a-b, the incorporation of the phosphoramidite 96c into oligonucleotides enabled the postsynthetic modification of these molecules and yielded oligomers containing *O*⁶-methylguanines, 2,6-diaminopurines and their *N*⁶-aminoalkyl analogues or fluorescent 6-(*N,N*-dimethylaminopropylidene)-2-aminopurine residues.^{13c}



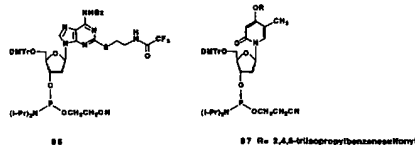
Along similar lines, *O*⁶-phenyl-2'-deoxyinosine was converted to a suitably protected phosphoramidite intermediate and inserted in a self-complementary dodecamer. The deprotected and purified oligomer was treated with either bis(aminoethyl)disulfide or bis(aminoethyl)disulfide yielding a DNA duplex having an aminoalkyl disulfide either at a specific location in consecutive base pairs on opposite strands. Upon reduction with dithiothreitol, the free thiol function formed an interstrand disulfide linkage during aerobic dialysis.¹⁷ Such cross-linked oligonucleotides should facilitate the study of enzyme-mediated unpairing during either transcription, replication or recombination.

The sequence-specific cross-linking of oligodeoxyribonucleotides has also been investigated by Webb *et al.*^{18,19} Their approach relied on the incorporation of the modified deoxyribonucleoside phosphoramidites 89a-b into oligonucleotides by solid-phase methods. The triacetyl nucleobases were converted to their corresponding ethyleneimine derivatives upon treatment with ethyleneimine. The resulting oligomers cross-linked with their complementary oligodeoxyribonucleotides albeit at a slow rate (*t*_{1/2} = 30 h at 22 °C). It was argued that the ethyleneimine function did not provide optimum "interstrand reach" to efficiently cross-link with target sequences.¹⁹

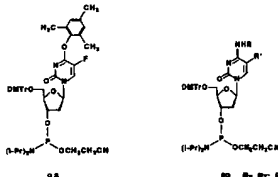
Kido *et al.*^{14b} described the emulsified synthesis of the aminoalkylated deoxyribonucleoside phosphoramidite 96 from 2-mercapto-2'-deoxyadenosine. The single insertion of 96 into oligonucleotides by solid-phase synthesis required a coupling time of 300 sec. Following deprotection and purification, the aminoalkylated oligomers were iodocrosslinked and characterized by enzymatic degradation to the nucleosides.^{14b} Oligonucleotides bearing a 2-(*N*-iodoacetylaminomethyl)thioadenosine residue were shown to crosslink and cleave complementary strands at adenines and guanines. It is believed that alkylation occurred at the N3 of adenine from the minor groove or with reactive groups of guanine in the major and minor grooves of the duplex. The efficiency of alkylation was low probably because of the free rotation of the reactive linker around the sulfur atom.^{14b} More rigid linkers may provide higher alkylation selectivity.

Of interest, the deoxyribonucleoside phosphoramidite 97 has been synthesized to enable the preparation of oligonucleotides containing 3-deazathymine residues.^{14c} It is anticipated that these modified oligonucleotides will be applied to experiments aimed at estimating the specificity of replication catalyzed by DNA polymerase I. Using nucleosides as models, it has been demonstrated

that 3-deaza-4-O-(2,4,6-trimethylphenylthio)thymine was easily converted to 3-deaza-thymine upon treatment with concentrated ammonium hydroxide at 55 °C.¹⁴¹



In an effort to determine the active-site nucleophile of DNA (cytosine-5)-methyltransferase *M.HaeIII*, an enzyme that recognizes dyad-symmetric sites (4(CGOC)) and carries out two sequential methylations of specific cytosine nucleobases, the construction of a suicide DNA duplex was undertaken.^{142a} One strand of the DNA duplex had a 5-methylcytosine residue at the expected *M.HaeIII* methylation site (3'-4(GGGTATCG-MedC)GGTACTC-5'). The complementary strand carried a 5-fluorocytosine nucleobase also at the expected *M.HaeIII* methylation site (5'-[CGCATAGG(5-FdC)CATGACG]-3'). These hexadecanucleotides were prepared by the incorporation of the 4-O-(2,4,6-trimethylphenyl)-5-fluoro-2'-deoxyuridine phosphoramidite 96 and the 5-methyl-2'-deoxyuridine phosphoramidite 99 by conventional solid-phase synthesis. Upon removal of the protecting groups with concentrated ammonium hydroxide, the 4-O-(2,4,6-trimethylphenyl)-5-fluorouracil residue was converted into a 5'-fluorocytosine nucleobase in quantitative yields.^{142b} The purified hexadecanucleotides were annealed and then mixed with *M.HaeIII* along with the cofactor *S*-adenosyl-L-methionine. The covalent DNA-*M.HaeIII* complex was purified and submitted to proteolysis. Sequencing of the proteolytic fragments revealed that Cyt71 of *M.HaeIII* formed a covalent bond with DNA during catalytic methyl transfer.^{142b}

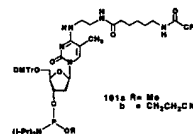


Schmidt *et al.*¹⁴³ independently described the preparation and the incorporation of the 5-fluoro-2'-deoxyuridine phosphoramidite 100a into synthetic oligonucleotides to investigate the catalytic mechanism whereby the DNA cytosine methyltransferase of *E. coli* K-12 methylates cytosine residues at

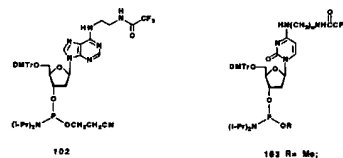
C-5 in a sequence-specific manner. It is important to mention that the 5-fluoro-2'-deoxyuridine phosphoramidite 100a can be inserted in oligodeoxynucleotides by solid-phase techniques without protecting its exocyclic amino function.¹⁴³

4.2. Ligand Incorporation from Aminoalkylated Purines and Pyrimidines

Urdén *et al.*¹⁴⁴ reported the synthesis of the deoxyribonucleoside phosphoramidites 101a-b from the corresponding 4-oxo-2-oxo-1,2,3,4-tetrahydropyrimidin-2-yl nucleosides. These derivatives led to the solid-phase synthesis of oligonucleotides having aminoalkyl functions at various locations within the oligonucleotide chains. Reporter groups such as biotin, fluorescein, and isoluminol were coupled to the purified aminoalkylated oligomers and their detection limits were assessed.^{144a-b} Furthermore, the reaction of the aminoalkylated oligomers with *p*-phenylene diisothiocyanate followed by incubation with either horseradish peroxidase or alkaline phosphatase afforded the corresponding enzyme-DNA conjugates.^{144c}

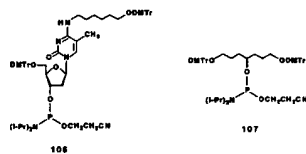


Cossick and Douglas¹⁴⁵ described the synthesis of the aminoalkylated deoxyribonucleoside phosphoramidite 102 and its incorporation into a dimer as a model to evaluate the formation of specific interstrand cross-links. It was argued that the hybridization of an oligonucleotide having a 4-N(2-aminoethyl)adenine nucleobase with a complementary target DNA or RNA sequence would position the nucleophilic aminoethyl function in the vicinity of a pyrimidine residue located on the complementary strand.¹⁴⁵ Activation of the 5,6-double bond of pyrimidines with bisulfites would trigger a nucleophilic attack at C-4 by the aminoethyl group and would result in the cross-linking of the two strands. No data supporting this concept have, as yet, been reported.

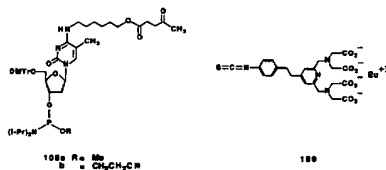


aminoalkylation of *N*⁶-*p*-toluenesulfonyl 2'-deoxyuridine derivatives with 1,6-diaminohexane.^{146a} The deoxyribonucleoside phosphoramidite 104 was incorporated at the 5'-end of an oligonucleotide anchored to a solid support and, after deprotection, the aminoalkylated oligonucleotide was biotinylated.^{146a} Along similar lines, the phosphoramidite 105 was prepared in a multistep synthesis from 3',5'-di-*O*-tert-butylidimethylsilyl-2'-deoxyuridine and mesitylenesulfonyl chloride.¹⁴⁷ The aminoalkylated phosphoramidite was repeatedly inserted at the 5'-terminus of a 2'-O-allyl oligoribonucleoside and the resulting aminoalkylated oligonucleoside was tetrahydropyranated in an attempt to facilitate the purification of RNA-protein complexes.^{147,148}

In an alternate approach, Barza *et al.*¹⁴⁹ described the synthesis of the deoxyribonucleoside phosphoramidite 106 from the corresponding 4-thiobenzoyl nucleoside and its incorporation into oligonucleotides toward the preparation of polybiotinylated probes. The synthesis of the phosphoramidite 107 was also reported for the same purposes. Incidentally, the coupling of 107 to solid-phase linked oligonucleotides occurred with an efficiency of 94-97%.¹⁴⁹



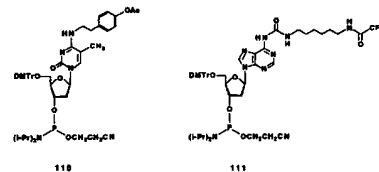
The synthesis of "branched" DNA molecules as amplification multimers in bioassays has been reported by Horu *et al.*¹⁵⁰ and by Chang *et al.*¹⁵¹ This type of branched DNA molecules are composed of two distinctive domains of oligonucleotide sequences. One domain consists of a target specific segment contiguous to an arrangement of branching nucleobases generated by the multiple incorporation of the levulinylated phosphoramidite 108a-b by standard solid-phase synthesis. The selective removal of the levulinyl groups unmasked hydroxy functions for the solid-phase synthesis of the second domain of oligonucleotides. Labelled probes were hybridized to this second set of DNA sequences thereby providing enhanced detection sensitivity.^{150,151}



In an effort to prepare Eu³⁺-labelled DNA probes, Send *et al.*¹⁵² inserted the deoxyribonucleoside phosphoramidite 103 fifty times at the 5'-end of a synthetic oligomer (50-mer). The purified oligonucleoside (100-mer) was then reacted with the Eu³⁺ chelate 109 at pH 10.5. The Eu³⁺-labelled probe permitted the detection of 200 picograms of phage lambda DNA by time-resolved fluorometry.¹⁵² It must be noted that a bathophenanthroline-Ru(II) complex derivatized as a 8-cyanoethyl-*N,N'*-dipropylphosphoramidite derivative, has also been applied to the solid-phase labelling of oligonucleotides.¹⁵³ Like Eu³⁺-labelled oligonucleotides, oligomers labelled with bathophenanthroline-Ru(II) complexes can be detected by time-resolved fluorometry.¹⁵³

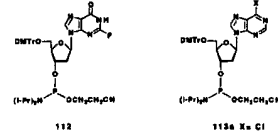
The deoxyribonucleoside phosphoramidite 110 was synthesized from 4-thiothymidine and tyramine¹⁵⁴ and inserted at specific locations within oligonucleotides by solid-phase synthesis. The tyramine residues of the deprotected oligonucleotides were radioiodinated by the standard "chloramine T" oxidation. The resulting oligomers have been useful as primers for PCR amplification and for the detection of viral DNA sequences.¹⁵⁴

The deoxyribonucleoside phosphoramidite 111 has been prepared from *N*⁶-phenacylcarbamoyl-2'-deoxyadenosine derivatives¹⁵⁵ and represents an addition to the repertoire of deoxyribonucleoside phosphoramidites dedicated to the preparation of aminoalkylated oligonucleotides.



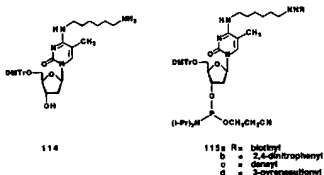
4.3. Ligand Incorporation via Halogenated Purines

Harris *et al.*¹⁵⁶ reported the synthesis and application of the halogenated deoxyribonucleoside phosphoramidites 112 and 113a-b in the solid-phase synthesis of modified oligonucleotides. Typically, the treatment of these solid-phase bound oligonucleotides with either D-(-)-phenylglycidol or with amines derived from (*z*)-*trans*-7,8-dihydro-*anti*-9,10-epoxy-4,9,10,11-tetrahydro-[*e*]-pyrene and (*z*)-*trans*-4,9-epithymine-*anti*-10,11-epoxy-4,9,10,11-tetrahydro-[*e*]-anthracene, under the recommended conditions, afforded the corresponding adducted oligomers.¹⁵⁶ This synthetic method is quite attractive for the preparation of oligonucleotides having structurally defined adducts in the quantities required for structural studies.

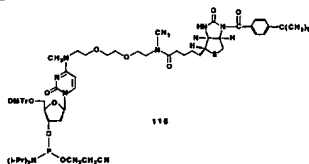


4.4. Modified Nucleotide Phosphoramidites and Direct Ligand Incorporation.

Protected deoxyribonucleoside phosphoramidites carrying various markers such as biotinyl, dimethylphenyl, dansyl and pyrenyl have been synthesized by Boger *et al.*¹³⁷ as a means to label synthetic oligonucleotides. Specifically, 114 was prepared in 85% yields from the aminoalkylation of the corresponding 4-thiodesoxycytidine with 1,6-diaminohexane. The reaction of 114 with either biotin *N*-hydroxysuccinimide ester, 1-fluoro-2,4-dinitrobenzoyl, dansyl chloride or pyrenesulfonyl chloride, followed by phosphorylation, yielded the labelled phosphoramidites 115a-d. These were activated with 1H-tetrazole and incorporated into oligonucleotides with high coupling yields (>97%) by standard solid-phase synthesis. Multiple labelling can be performed on the same oligonucleotide to provide increased detection sensitivity to *in situ* hybridization experiments.¹³⁷



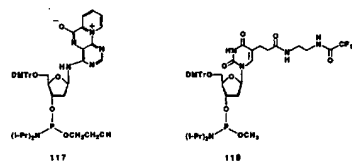
This approach has been further refined by the synthesis of the biotinylated deoxyribonucleoside phosphoramidite 116.¹³⁸ Relative to 115a, the phosphoramidite 116 carries a larger and more polar spacer arm to facilitate biological applications. Furthermore, 116 is readily soluble in acetonitrile and has been singly or repeatedly inserted at either the 3', 5' or at both termini of 2'-O-allyl oligonucleotides.¹³⁸ The deprotection of DMT-containing oligonucleotides with concentrated ammonium hydroxide under standard conditions, proceeded without significant loss of biotin. It has been shown that purified tetra-biotinylated oligomers prepared from the incorporation of 116 had reduced non-specific binding to streptavidin-agarose. The tandem incorporation of four biotin residues gave reproducibly superior binding when compared to oligonucleotides containing only one or two biotin residues.¹³⁸



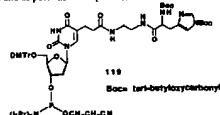
Milewicz *et al.*¹³⁹ reported the synthesis of the deoxythymine phosphoramidite 117 as a means to generate fluorescent oligonucleotides that could be applied to the non-isotopic detection of specific DNA sequences or the study of nucleic acid thermodynamics. Due to the sensitivity of the thymine residue to nucleophilic bases, nucleobase protecting groups different than the benzoyl and isobutryl groups had to be used to allow complete oligonucleotide deprotection with concentrated ammonium hydroxide at ambient temperature. The solid-phase synthesis of hexadecanucleotides having a single deoxythymine residue has been achieved and deoxythymine-containing oligomers were detected as strong fluorescent bands by polyacrylamide gel electrophoresis analysis.¹³⁹

4.5. Ligand Incorporation at C-5 of Pyrimidines.

The reaction of 5-chloromercaptyl-2-deoxyuridine with methyl acrylate led to the synthesis of the aminoalkylated deoxyribonucleoside phosphoramidite 118 and its subsequent incorporation into oligonucleotides.^{140a} Following deprotection and purification, the oligomers were then derivatized with either *N*-succinimidyl-1-pyrenebutyrate, 1-pyrenesulfonyl chloride, fluorescein-5-isothiocyanate, sulfoxsuccinimidyl-6-(biotinamido)hexanoate, *N*-hydroxysuccinimidyl-4-antirrhizumone-2-carboxylate or the *N*-hydroxysuccinimide ester of 4-carboxy-4'-methyl-1,2-bipyridine.^{140a} Incidentally, bipyridine-labelled oligomers reacted with $Ru(bpy)_3(1H_2O)_2^{+2}$ to yield oligonucleotides covalently attached to $Ru(bpy)_3^{+2}$ derivatives.^{140b} These modified oligonucleotides displayed normal hybridization behavior and ruthenium(II) has been known to effect light-induced DNA strand scission.^{140c}

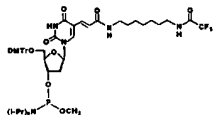


It is generally accepted that RNA hydrolysis, catalyzed by bovine pancreatic ribonuclease A, requires the participation of two imidazole groups located on histidine residues 12 and 119 in the active site.^{140d} In this context, Bashkin *et al.*^{140e} proposed the synthesis of ribonuclease mimics by combining the RNA hydrolysis activity of imidazole with the ability of oligonucleotides to bind to RNA with sequence specificity. The deoxyribonucleoside phosphoramidite 119 has been prepared and incorporated at one specific location into oligonucleotides via standard solid-phase phosphoramidite methodology. These modified oligonucleotides are now being evaluated as antisense molecules in the control of gene expression and as potential therapeutics.

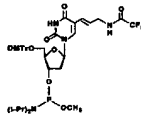


The synthesis of the aminoalkylated deoxyribonucleoside phosphoramidite 120 and its insertion in synthetic oligonucleotides has been reported by Jablonick *et al.*¹⁶³ and others.¹⁶⁷ These modified oligomers were then reacted with di-(*N*-succinimidyl)suberate and coupled with alkaline phosphatase.^{163,162} The resulting conjugates efficiently hybridized to complementary target sequences and retained the full enzymatic activity associated with the alkaline phosphatase entity. Hybrids were detected with a dye precipitation assay at a level of 10^6 molecules (2×10^{-18} M) of target DNA. Purified oligonucleotides carrying two aminoalkylated nucleotides could alternatively be reacted with fluorescein isothiocyanate and used as primers in DNA sequencing according to Brumbaugh *et al.*¹⁶³

It is to be noted that the incorporation of the phosphoramidite 121 into oligonucleotides during solid-phase synthesis also produced aminoalkylated oligomers which, upon deprotection, were suitable for reaction with the *N*-hydroxysuccinimide ester of *N*-biotinyl-5-aminocaproic acid.¹⁶⁴ Following purification, the biotinylated oligonucleotides were hybridized to target DNA immobilized on microtiter plates and were detected with a streptavidin-biotinylated horseradish peroxidase complex. Oligonucleotides carrying biotin labels near or at the end of the sequences were more effective probes than those oligomers bearing internal biotin labels.¹⁶⁴

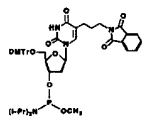


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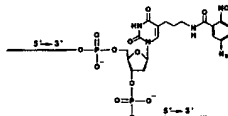


121

In a strategy to investigate protein-DNA interactions Gibson and Benkovic¹⁶⁵ described the synthesis of the deoxyribonucleoside phosphoramidite 122 from 5-iododeoxyuridine and its integration at the terminus of two nucleotides. The deprotected oligomers were treated with the cross-linker (*N*-hydroxysuccinimidyl)-5-azido-2-nitrobenzoate yielding the adduct 123. Oligomers carrying the photolabile group adjacent to the 3'-end have served as primers for template-directed DNA synthesis with either the Klenow fragment of *E. coli* DNA polymerase I, bacteriophage T4 DNA polymerase, or AMV reverse transcriptase. Irradiation of the primer elongation mixture with 302 nm light produced covalent complexes between DNA and the polymerases. Cross-linking with AMV reverse transcriptase was shown to occur predominantly in the "B-subunit".¹⁶⁵



122



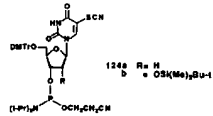
123

Along similar lines, the incorporation of the deoxyribonucleoside phosphoramidite 122 into oligomers has been achieved as a means to generate DNA duplexes biotinylated at specific locations and determine the structural requirements for the exonuclease and polymerase activities of prokaryotic and phage DNA polymerases.¹⁶⁶ It was demonstrated that, in the presence of avidin, the exonuclease and polymerase activities of the Klenow fragment required the primer termini to be at least fifteen and six base pairs downstream from the biotin-avidin complex, respectively. These results showed that the polymerase and exonuclease sites of the Klenow fragment were physically distinct in solution and displayed different substrate structural requirements for activity.¹⁶⁶

The insertion of 122 in oligonucleotides also allowed the derivatization of aminoalkylated oligomers with many or dantyl chlorides.^{166,167} Annealing the fluorescent oligomers with complementary oligonucleotides generated duplexes exhibiting fluorescence emissions that increased in intensity upon binding with the Klenow fragment of DNA Polymerase I. By varying the position of the fluorescent label within the DNA duplexes and observing fluorescence emissions, strong enzyme-DNA contact points were identified.¹⁶⁸

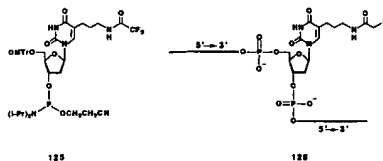
Bradley and Hama¹⁶⁹ described the synthesis of 5-thioisopurano-2'-deoxyuridine and uridine phosphoramidites (124a-b) for the functionalization of oligonucleotides without altering Watson-Crick base-pairing. Modified oligonucleotides resulting from the incorporation of 124a were deprotected under standard conditions without affecting the 5-thioisopurano function. Upon treatment with DTT at 55 °C, the thioisopurano group was reduced to a 5-mercapto function which was then reacted with either *p*-azidobenzoyl bromide or 5-iodoacetamidofluorescein to yield functionalized oligonucleotides suitable for the study of protein-nucleic acid interactions.¹⁶⁹ It must be understood that a variety of functional groups could also be attached to such modified oligonucleotides.

In a different context, dodecamers containing 4-biotinacetylthymine residues and the recognition site d(GATATC) of the endonuclease *EcoRV* or *EcoRV* methyltransferase were found to photochemically cross-link to either enzymes.¹⁶⁹

124a R = H
124b R = OSi(Me)2Bu-t

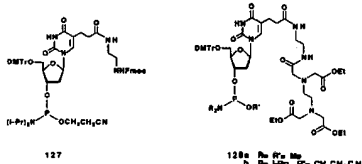
The protected aminoalkylphosphoramidite 125 was synthesized by Meyer *et al.*¹⁶⁷ and applied to the solid-phase synthesis of an oligonucleotide carrying an internal 5-(3-aminopropyl)uracil residue. The deprotected oligomer was iodocoupled with *N*-hydroxysuccinimidyl iodocoumarate affording 126. The incubation of 126 with its complementary DNA target generated observable interstrand cross-linking within 48 h at 37 °C. It was shown that the cross-linking reaction occurred by alkylation of a guanine residue of the complementary strand. Heating the DNA duplex at 51 °C resulted in the depurination of the alkylated guanine residue and scission of the target strand at the depurination site.¹⁶⁷ In addition, the modified oligonucleotide selectively alkylated the target strand without random alkylation of non-complementary nucleic acids. This feature is quite attractive for the application of these oligonucleotides as chemotherapeutic agents.

Similarly, Fovais *et al.*¹⁶⁷ reported the synthesis of the deoxyribonucleoside phosphoramidite 127 and its incorporation at the 5'-end of an oligonucleotide by solid-phase synthesis. The deprotected and modified oligonucleotide was treated with *N*-hydroxysuccinimidyl bromocoumarate to yield the corresponding *N*-bromocoumarylated oligodeoxyribonucleoside. This oligomer hybridized to adjacent



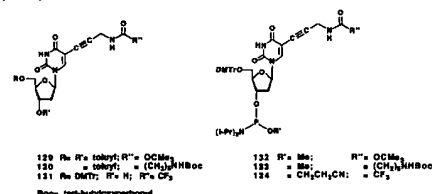
inverted purine tracts on double-helical DNA by triple-helix formation and alkylated single guanine residues on opposite strands at 37 °C (pH 7.4). After depurination, double-strand cleavage at a single site within plasmid DNA occurred in yields greater than 85%.¹²⁵ This methodology may become a valuable tool for the sequence-specific cleavage of large DNA molecules.

In their efforts to develop sequence-specific DNA cleaving agents, Dreyer and Dervan¹²⁶ reported the synthesis of a DNA hybridization probe carrying an EDTA-functionalized derivative of thymidine. In the presence of Fe(II), O₂ and DTT the modified probe led to the cleavage of a complementary single-stranded DNA segment.^{126a} The EDTA-functionalized deoxyribonucleoside was incorporated into the hybridization probe via the phosphoramidite 128a during routine solid-phase synthesis.^{126a,b} The phosphoramidites 128a-b were similarly inserted in triple-helix forming oligodeoxynucleotides which mediated the single site-specific cleavage of double-helical DNA *in vitro*.^{126c,d}



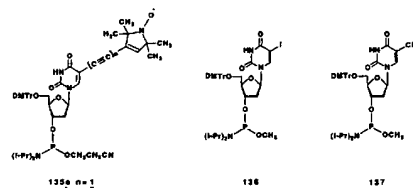
The derivatization of deoxyuridines at C-5 with masked primary aliphatic amino groups has also been exploited by Crickbank *et al.*¹²⁷ and Hananishidz *et al.*¹²⁸ Their strategies involved the reaction of a suitably protected 5-iododeoxyuridine with a *N*-blocked propargylamine, bis-(triphenylphosphine)palladium chloride, and cuprous iodide to yield the corresponding alkynyl nucleoside 129¹²⁷ or 131.¹²⁸ Additionally, the alkynyl nucleoside 130 was prepared by reacting the deprotected amino function of 129 with the *p*-nitrophenyl ester of 6-*tert*-butylpropargylcarbamoylaminobenzoic acid and DBU.¹²⁷ The alkynyl nucleosides 129-131 were converted to their corresponding phosphoramidites (132-134) and inserted in oligonucleotides by standard solid-phase synthesis.^{129,130} Following the removal of the phosphate protecting groups, the support-linked oligomers were treated with trifluoroacetic acid/ethanedithiol (9:1) for 5 min to cleave the *tert*-butylpropargyl protecting group(s) without extensive depurination.¹³⁰ The purified aminoalkylated oligonucleotides were

conjugated to fluorescein^{129,130} and the resulting conjugates were hybridized with complementary polyadenylated mRNAs.¹³⁰ Probes having short linker arms (derived from 132) did not hybridize as efficiently as the probes carrying larger linker arms (derived from 133). Furthermore, 5'-singly labeled probes hybridized better than the multilabeled ones.¹³⁰



Boch *tert*-butylpropargylcarbamoyl

Using a similar synthetic methodology, Speltzstein *et al.*¹³¹ prepared the deoxyribonucleoside phosphoramidite 135a for the site-specific incorporation of a paramagnetic probe into a self-complementary oligonucleotide. The bulky and hydrophobic nitrobenzyl moiety did not induce disruption of DNA secondary structures and was sufficiently constrained to permit the correlation of its motion with that of the carrier DNA. However, the length of the alkynyl arm (from the incorporation of 135b) affected the rotation of the nitrobenzyl moiety about the alkynyl axis and should be taken into consideration when monitoring sequence-dependent structural features.¹³² These probes may, nonetheless, be useful in investigations pertaining to the dynamics of unusual DNA structures and/or sequences of biological relevance.



The study of macromolecular structures can be performed by X-ray scattering methods both in solution and in the solid state. The presence of a heavy atom label facilitates the elucidation of the geometric and dynamic structure of the molecule. In this context, Sheardy and Seeman¹³³ reported the

4.6. Ligand Incorporation at C-8 of Purines

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The derivatization of oligonucleotides, particularly, at the internucleotidic phosphodiester linkages or at the nucleobases has inherent limitations. Functional groups attached to these entities may interfere with base pairing and/or stacking interactions. The functionalization of oligonucleotides

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CONCLUDING REMARKS

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REIVINDICACIONES

1. ARN de estructura de cadena doble con por lo menos un extremo romo, en donde cuyo por lo menos extremo romo además comprende una modificación química, en donde dicha modificación química es hidroxipropilfosfodiéster.

2. El ARN de estructura de cadena doble de acuerdo con la reivindicación 1, caracterizado porque ambas cadenas comprenden extremos 3' que comprenden una modificación química, en donde dicha modificación química es hidroxipropilfosfodiéster.

3. El ARN de estructura de cadena doble de acuerdo con la reivindicación 1, caracterizado porque comprende dos extremos romos.

4. El ARN de estructura de cadena doble de acuerdo con la reivindicación 1, caracterizado porque el ARN de estructura de cadena doble comprende una cadena de 19 pares base de ribonucleótidos contiguos.

5. Una composición farmacéutica caracterizada porque comprende una cantidad efectiva del ARN de estructura de cadena doble de acuerdo con las reivindicaciones 1 a 4.

6. Un método *in vitro* para identificar y/o caracterizar agentes farmacológicos que actúan en por lo menos una proteína objetivo, caracterizado porque dicho método comprende los pasos de:

-poner en contacto una célula eucariótica capaz de expresar por lo menos un gen endógeno que codifica la proteína(s) de interés con:

(a) por lo menos una molécula de ARNds de acuerdo con las reivindicaciones 1 a 5, la cual es capaz de inhibir la expresión del gen ó genes que codifican la proteína o las proteínas de interés; y

(b) una sustancia de prueba o una colección de sustancias de prueba en donde las propiedades farmacológicas de dicha sustancia de prueba o dicha colección van a ser identificadas y/o caracterizadas.