

ABSTRACT

Title of Document: SELECTIVE DELIVERY OF A QUINONE
METHIDE PRECURSOR BY PEPTIDE
NUCLEIC ACIDS

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Quinone methides (QMs) are electrophilic intermediates that can be generated in vivo to alkylate DNA and function as anti-cancer drugs. Previously, DNA-QM conjugates have shown the ability to selectively deliver a QM to specific sequences of DNA. Peptide nucleic acids (PNAs) conjugates of QM are now being developed since PNA binds DNA with higher affinity than natural DNA. Synthesis of PNA oligomers and conjugation of the PNA to QM precursor are reported here.

Synthesis of peptides was used to study the optimum conditions for preparation of the ultimate peptide-PNA conjugate. Both peptides and peptide-PNA have been synthesized after optimizing solid-phase techniques. Conditions for coupling a quinone methide precursor (QMP) and peptide-PNA conjugates were also evaluated. 8-Amino-3,6-dioxaoctanoic acid that links PNA and QMP is essential for coupling. MOPS buffer containing the peptide-PNA and an acetonitrile/dimethylformamide

mixture containing QMP were combined for coupling. Finally, reactive QM derivative of peptide-PNA-QM was studied.

SELECTIVE DELIVERY OF QUINONE METHIDE PRECURSOR BY PEPTIDE
NUCLEIC ACIDS

By

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Professor Ashton Cropp

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Abbreviations

A - adenine

BPDE - 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene

Bzl - O-benzyl

C - cytosine

CHCA - α -cyano-4-hydroxycinnamic acid

DCC - N,N'-dicyclohexylcarbodiimide

DCHA - dicyclohexylamine

DCM - methylene chloride

DCU - dicyclohexylurea

DIPEA - N,N'-diisopropylethylamine

DMF - N,N-dimethylformamide

DNA - deoxyribonucleic acid

dRb - deoxybose

EDCI - 1-ethyl-3-(3'-dimethylaminopropyl)carbodiimide

ESI - electrospray

Fmoc - 9-flourenylmethoxycarbonyl

G - guanine

HATU - *O*-(7-aza-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate

HBTU - 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate

HCTU - 2-(6-chloro-1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium

hexafluorophosphate

HF - hydrofluoric acid

MALDI - matrix-assisted laser desorption/ionization

MBHA - 4-methylbenzhydramine hydrochloride

MES - 2-(N-morpholino)ethanesulfonic acid

Mini PEG - 8-amino-3,6-dioxaoctanoic acid

Mops - 3-(N-morpholino)propanesulfonic acid

MS - mass spectrum

Mts - N^α-Boc-N^ε-mesitylenesulfonyl

NMP - N-methylpyrrolidone

PNA - peptide nucleic acid

RNA - ribonucleic acid

RP-HPLC - reverse phase high performance liquid chromatography

QM - quinone methide

QMP - quinone methide precursor

SA - sinapinic acid

SPPS - solid-phase peptide synthesis

ssDNA - single strand DNA

T- thymine

TBDMSCl - tert-butyldimethylsilyl chloride

t-Boc - *t*-Butyloxycarbonyl

TEA - triethylamine

TFA - trifluoroacetic acid

TFMSA - trifluoromethanesulfonic acid

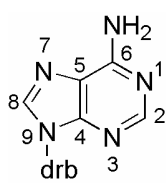
UV-Vis - ultra-violet visible spectroscopy

Z - benzyloxy-carbonyl

Chapter 1: Introduction

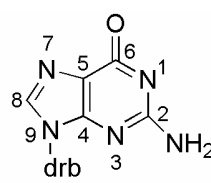
1.1 Importance of DNA alkylation

Double helix DNA consists of two twisted polymers with phosphoribose backbones (**Figure 1.1**).¹ Four bases, adenine, thymine, guanine and cytosine are linked to their backbones (**Scheme 1.1**).



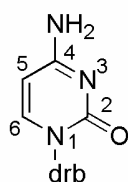
Adenosine

1.1



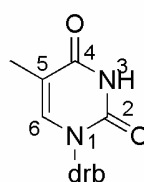
Guanosine

1.2



Cytosine

1.3



Thymidine

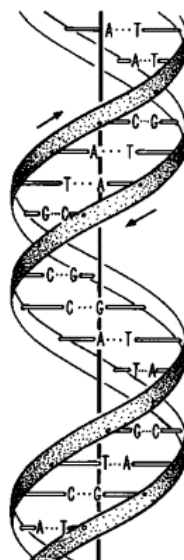
1.4

Scheme 1.1 Structures of DNA bases adenine, thymine, guanine and cytosine

DNA is constantly modified by intracellular and extracellular chemicals. The modification of DNA may result in covalent changes and cause severe diseases like cancer. Many methods being developed to DNA level which may cure cancer rely on DNA modification. DNA alkylation is one type of DNA modification.

Some natural products can alkylate DNA. These compounds are active in blocking the growth of tumor cells, but most of these compounds also show lack selectivity toward tumor cells. Finding more specific alkylating agents or selective delivery of

alkylation agents to cells is an effective method to reduce the side-effect of these compounds.

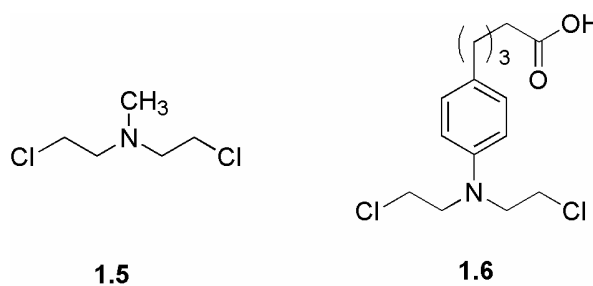


A schematic illustration of the double helix.

Figure 1.1 DNA double helix¹

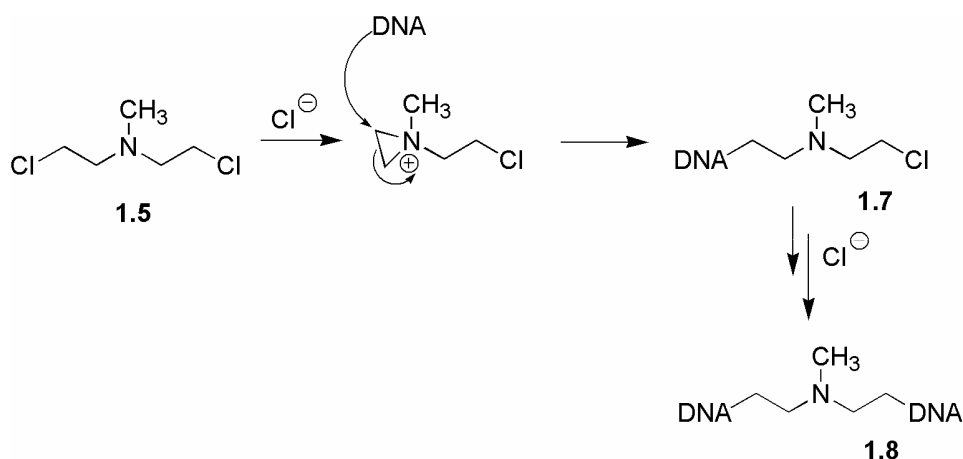
1.2 DNA alkylation agents

The nitrogen mustards are natural DNA alkylating agents and have been intensively studied as a class of anti-cancer drugs.² Bis(2-chloroethyl)methylamine (**1.5**) and chlorambucil (**1.6**) are two typical molecules of nitrogen mustards (**Scheme 1.2**).



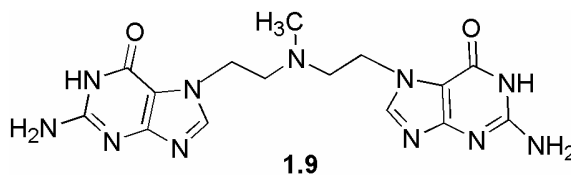
Scheme 1.2 Structure of nitrogen mustards

Bis(2-chloroethyl)methylamine shows high activity by inducing DNA interstrand cross-links (**Scheme 1.3**). The reaction is promoted by a near neighbor effect. The three-member ring intermediate is formed by losing a chloride and then attacked by nucleophiles from DNA. Mono-DNA adduct (**1.7**) is generated first. Bis-DNA adduct (**1.8**) can then be generated by a second loss of chloride.



Scheme 1.3 DNA alkylation by bis(2-chloroethyl)methylamine

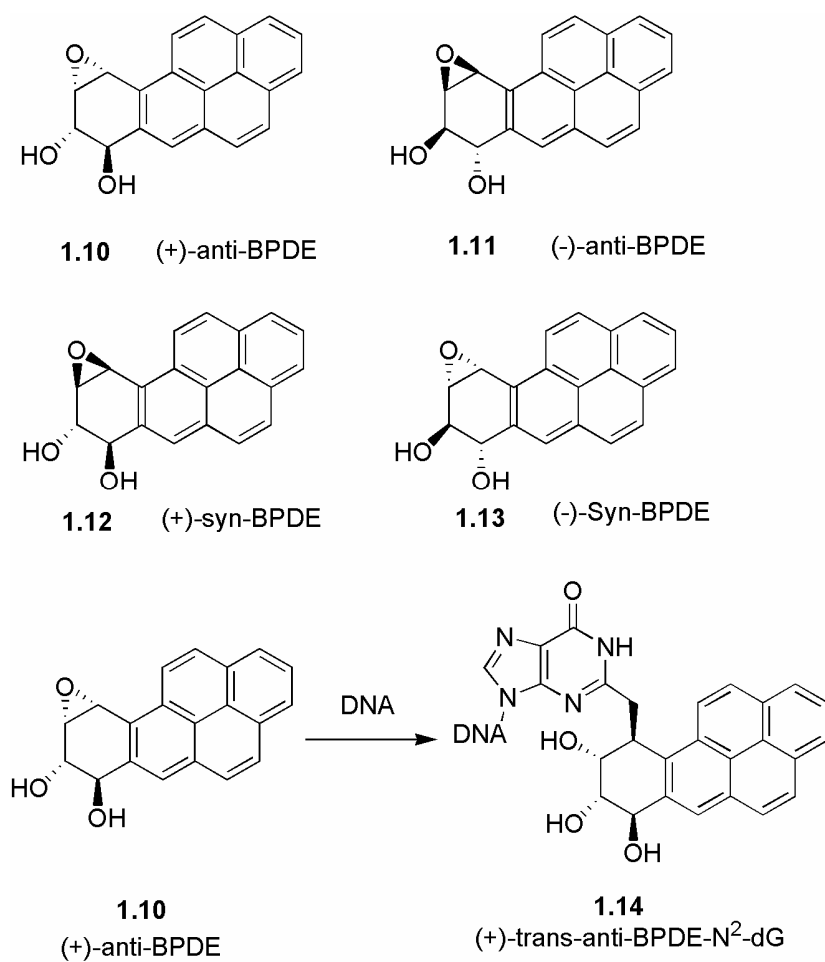
Compounds **1.9** in which two guanine residues are bridged via their respective N7 atoms by one mechlorethamine was isolated from mechlorethamine-treated yeast RNA.^{3, 4} The structure of **1.9** shows that the cellular target of DNA is alkylated primarily at the N7 position of guanine compared to lesser reaction at the N3 position of adenine (**Scheme 1.4**).



Scheme 1.4 N7 adduct of bis(2-chloroethyl)methylamine

Although DNA interstrand and DNA-protein crosslinks can be produced from bis(2-chloroethyl)methylamine, the DNA interstrand crosslink formed between the two complementary strands of DNA is believed to be the major cytotoxic lesion.⁵

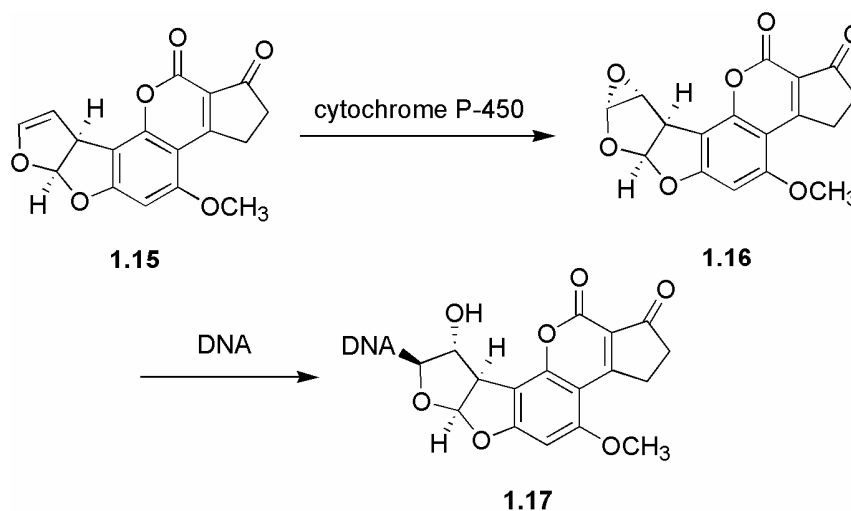
Epoxide-containing agents are another kind of DNA alkylating agents. Four stereoisomers 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene BPDE (**1.10** - **1.13**) generated in vivo shows the ability of epoxide-containing agents to alkylate DNA (**Scheme 1.5**).^{6,7}



Scheme 1.5 Epoxide-containing DNA alkylating agents

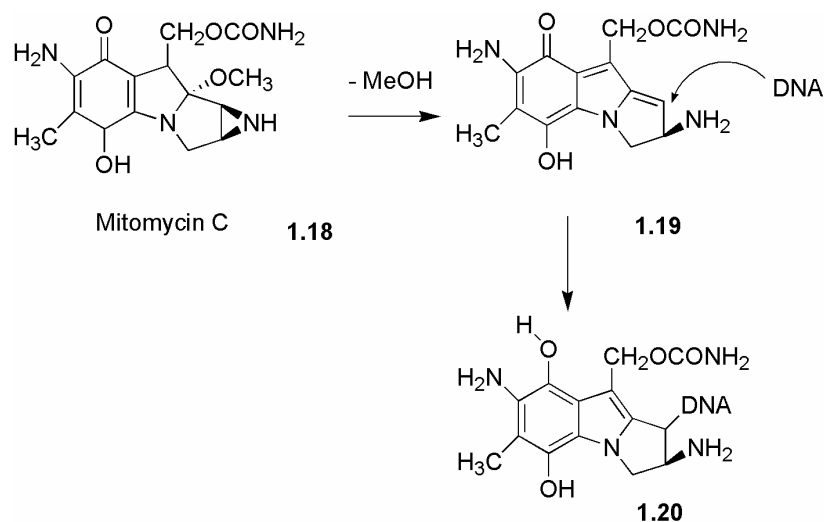
These compounds are highly active due to the benzylic epoxide. Several adduct are formed while N² of deoxyguanosine adduct is predominant.⁷ (+)-Anti-BPDE-DNA adduct is found to inhibit RNA⁸ and DNA⁹ polymerases.

The generation of epoxide via cytochrome P-450 in vivo is a typical theme in biological systems. The oxidation of aflatoxin (**1.15**) is a good example. The epoxide intermediate (**1.16**) formed via cytochrome P-450 oxidation will alkylate DNA. The DNA residues most susceptible to alkylation by these agents include dG N7, dA N⁶, and dC N3.¹⁰



Scheme 1.6 DNA alkylation by aflatoxin

Mitomycin C (**1.18**) is from a family of aziridine-containing natural products. It is used in cancer treatment by alkylating DNA (**Scheme 1.7**).¹¹ These effects are dependent on reductive bioactivation of mitomycin C. Generated by the reduction of mitomycin C, 2,7-diaminomitosenone (**1.19**) is the major intermediate. The intermediate **1.19** alkylates DNA in cells and forms an adduct at the N7 position of guanine.

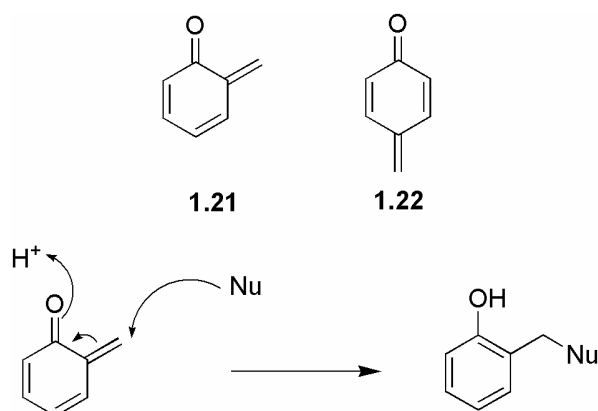


Scheme 1.7 Generation of DNA adduct from mitomycin C via QM

The intermediate **1.19** plays an important role in the alkylation process and it is also a kind of compounds called quinone methides (QMs).

1.3 Quinone Methides (QM)

QMs are a class of compounds that contain a methylene in place one of the oxygen atoms in quinone.^{12, 13} There are two major types of QMs: *o*-QM (**1.21**) and *p*-QM (**1.22**) (Scheme 1.8).

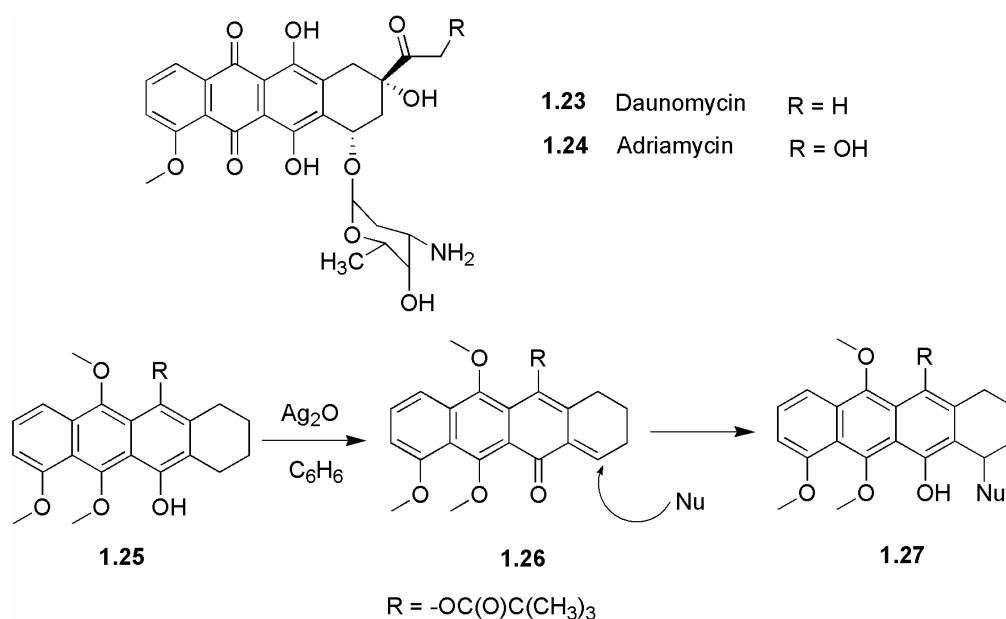


Scheme 1.8 Structure of quinone methide and its adductive product

QM is a strong electrophile and can be attacked by nucleophile from the methylene group, regenerating the aromatic ring. Due to its high reactivity, QM is usually generated in situ and used immediately. QMs are involved not only in the traditional organic synthesis, but also in a variety of biochemical reactions.¹⁴⁻¹⁷

QMs can be generated from thermal methods,¹⁸ UV light,^{19, 20} desilylation of silyl ethers by fluoride,²¹ oxidation by Ag₂O and PbO₂,^{22, 23} and biological methods.²⁴

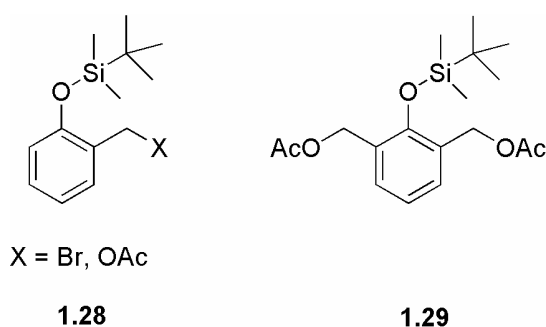
Anthracycline is an important class of anti-tumor antibiotics. Daunomycin (**1.23**) and adriamycin (**1.24**) are two of these anti-tumor structures (**Scheme 1.9**).²⁵ The mechanism of their high biological activity is proposed via QM mediates. In order to verify the idea, Ag₂O oxidation was used to convert the daunomycin analog (**1.25**) to generate QM intermediate (**1.26**). **1.26** is attacked by nucleophiles such as EtOH, H₂NPh and NaCN.²⁵ Then it is reasonable to think that QM is the essential intermediate in alkylating DNA. The biological activity of daunomycin and adriamycin may go through the QM intermediate.



Scheme 1.9 Daunomycin, adriamycin and their analog

The adriamycin analog (**1.25**) was synthesized to evaluate the function of QM intermediate during the reaction.

Desilylation of silyl ethers by fluoride is also extensively used to study the generation and react of QMs with DNA.²⁶⁻²⁹ Compounds **1.28** and **1.29** represent this type of quinone methide precursor (QMP) which are used to help understand the property of QM (**Scheme 1.10**).



Scheme 1.10 Models of silyl ether protected QMP

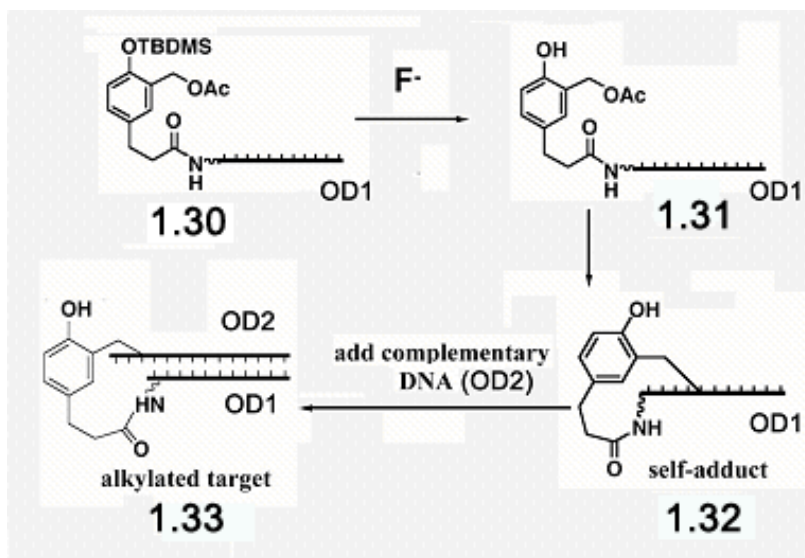
The hydroxyl group is protected by silyl ether and a good leaving group is linked to the benzylic position. The generation of QM is triggered by adding fluoride. This method generates QMs which can be used in situ quickly and effectively.

Since there are A, T, G and C nucleobases in DNA, lots of sites may function as target to alkylation. The Rokita group has studied the kinetic and thermodynamic property of the adducts from unsubstituted QMs and individual nucleobases as well as DNA sequences.²⁶ The QM intermediates often shows a selectivity for some nucleophiles of DNA.³⁰ The reactions between QM and weak nucleophiles such as N1, N² of dG and N3 of dC are irreversible, while the QM adducts generated with strong nucleophiles such as N1 of dA, N3 of dC and N7 of dG show reversibility. The kinetic products of QM alkylation can regenerate QM and extend the cellular activity.

The substituents on QMs affect the formation and stability of their nucleophilic adducts tremendously.³¹ When an electron-withdrawing group is present, its dC N3-QM adduct was stabilized. However, a related adduct with an electron-donating methyl group on the QM is very labile and regenerates its QM rapidly. By changing different electronics of the QM functional groups, the reversibility of QM-nucleobases adducts show them be the desired balance of liability and stability.

The lack of selectivity toward DNA sequences is a major problem for all the DNA alkylating agents. In order to increase the efficiency of QMs and reduce the side-effect in normal cells, selective delivery of QMP is an essential topic.

Rokita group also studied the selective delivery of QMP.²⁷ Selective alkylation of a chosen sequence of DNA can be accomplished by linking QMP to the complementary DNA.



Scheme 1.11 Selective delivery of QMP by DNA sequence²⁷

After QMP was coupled with a DNA sequence (**OD1**), the acetate derivative (**1.31**) was generated when fluoride was added to the conjugate (**1.30**). QM intermediate was

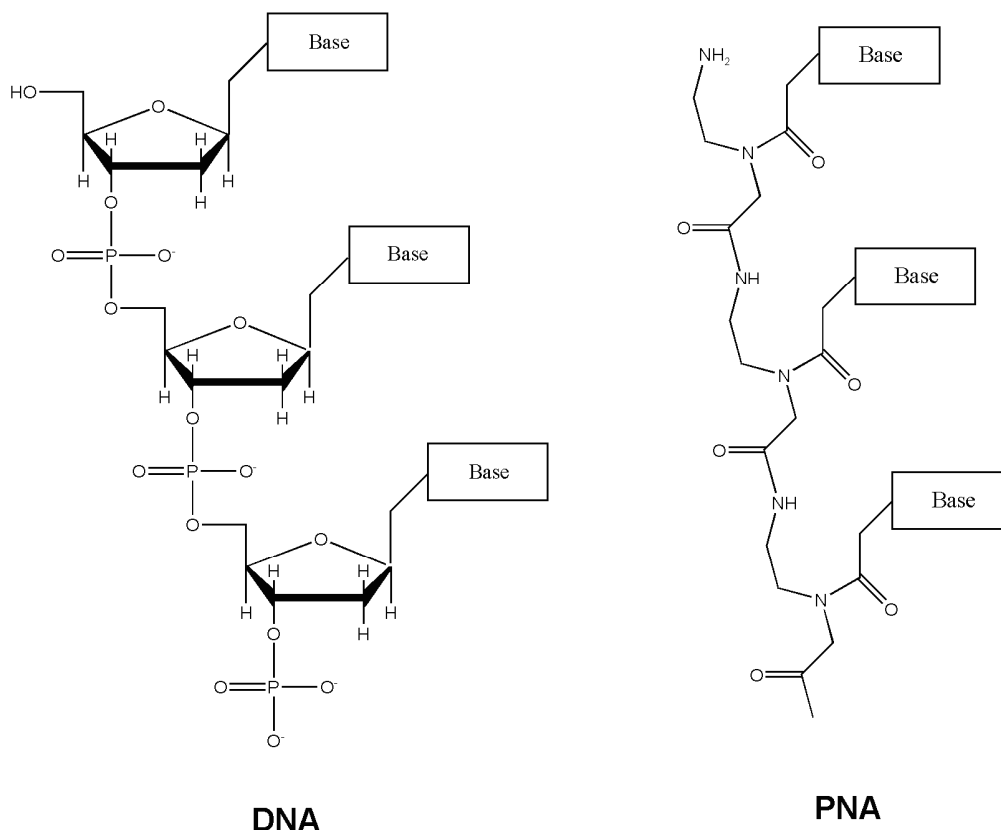
attacked by DNA and formed DNA-QM self-adduct (**1.32**). Due to the reversibility of the self-adduct, target DNA sequence (**OD2**) was alkylated (**1.33**) when the complementary DNA was mixed with **1.32** (**Scheme 1.11**).²⁷

However, the use of DNA as selective delivery agents has its limitation. The degradation of oligonucleotides caused by cellular nucleases and lack the membrane permeability makes other sequence directing agents important. Peptide Nucleic Acids (PNAs) are one of the choices gaining lots of interest recently.

1.4 PNAs Function as sequence specific delivery agents

First designed by scientists from Denmark,³² PNA is synthesized and used in binding with both DNA and RNA in a site-selective manner by Watson-Crick base-pairing. PNA is a chemical similar to DNA and RNA but differs in its “backbone” (**Scheme 1.12**). The backbone of PNAs is composed of repeating N-(2-aminoethyl)-glycine units. Through the methylene carbonyl bonds, the purine (A, G) and pyrimidine (C, T) bases are linked with the backbone. Several important properties of PNA make them a powerful tool in sensitive DNA diagnostics and gene therapeutics.

The original purpose of synthesizing PNA was to recognize double-stranded DNA. The backbone of PNA is neutral so PNA/DNA duplex is more stable than DNA/DNA.³³



Scheme 1.12 Backbones of DNA and PNA

PNA is also capable of inhibiting translation *in vivo* and may suitable as a DNA probe.^{34, 35} PNAs are shown to resist to nucleases and proteases *in vivo*.³³ If OD1 in Scheme 1.11 is replaced by PNA sequence, the PNA oligomers can selectively deliver QMP to cellular environment.

The structure of PNA monomers also makes them easily employed in synthesis of PNA oligomers. The PNA monomers have both amine terminus and carboxylic acid terminus. Because of the similarity of PNA components and amino acids, PNA can be easily synthesized by standard solid phase peptide synthesis (SPPS)^{36, 37} which is widely used in peptide synthesis.

First developed by Merrifield R.B. in early 1960s,³⁶ SPPS allows the synthesis of both natural occurring and artificially designed peptides. SPPS is much quicker than

classical synthesis since it avoids the complicate separation procedures that are associated with the liquid phase peptide synthesis.

After PNA oligomers are synthesized through SPPS, QMP can be coupled with PNA chain. Once the selective delivery agents are determined, it is important to find a target in cells.

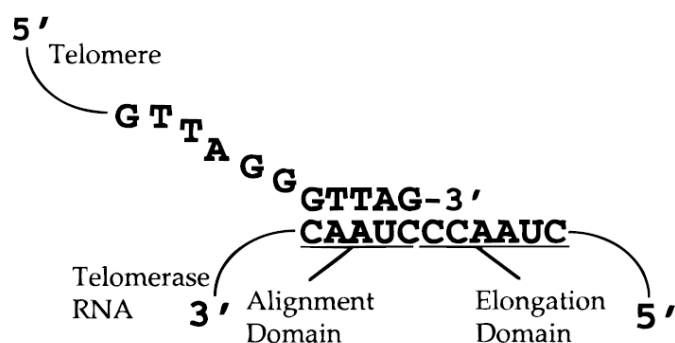
1.5 Function of telomerase and why choose telomerase as a target

Telomeres are highly repeated DNA-protein structures (5'-TTAGGG-3' in vertebrates) at the ends of linear chromosomes.³⁸ Human telomeres contain about 5-15 kilobases of a tandem repeat sequence. They distinguish the natural termini from random breaks and protect the loss of important genes in chromosomes. These structures also help to stabilize DNA during replication.³⁹ Telomeres keep shortening after each cell cycle.⁴⁰ Once the length of telomeres decreases to minimum threshold, the protective telomere caps become destabilized and result in replicative senescence or the death of cells.⁴¹

In some human cell types such as adult germline cells and stem cells, telomeres are not always shortening with the proliferation of cells due to the existence of telomerase.⁴² Telomerase is an enzyme that adds specific DNA sequence repeats ("TTAGGG" in all vertebrates) to the 3' end of DNA strands in the telomere regions.⁴³ In most normal cells, telomerase activity is almost undetectable.⁴⁴ While telomerase activity is found in most human tumors.⁴⁵ The length of the telomeres in tumor cells does not continuously shorten with the successive cell divisions because of the presence of active telomerase. Telomerase is then thought to be an essential

factor in the “immortal”⁴⁶ property of cells by adding the repeating sequences to the telomeres (**Scheme 1.13**).

Thereby, telomerase is a potential target for the anti-tumor drugs. The length of telomere in the cells will shorten corresponding to the cells divisions if the telomerase can be inhibited by a drug.



Scheme 1.13 Binding of the end of telomere by human telomerase⁴⁷

Several methods have been developed to deactivate telomerase.⁴⁸ Oligonucleotide-based therapeutics is just one of the potential methods to develop anti-cancer drugs by binding to the appropriate site inside the telomerase. There are two subunits in human telomerase: hTR and hTERT. The RNA hTR acts as a template for replication and the protein hTERT catalyzes nucleotide polymerization.³⁷ The hTR has 451 nucleotides, and nucleotides 45-65 (5'-CTAACCTAAC) provide an appropriate binding site for direct inhibition of telomerase activity. By using a peptide nucleic acid (PNA), 5'-GTTAGGGTTAG-3' is found to inhibit telomerase effectively.⁴⁷

Because of the use of QMPs and PNA oligomers, we will study the synthesis of PNA oligomers and peptide-PNA conjugates by SPPS. Optimal conditions for coupling peptide-PNA conjugates with QMP are also discussed in detail. Finally, possibility of the formation of self-adduct by peptide-PNA-QMP conjugates will be

examined. The synthesis of PNA oligomers and peptide-PNA-QMP self-adduct provides a new method to selective delivery of QMP to target RNA in cells.

Chapter 2: Synthesis of peptide-PNA conjugate

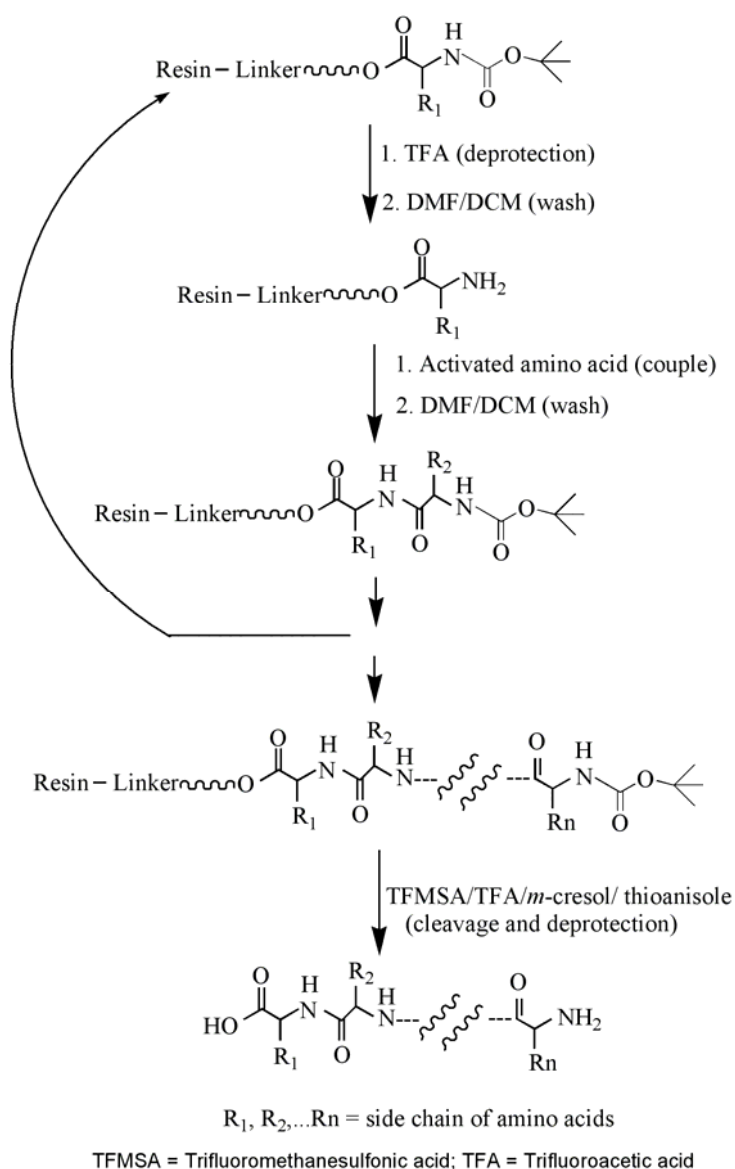
2.1 Introduction to Solid-Phase Peptide Synthesis (SPPS)

Natural peptides have multiple functions as hormones, cytokins and enzymes in living cells. The biosynthesis and function of peptides in cells are important to understand the cause of various diseases. In order to study the biological property and activity of peptides, a large amount of different length and types of peptides were synthesized. Liquid phase peptide synthesis is used in making short peptides. For long peptides, solid phase peptide synthesis (SPPS) is favored by eliminating the difficulty of purifying the products in every step. One of the best properties of SPPS is the easy separation of byproducts during synthesis. SPPS³⁶ has been a standard technique in construction of peptides effectively for almost 50 years.

Reaction was carried out in a fritted funnel inserted into a side arm flask through a single hole rubber septa (**Appendix**). A T-valve was at the bottom of the funnel. The side-arm of the T-valve was connected to a Schlenk line. The reaction is taken in the funnel by bubbling nitrogen through the Schlenk line. When the reaction is complete, the remaining starting material and byproducts are washed away by the appropriate solvents such as methylene chloride (DCM) or N,N-dimethylformamide (DMF) through a filter. The intermediate linked on the insoluble resin is the only species remaining in the funnel for next cycle.

The solid-phase process consists of several steps repeated in a cyclic fashion (**Scheme 2.1**). The activated carboxylic acid terminus of the first amino acid is

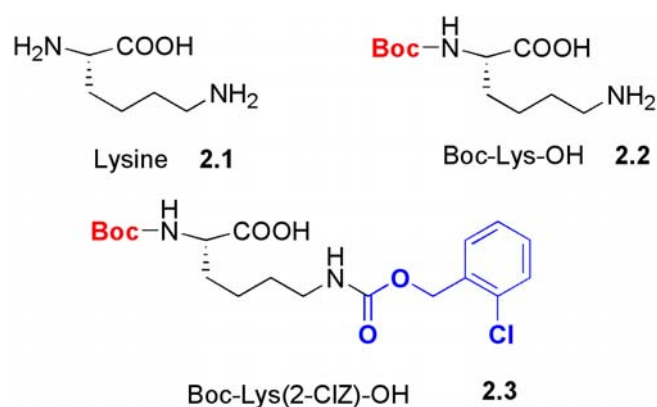
coupled with the free amine groups from the resin. The protected amine terminus is then deprotected by corresponding deprotection reagents. When the byproducts are washed away, the free amine group from the first amino acid is ready to react with the following amino acids. By repeating the coupling, deprotecting and washing procedures, a desired peptide chain is formed at the solid resin. Finally, removal of the peptide chain from the resin is achieved by swelling resin in cleavage solution.



Scheme 2.1 General procedure of SPPS in Boc Strategy

Several steps are important to improve the yields and decrease the byproducts.⁴⁹

First is the protecting group for the N-terminal of the amino acids. In order to avoid polymerization of the amino acid itself, the N-terminal of amino acids is protected before the coupling. The protecting reagents are called ‘temporary’ protecting group compared to ‘permanent’ protecting group which protects the possible amine groups at the side chain of amino acids. The temporary protecting groups are removed after the coupling in every cycle, while permanent protecting groups are not removed until the peptide is removed from the resin by cleavage solution. Use of ‘temporary’ and ‘permanent’ groups is shown in lysine for *t*-Butyloxycarbonyl (*t*-Boc)³⁶ SPPS (Scheme 2.2).

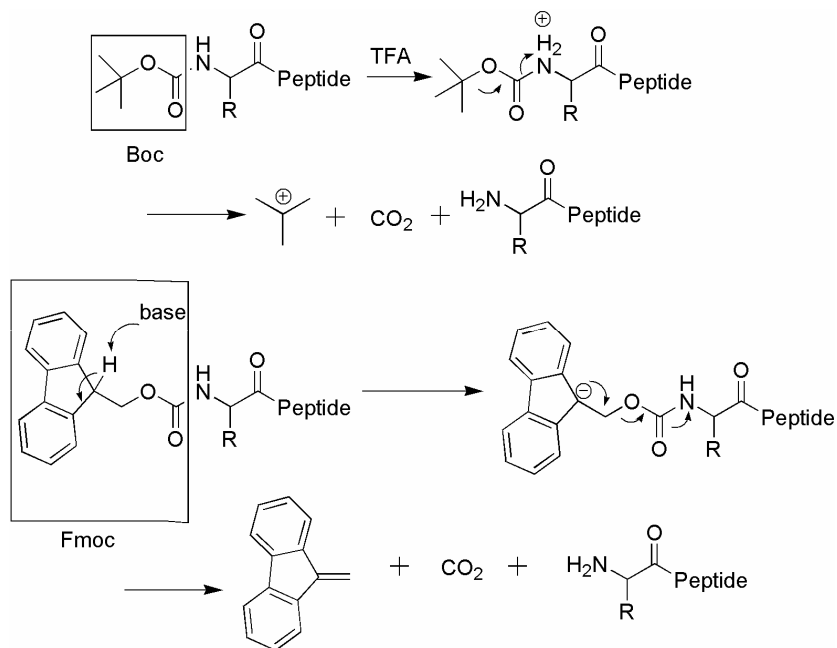


Scheme 2.2 Structures of lysine and ‘temporary’ (red), ‘permanent’ (blue) groups protected lysine

If lysine (2.1) is used directly in SPPS, lysine dimer or trimer may be formed. So Boc-Lys-OH (2.2) is introduced, the polymerization of lysine will be avoided since the α -amine group is protected by the Boc temporary group. The Boc group can be removed by trifluoroacetic acid (TFA) effectively. Another problem rises with use of only a Boc group in 2.2. A free amine group at the side chain of lysine will also react

with carboxylic acid from other amino acids. To avoid this problem, Boc-Lys(2-ClZ)-OH (**2.3**) was developed. The free amine group on the side chain of lysine is protected with 2-chlorobenzoyloxycarbonyl group. The 2-chlorobenzoyloxycarbonyl group is called a ‘permanent’ protecting group because it is stable to the TFA treatment. The 2-chlorobenzoyloxycarbonyl is only sensitive to cleavage solution such as hydrofluoric acid (HF) and trifluoromethanesulfonic acid (TFMSA). After every TFA deprotection cycle, Boc group that protects α -amine is removed while 2-chlorobenzoyloxycarbonyl is still remains and protects the amine of the side chain. When the synthesis is finished, the cleavage solution (HF or TFMSA for Boc protocol) will remove the ‘permanent’ protecting group and remove the peptides from the resin.

t-Boc and 9-flourenylmethoxycarbonyl (Fmoc)⁵⁰ are the two most used ‘temporary’ protecting groups in SPPS (**Scheme 2.3**).



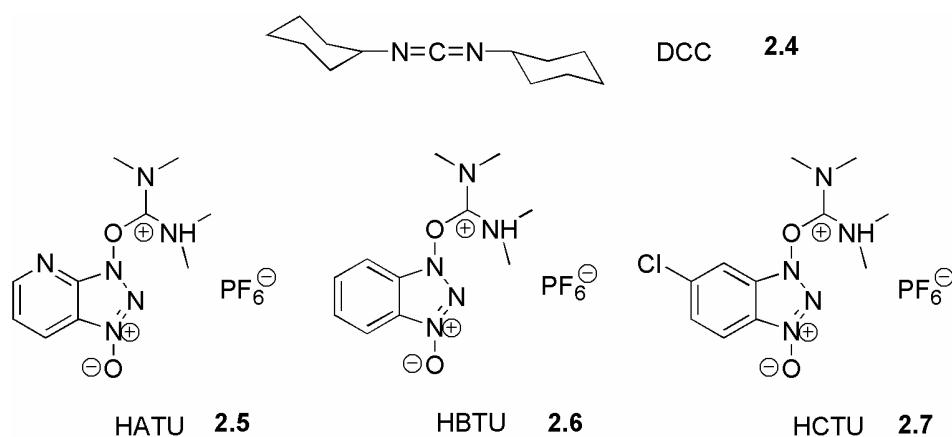
Scheme 2.3 *t*-Boc and Fmoc protecting groups in SPPS and the mechanism of deprotection

In *t*-Boc protocols, the 'temporary' protecting group is removed by TFA after each coupling. When the desired peptides have been synthesized on the resin, HF or TFMSA are used to remove the peptide from the resin. When the protecting group is Fmoc, 20% piperidine in DMF is used to remove Fmoc protecting group. Finally, the peptides are removed from the resin by TFA.

Fmoc protocol avoids repeated use of TFA needed for the Boc method. Fmoc is used in most peptides synthesis due to the milder condition required. Boc protocol is still used in the synthesis of some basic liable peptides and long amino acid peptides.

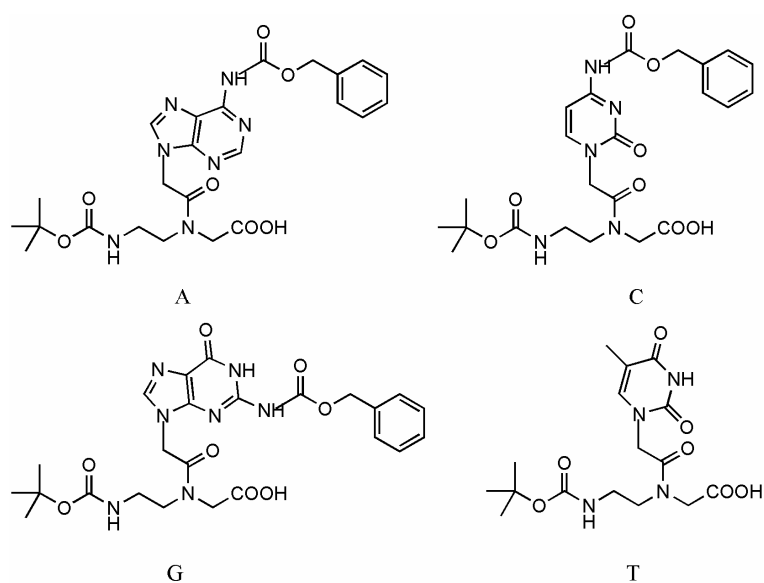
Coupling efficiency is an important factor in SPPS. If the average coupling efficiency is 90%, after 10 cycles, the final yield is only about 35% and lots of byproducts are produced. One way to make the reaction go to completion with high efficiency is to activate the carboxylic acid terminal of the amino acids first and then couple with the amine groups. Many kinds of activating agents have been discovered including *N,N'*-dicyclohexylcarbodiimide (DCC),⁵¹ 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), *O*-(7-aza-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) and 2-(6-Chloro-1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HCTU) (**Scheme 2.4**).

DCC is widely used because of its high activity and low cost. HATU, HBTU and HCTU are commonly used in activating amino acids since the byproduct dicyclohexylurea (DCU) is insoluble in DCM. HATU is the most expensive reagent among the three hexafluorophosphates, while it works the best. It is usually used when HBTU and HCTU are failed in reactions.



Scheme 2.4 Structure of DCC, HATU, HBTU and HCTU

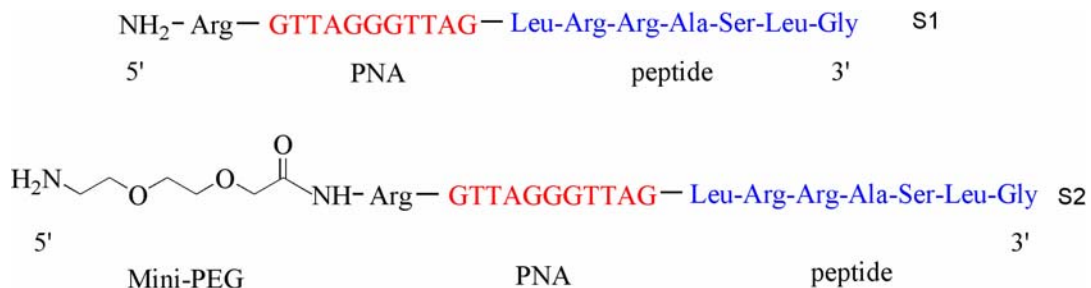
SPPS is also used in synthesizing PNA oligomers³⁵. **Scheme 2.5** shows the benzyloxy-carbonyl (Z) protected PNA monomers which can be used in SPPS through the Boc protocol. Boc protocol is found to work better than Fmoc in PNA synthesis.⁵²



Scheme 2.5 Z-protected PNA- A,T,G and C monomers

Because of the neutral property of the PNA backbone and its availability through SPPS, we design new peptide-PNA conjugates **S1** and **S2**. Conjugate **S1** mainly consists of two parts: a seven amino acids peptide and a PNA sequence conjugate

(**Scheme 2.6**). Compound **S2** attaches an additional 8-amino-3,6-dioxaoctanoic acid (mini-PEG) at the 5' terminus of arginine.



Scheme 2.6 composition of compounds **S1** and **S2**

Several reasons are considered to design compounds **S1** and **S2**:

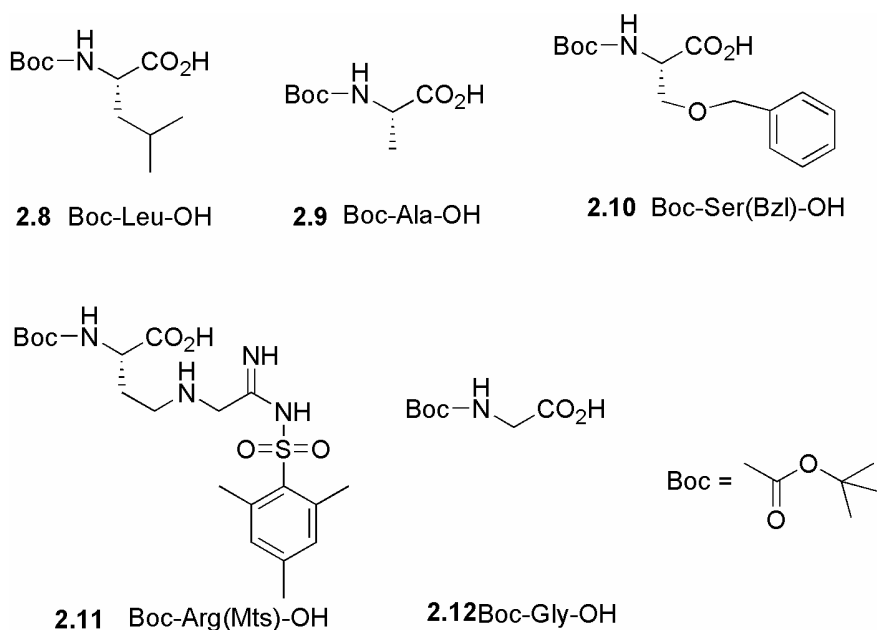
- a) The PNA and peptide sequence can be synthesized by SPPS and QM precursor can be linked with the Arg at the 5' terminus of the PNA sequence by standard protocol.²⁷
- b) Kemptide⁵³ (5'-LRRASLG-3') is readily phosphorylated by protein kinase A (PKA) at the Ser site. Thus the LRRASLG sequence in compound **S1** and **S2** can function as a phosphorylation site in place of the standard 5'-phosphorylation of DNA.
- c) PNA (5'-GTTAGGGTTAG-3') complementary to the telomerase RNA sequence can bind with the RNA in telomerase tightly and may inhibit the function of telomerase effectively.⁴⁷
- d) The amine group at the 5' terminus of the peptide-PNA conjugate S1 may be hindered by the peptide and PNA chain. The bifunctional mini-PEG⁵⁴ was attached at the 5' terminus of the conjugate as a linker to elongate the free amine group. This amine group may react with our DNA alkylating agent quinoe methide precursor (QMP).

- e) The use of PNA in vivo is hampered by its poor water solubility. Hydrophilic mini-PEG helps the solubility of conjugate in water due to the hydrogen bond between water molecules and the oxygen atoms in the mini-PEG chain.

2.2 Result and Discussion

2.2.1 Conditions for the synthesis of peptides

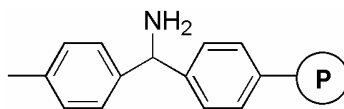
Conditions for *t*-Boc proctol in SPPS were evaluated by synthesizing the 7mer and 8mer peptides 5'-LRRASLG-3' (**S3**) and 5'-RLRRASLG-3' (**S4**). The protected natural amino acids that were necessary for reaction, Boc-leucine (Boc-Leu-OH), Boc-Alanine (Boc-Ala-OH), Boc-O-benzyl-L-serine (Boc-Ser(Bzl)OH), N^α-Boc-N^ε-mesitylenesulfonyl-L-arginine (Boc-Arg(Mts)-OH), Boc-Glycine (Boc-Gly-OH) are illustrated in **Scheme 2.7**.



Scheme 2.7 Structures of the amino acids used in SPPS

Several factors contribute to the successful synthesis of peptides. There are no specific requirements for the gas used in peptide synthesis. Several groups suggest the use of nitrogen gas in PNA synthesis.^{34, 37} Pure nitrogen gas was used to eliminate the effect of water from air. So nitrogen gas was used in these experiments to achieve best result.

4-Methylbenzhydramine hydrochloride (MBHA) resin (**Scheme 2.8**) was used in reaction.



Scheme 2.8 Structure of MBHA resin

Two types of MHBA resins were tried in SPPS. Because PNA synthesis works better at low substitution (0.1-0.3 mmol/g),⁵² we decided to use low substitution (0.1 mmol/g) resin to synthesize the 7mer peptide **S3** at first. When **S3** was coupled with resin, following PNA oligomers could be linked with the peptide. I found that the low substitution resin did not work in the reaction. This may be caused by the low concentration of amino acids in solution or low coupling efficiency. Typically, 5 equivalents of each amino acid in 4 mL DMF was used in the coupling steps to ensure the reaction complete. When 100 mg of low substitution resin was used, the amount of resin is approximately 10 μ mol. 50 μ mol of amino acid was activated in 4 ml DMF with HBTU. The concentration of amino acid is 12.5 μ M, but typically the concentration of amino acid used in coupling should be 100 μ M. The low concentration of amino acids may have caused the failure of coupling.

The resin was then changed to a standard substitution (0.7 mmol/g). When 100 mg (70 μ mol) resin was used, 350 μ mol amino acids (5 eq. to resin) were applied in 2.5

ml DMF. The coupling was monitored by Kaiser test⁵⁵ and worked well. This shows the concentration 140 μmol is suitable for the SPPS.

The carboxylic acid group from amino acids was activated by HBTU for two minutes. When five equivalents of the amino acid (compared to resin), 4.5 equivalent HBTU and ten equivalent *N,N'*-Diisopropylethylamine (DIPEA) were mixed in DMF for two minutes, the color of solution changed to slightly yellow. The resulting solution was added and coupled with resin.

Various coupling times were also tried. Normally, the time should be no more than one hour. In several papers, about 30 minutes were used to finish coupling.^{37, 56} During my experiment, 20 minutes and 30 minutes showed no difference in coupling. Less than 20 minutes was not tested.

A procedure named 'capping' was also performed after the first coupling. It is reasonable to think that after the completion of the first coupling of the amino acid to the resin, there are still free amine groups at the surface of the resin. Capping solution (5 ml, acetic anhydride/pyridine/ *N*-Methylpyrrolidone = 2/1/1) was added to the resin only after the first coupling to ensure the consumption of all free amine groups. Capping between the first and second coupling will decrease the occurrence of byproducts.⁴⁹

95% TFA (TFA/*m*-cresol=95/5) can deprotect *t*-Boc group in 5 minutes.⁴⁹ In our experiment, the same method was used. *t*-Boc protected product was swelled in 95% TFA twice (4 minutes each time) to remove *t*-Boc completely. When the Kaiser test⁵⁵ showed blue, this means the completion of deprotection. Kaiser test was used after

every coupling and deprotecting procedure to verify the completion of reaction. This is considered an effective way to monitor every step of reaction.

In literature, cleavage solution is used to remove the peptide from the resin.⁴⁹ The cleavage solution (trifluoromethanesulfonic acid (TFMSA)/TFA/*m*-cresol/ thioanisole = 2/6/1/1) was cooled to -20°C before using. The cleavage time is one hour. All the other reagents, solvents and procedures were handled under room temperature. At first, I followed the time from literature. The resin was treated by cleavage solution one hour. The yield was only about 10%. Then the procedure was revised. The resin was first treated with cleavage solution for one hour, the resulting solution was collected. Then the resin was treated with another part of new cleavage solution for one hour. The resulting solution was collected and combined with the previous solution. Experiment shows that resin treated by cleavage solution twice (one hour everytime), the yield is more than 50%.

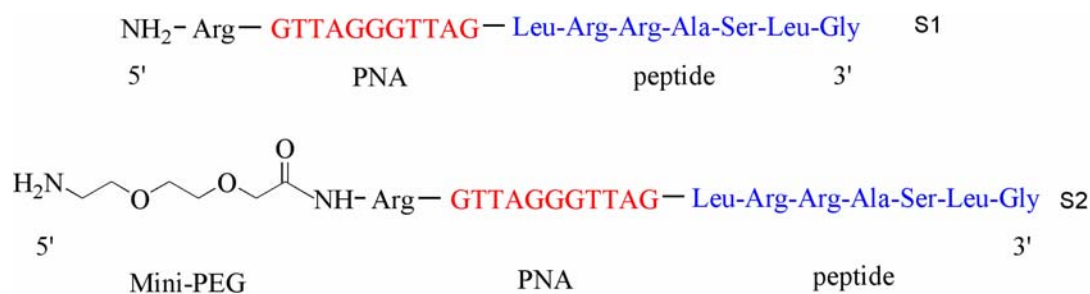
If the coupling could not be finished in one day, the N-terminal of final amino acid should be *t*-Boc protected and the resin be kept under vacuum.

After the peptides were precipitated from cold diethyl ether, the peptides were purified by C18 reverse-phase high performance liquid chromatography (RP-HPLC). The eluents are 0.1% TFA in water and 0.1% TFA in acetonitrile.

2.2.2 Synthesis of peptide-PNA conjugate

Basic procedure of synthesizing peptide-PNA conjugates are derived from the peptides synthesized above. However, after the PNA monomers were used, several changes were applied.

Peptide-PNA conjugates (**S1**, **S2**) were also successfully synthesized (**Scheme 2.9**).

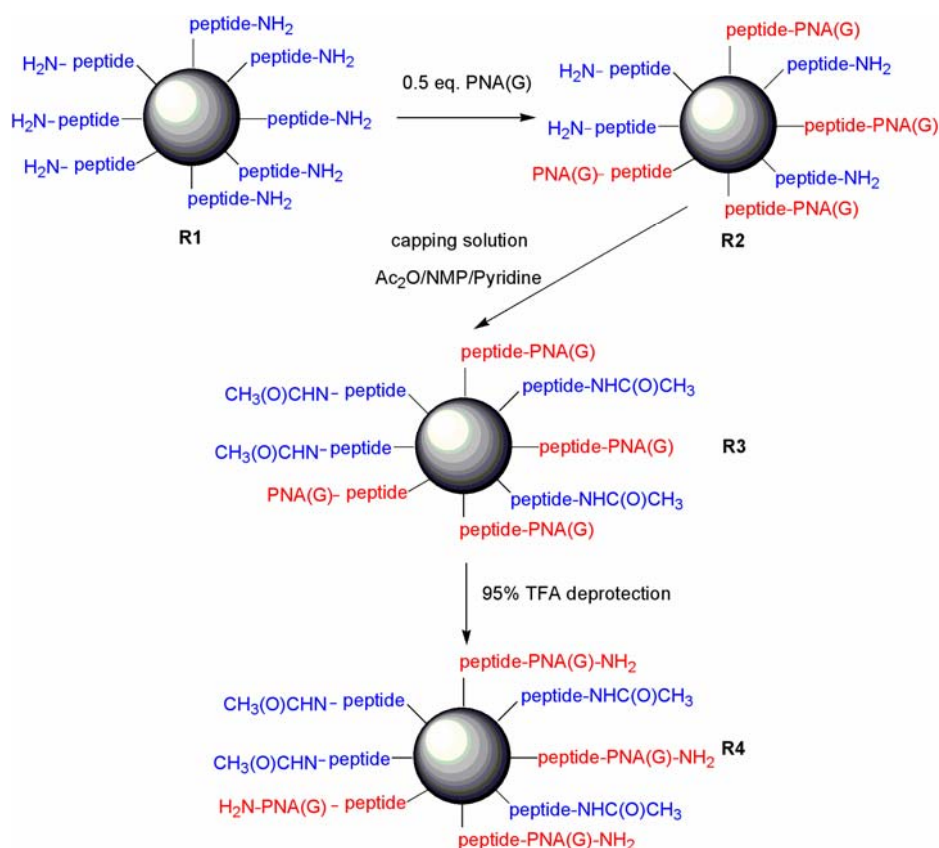


Scheme 2.9 Peptide-PNA conjugates **S1, S2**

PNA monomers and oligomers are easily to attach at the surface of glasswares.⁵² Thus plastic tubes were used to hold all PNA solutions. Plastic vials were applied to collect the peptide-PNA conjugates solution which were purified from RP-HPLC. Different from most amino acids, PNA monomers are difficult to dissolve in DCM or N-methylpyrrolidone (NMP) which are widely used in peptide synthesis. DMF works as a good solvent for PNA dissolution.

To activate the PNA monomers, HATU was added to DMF solution of PNA monomers. Typical procedure for activating PNA monomers is to dissolve PNA monomers and HATU in DMF. Then DIPEA is added to activate the carboxylic acid from amino acids. The only difference is PNA monomer G. Since PNA monomer G is difficult to dissolve in DMF, PNA monomer G was first dissolved in DMF by vortex for 5-10 minutes. HATU and N N-Diisopropylethylamine (DIPEA) were then added to the DMF solution containing PNA monomer G.

Resin used in peptide synthesis is 0.7 mmol/g while the recommended concentration of resin for PNA synthesis is 0.1mmol/g to 0.3mmol/g.⁵² The peptide chain coupled to the resin (100 mg resin, the amount of peptide on it is 70 μmol , theoretically) was deprotected by TFA (**R1, Scheme 2.10**) and ready for coupling PNA.



Scheme 2.10 Capping after the first PNA(G) monomer was coupled with peptide.

The unreacted peptide (blue in **R2**) was capped with capping solution and terminate the reaction

35 μ mol (0.5 equivalent) of PNA monomer in 1 ml DMF was used in the first PNA(G) coupling to download the substitution of resin. Since this amount of PNA monomer (35 μ mol) is not sufficient to couple with all the free amine groups from the previous amino acid (70 μ mol), only part of the free amine groups from peptides were reacted with PNA(G) (**R2**, red colored). The other free amino groups were remaining. Then capping solution (acetic anhydride/pyridine/ N-Methylpyrrolidone = 2/1/1) was used to terminate the amine groups. After the capping procedure, only Boc-PNA-peptide-resin (red in **R3**) and AcO-peptide-resin (blue in R3) should exist. After the

resin was swelled in 95% TFA, the Boc-PNA-peptide-resin can be converted to NH₂-PNA-peptide (red in **R4**) while the AcO-PNA-peptide (blue in **R4**) would not change. When the second activated PNA monomer was mixed with resin, only NH₂-PNA-peptide could react with the second PNA monomer while the AcO-PNA-peptide kept unchanged. From the second PNA monomer, regular cycles were taken to couple corresponding PNA monomers to the resin.

By applying SPPS, peptides **S3**, **S4** and peptide-PNA conjugates **S1**, **S2** were successfully synthesized.

2.2.3 Conclusion

Through the synthesis of peptides, we found the concentration of amino acids for coupling should be 50 μ M or higher. O-benzyl (Bzl) is suitable for protecting serine side chain and N^ε-mesitylenesulfonyl (Mts) is good for protection of the Arginine side chain. 0.7 mmol/g resin is good for the synthesis of peptides and peptide-PNA conjugates.

2.3 Experimental

2.3.1 General method:

Beckman Avanti J-25I centrifuge was used to separate the peptides and peptide-PNA conjugates from diethyl ether. Labconco lyophilizer was used to lyophilize peptides and peptide-PNA samples. RP-HPLC separation was accomplished by JASCO PU980. The column employed was Varian C-18 column (Microsorb-MV 300, 5 μ m particle size, 250 mm \times 4.6 mm). High resolution mass spectra were determined

with SHIMADZU-KRATOS Axima-CFR MALDI-TOF and JEOL AccuTOF-CS ESI-MS.

2.3.2 Materials

Solvents, starting materials, and reagents of the highest commercial grade were used without further purification. Aqueous solutions were prepared with distilled, deionized water with a resistivity of 18.9 M Ω . All the Boc-protected amino acids and 4-methylbenzhydrylamine (MBHA) resin (0.7mmol/g) were purchased from Advanced ChemTech. The following Boc-protected amino acid derivatives were used: Boc-Arg(Mts)-OH and Boc-Ser(Bzl)-OH. Mini-PEG was purchased from Peptide International. DMF (peptide synthesis), DCM, TFA, Ac₂O, DIPEA, NMP, TFMSA, α -cyano-4-hydroxycinnamic acid (CHCA) and sinapinic acid (SA) were purchased from Acros. Kaiser test kit was obtained from Aldrich.

Kaiser Test.⁵⁵

The Kaiser Test kit includes 6% ninhydrin solution in ethanol, ~80% phenol solution in ethanol and potassium cyanide solution in pyridine. Three drops of solution from each bottles was added to a test tube which contains about 1 mg of resin, then the solution was heated for 5 minutes at 100°C. If the solution turns purple, free amine groups are present. If the color does not change, free amine groups are absent.

Peptide Synthesis (for first 7 amino acids in the sequence) The peptides and peptide-PNA conjugates were synthesized following the revised *t*-Boc SPPS chemistry.³⁴

Reaction was carried out in a 20 ml fritted funnel inserted into a 250 ml side arm flask through a single hole rubber septa. A T-valve was at the bottom of the funnel.

The side-arm of the T-valve was connected with the Schlenk line. By forcing the nitrogen from the T-valve and frit, solvent and resin can be fully mixed by bubbling. When the vacuum was attached with the side arm of the 250 ml flask, the solvent in the vial was forced down to the flask and separated with the resin.

Standard substituted MBHA resin 100 mg (0.7 mmol/g, 70 μ mol) was swelled in DMF for 3 hours at room temperature. Then the DMF was removed and the resin was washed by 3 ml DCM for 1 minute twice.

2.5 ml DMF was added to the vial that contains the desired amino acids (5 eq., 350 μ mol) and HBTU (4.5 eq. 315 μ mol). DIPEA (10 eq. 700 μ mol) was then added to the resulting solution. The solution was vortexed for 2 minutes to activate the carboxylic terminus and then added to the fritted funnel at room temperature. The solvents were mixed with resin by bubbled nitrogen at room temperature for 20 minutes and removed by vacuum. The resin was then washed by 3 ml DMF for 1 minute twice and 3 ml DCM for 1 minute twice. Kaiser test was performed to confirm the efficiency of coupling. If Kaiser test shows blue, the coupling was repeated.

The completion of the first amino acid coupled with resin was monitored by Kaiser test. Ac₂O/NMP/Pyridine (5 ml, v/v=1/2/2) capping solution was added to the resin to cap the remaining free amine groups and reaction was mixed by bubbling N₂. The capping solution was removed after 1 hour incubation at room temperature by passing the liquid through filter. 3ml DMF was used to wash the resin for 1 minute twice. After removing DMF, 3 ml DCM was applied to wash resin for 1 minute twice.

The Boc protecting group was removed before the second coupling by mixing with 1.5 ml 95% TFA/m-cresol (v/v=95/5) for 4 minutes twice. 3 ml DCM was added

once and used to wash the resin for 1 minute four times after the 95% TFA was removed. Additional 3 ml DMF washing for 1 minute twice followed by 3 ml DCM washing for 1 minute twice were carried out to fully remove the TFA. The presence of amine group was tested by Kaiser test.

When deprotection was finished, the second amino acid (350 μmol), HBTU (315 μmol) and DIPEA (700 μmol) was mixed for the second coupling. The procedure was repeated until all the amino acids were added consequently. The product was purified by RP-HPLC (**Figure 2.1**) and confirmed by both MALDI-TOF (**Figure 2.2**) and ESI-MS (**Figure 2.3**).

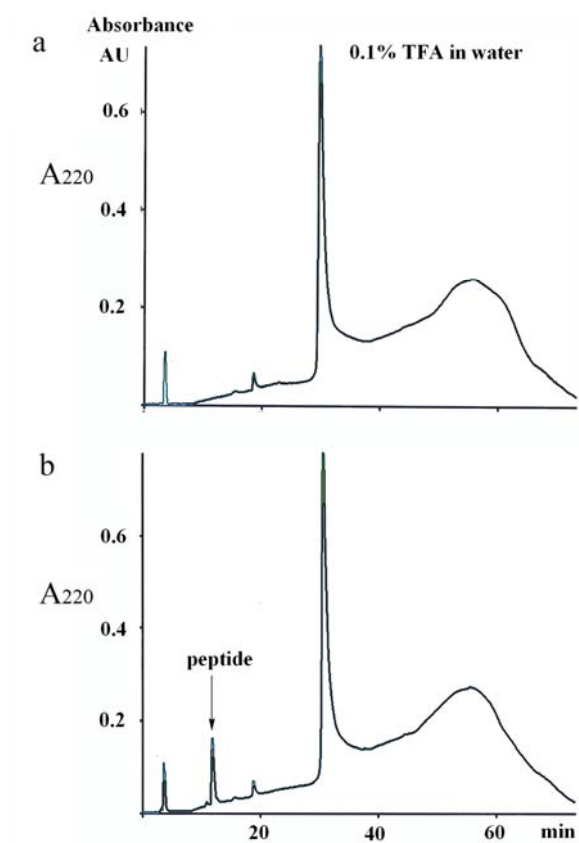


Figure 2.1 RP-HPLC of a) blank injection; b) 7mer peptide (5'-LRRASLG-3') at 220 nm, the eluent of peptide appeared at 16 minute

MALDI of Kemptide

5'-LRRASLG-3'

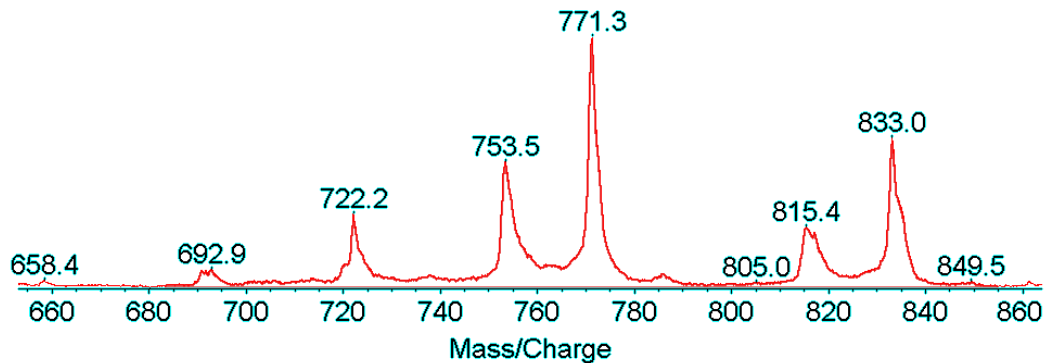


Figure 2.2 MALDI of 7mer peptide (5'-LRRASLG-3'), Calculated mass: 770.9,

MALDI found: 771.3, CHCA as matrix

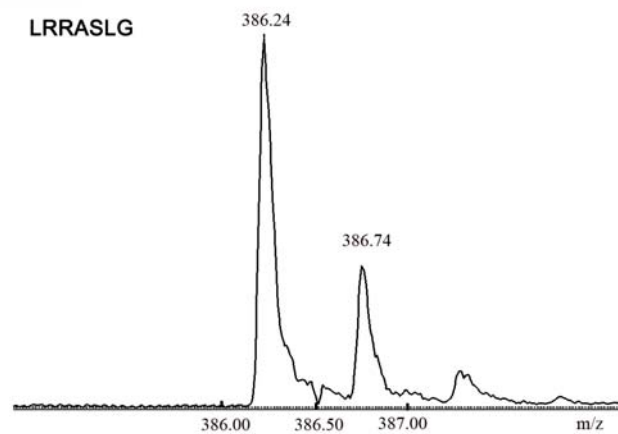


Figure 2.3 ESI-MS of 7mer peptide (5'-LRRASLG-3'), Calculated mass: 770.9, $[M+2H^+] = 386.45$, ESI-MS found: 386.24. (In this MS spectrum, the difference of main peak and 2nd peak is 0.5. The difference is caused by an isotope effect. Since this effect is an integer, a minimum of 2 charges must be present: $1.0/0.5 = 2$, there are two charges on the peptide. If the difference is 0.33, there are three charges on the peptide)

Peptide-PNA Conjugate Synthesis (for the following PNA in the sequence):³⁴

After the 7 amino acids 5'-LRRASLG-3' peptide was coupled onto the resin, the reactive amines of the nascent oligomer was decreased from 0.7 mmol/g to 0.1 mmol/g because the PNA coupling works well at low loading resin.⁵² (40 mg peptide-preloaded resin was used in peptide-PNA synthesis. The amount of free amine group was 28 μ mol ($0.7 \text{ mmol} \times 40 \text{ mg} = 28\mu\text{mol}$). Then half amount of PNA(G) monomer was used in peptide-PNA synthesis (14 μ mol) to download the substitution of resin. The unreacted free amine group was then terminated by capping solution. So therotically, the substitution should be 0.35 mmol/g. However, considering the yield of peptides was lower in the coupling reaction, we assumed the substitution was 0.1 mmol/g. It is a pretty rough estimation. The following PNA coupling worked well depends on this assumption.)

40 mg peptide-preloaded resin was used. The first PNA monomer (14 μ mol) and HATU (13 μ mol) was dissolved in DMF. DIPEA (30 μ mol) was transferred to the resulting solution. The solution was fully mixed by vortex mixing. Then the activated PNA monomer was mixed with H₂N-LRRASLG-resin. Capping (Ac₂O/Pyridine/NMP = 2/1/1) was applied again after the first coupling finished.

When the first PNA coupling and capping was finished, 1.5 ml 95% TFA/m-cresol (v/v=95/5) was incubated for 4 minutes twice to remove the Boc group. When the TFA was removed by vacuum, 3 ml DCM was used to wash the resin for 1 minute 4 times. 3 ml NMP washing for 1 minute twice and 3 ml DCM washing for 1 minute twice were carried out to wash resin. When the presence of free amine group was confirmed by the Kaiser test (the color turns to blue), following activated PNA solution was prepared and added.

1.2 ml DMF was added to the vial that contained PNA monomers (30 μmol) and HATU (27 μmol). Then DIEA (60 μmol) was added to the previous solution. The solution was vortexed for 1.5 minutes to activate the carboxylic terminus and was added to the fritted funnel. Mixed by bubbled nitrogen at room temperature for 20 minutes, the solvents were removed by vacuum. The resin was then wash by DMF for 1 minute (2 \times 3 ml) and DCM for 1 minute (2 \times 3 ml).

The procedure was repeated until all the PNA monomers were added consequently. When all the synthesis was finished, methanol was used to fully wash the resin for 1 minute (1 \times 3 ml). The resin was then dried under vacuum overnight before cleavage.

Converting Mini-PEG. Dicyclohexylamine (DCHA) salt to Mini-PEG

244 mg (0.5 mmol) of Mini-PEG • DCHA was suspended in 10 ml of ethyl acetate in a 100 mL erlenmeyer flask with a magnetic stir bar. When product was dissolved, Ca. 5 ml ice-cold 1 N HCl was added and the pH was adjusted to pH = 2. A white precipitate formed. The precipitate was separated by filtration and was washed with 2 x 20 ml ethyl acetate. All ethyl acetate layers were combined and washed with 2 x 20 ml of brine. Organic layer was then collected and dried over MgSO_4 for 30 minutes. The drying agent was removed by filtration. The free acid of Mini-PEG at the bottom of flask was collected after removing the ethyl acetate in a rotary evaporator (Yield = 90.0%).

Link Mini-PEG with the peptide-PNA chain

1.0 ml DMF was used to dissolve Mini-PEG (25 mg, 94.9 μmol). HATU (85.0 μmol) and DIPEA (950 μmol) were added to previous solution. After two minutes,

the resulting solution was added to resin to couple Mini-PEG to resin and followed standard SPPS procedure.

Cleaving the peptide from resin

1.5 mL cleavage solution was used for 50 mg resin. The cleavage solution consists of 1 part thioanisole, 1 part *m*-cresol, 2 parts TFMSA and 6 parts neat TFA. The solution was cooled under -20°C before using. It is found that freshly opened TFMSA has the best result³⁴. Long stored TFMSA may turn to brown or black and shows poor cleavage efficiency.

50 mg of resin and 1.5 ml cleavage solution were incubated by barely bubbled nitrogen in the fritted funnel for 1 h at room temperature. The black cleavage solution was separated with the resin by vacuum and collected. Additional 1.5 ml cleavage solution was add to the funnel and was bubbled another 1 h. After the additional cleavage solution, 2 parts of neat TFA (3 ml) was used to wash the resin. All the solutions were combined. TFA was removed by continuously blowing nitrogen to the bottle. Finally the peptide was precipitated by mixing the cleavage solution with 50 ml cold diethyl ether. The precipitant and solution was centrifuged at 5000 rpm for 5 min. Additional 5 ml cold ether was used to wash the solid peptide-PNA conjugate 3 times. The solid at the bottom of the vials was redissolve in HPLC grade acetonitrile and transferred to plastic bottles. The crude peptide-PNA conjugates solution was finally dried by lyophilizer. Molecular weight was got from MALDI-TOF. For peptide-PNA conjugate **S1** (NH₂-Arg-GTTTAGGGTTAG-LRRASLG), calculated mass: 3999.5, MALDI found mass: 3999.0 (**Figure 2.4**); for peptide-PNA conjugate

S2 (NH₂-MiniPEG-Arg-GTTTAGGGTTAG-LRRASLG), calculated mass: 4144.7,
MALDI found: 4143.3 (**Figure 2.5**).

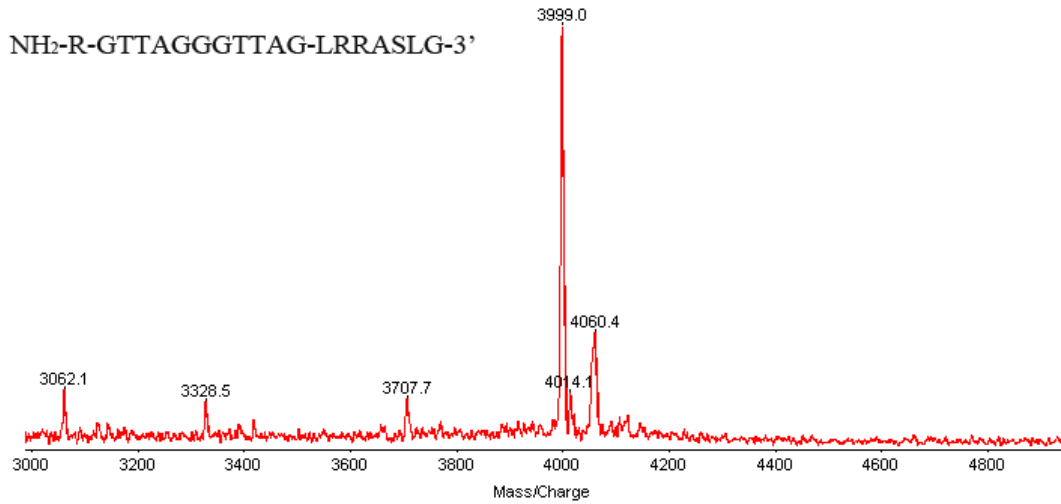


Figure 2.4 MALDI of Peptide-PNA-Arg-NH₂ conjugate. Calculated mass: 3999.5,
MALDI found: 3999.0

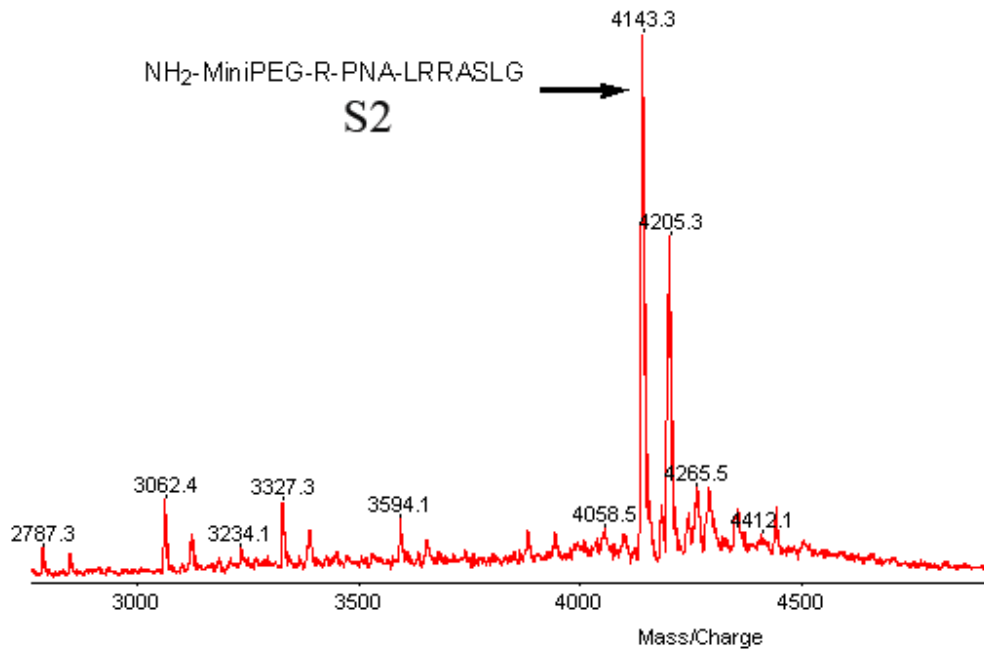


Figure 2.5 MALDI of peptide-PNA-Arg-MiniPEG conjugate, calculated mass:
4144.7, MALDI found: 4143.3

Purification and analysis

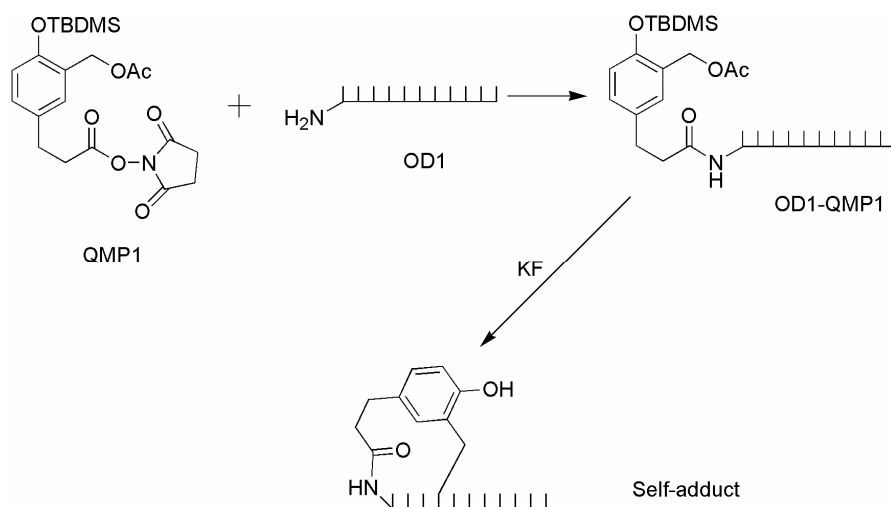
Reverse phase-high performance liquid chromatography (RP-HPLC) at 220 nm (for peptides) and 260 nm (for peptide-PNA conjugates) used a TFA/acetonitrile gradient to detect and purify the crude peptide. Solvent A: 0.1% TFA/H₂O and solvent B: 0.1% TFA/acetonitrile with a gradient of 0-35% of B in 35 min, at a flow rate 1.0 ml/min was used. The column employed was Varian C-18 column (Microsorb-MV 300, 5 µm particle size, 250 mm × 4.6 mm).

Chapter 3: Synthesis of QMP and Coupling between QMP and Peptide-PNA Conjugate

3.1 Introduction

Quinone methide intermediates (QMs) are good DNA alkylation agents. However, their poor selectivity of alkylation limits their use in biological systems and could cause severe side-effects. However, the Rokita group developed a general method for selective alkylating DNA based on QM (**Scheme 3.1**).²⁷ DNA-QMP conjugate was formed when *N*-succinimidyl-3-(3-acetoxymethyl-4-*tert*-butyldimethylsilyloxyphenyl)propionate (**QMP1**) was coupled with ssDNA (**OD1**). Generation of a OD1-QM self-adduct was triggered by adding KF. After the complementary ssDNA (**OD2**) was added, **QMP1** was delivered to the targeted DNA (**OD2**) via Watson-Crick base pairing between the two complementary **OD1** and **OD2**. The formation of self-adduct is reversible and the QM could be transferred to **OD2** without the existence of toxic KF.

Conjugate OD1-QMP1 was formed by coupling **QMP1** and DNA oligonucleotide (**OD1**) (**Scheme 3.1**). In the procedure, **OD1** functions as a vehicle to deliver **QMP1** to target DNA. When **QMP1** was mixed with **OD1**, the *N*-succinimidyl ester was attacked by the amine group from **OD1** and formed the conjugate OD1-QMP1.



Scheme 3.1 Selective delivery of QMP by DNA sequence

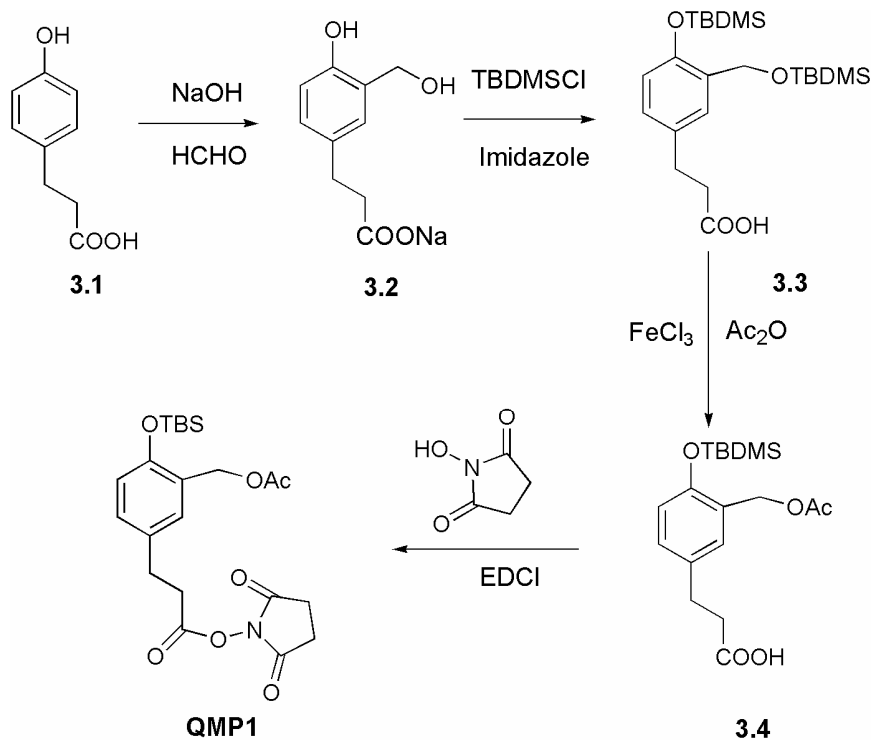
If the DNA is replaced by PNA oligomers, applications may extend to cells. PNA oligomers are resistance to degradation by nucleases and proteases, bind with high sequence specificity to complementary DNA, and form PNA/DNA duplex with higher stability than DNA/DNA duplex. Conjugating various molecules to PNA termini has been studied by several groups.⁵⁷⁻⁵⁹ Appella's group showed that by conjugating biotin to a PNA oligomer, fast and simple detection of certain DNA can be accomplished without the use of PCR in regular detection.⁵⁶ Target DNA can be detected at a 10 zmol (zmol = 10^{-21} mol) limit. Nielsen also published that conjugation of PNAs to a simple lipophilic ligand, such as an adamantyl group, can dramatically increase cellular uptake properties compared to PNA itself.⁵⁷

Synthesize the QMP1-peptide-PNA conjugates under a variety of conditions are described below.

3.2 Results and Discussion

3.2.1 Synthesis of QMP1

The synthesis of **QMP1** was slightly revised from a literature protocol (**Scheme 3.2**).²⁷



TBDMSCI = *tert*-butyldimethylsilyl chloride

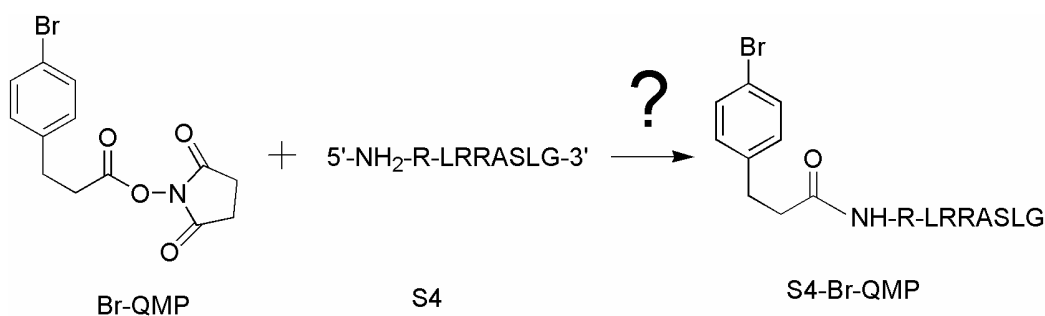
Scheme 3.2 Synthesis of *N*-succinimidyl-3-(3-acetoxymethyl-4-*tert*-butyldimethylsilyloxyphenyl) propionate

Previously, after treating **3.1** with NaOH and HCHO, crude products were directly added to the DMF solution of TBDMSCI and imidazole. Product **3.3** was purified by flash column chromatography in literature.²⁷ Practice showed that the polarity of **3.3** and 3-[4'-*tert*-butyldimethylsilyloxy-3',5'-bis(*tert* butyldimethylsilyloxymethyl)]propionic acid is similar. The separation by flash column chromatography failed. **3.2** was then dissolved in 10 ml water, the pKa of the aqueous of solution was adjusted to pH 2 - 3 by addition of 1M HCl. Because the pH of 3-[3-hydroxymethyl-4-hydroxyphenyl]propionic acid is 3 - 4, Salt **3.2** should be converted to the

corresponding acid. 50 ml ethyl ether was used to extract the acid from water. And then the corresponding acid was purified by chromatotron (hexane/ethyl acetate = 2/1). This method avoids the difficulty of separating compounds **3.3** and 3-[4'-*tert*-butyldimethylsilyloxy-3',5'-bis(*tert* butyldimethylsilyloxymethyl)] propionic acid. The yield is 21.2%.

3.2.2 Conditions for coupling between a QMP1 analog and peptides

In order to evaluate the conditions for coupling between **QMP1** and peptide-PNA conjugate, *N*-succinimidyl-3-(3-bromophenyl)propionate (**Br-QMP**) and peptide (RLRRASLG) **S4** were synthesized and coupled in various conditions (**Scheme 3.3**). **Br-QMP** was used because a) it is easily made from commercially available 3-(3-bromophenyl) propionate acid and *N*-hydroxyl succimide; b) the bromide at the aromatic ring will generate double peak in mass spectrometer due to the isotope distribution of bromide in nature. This signature of bromine-containing conjugate makes it easy to judge whether the coupling happens.



Scheme 3.3 Coupling between **Br-QMP** and peptide **S4**

First attempt to couple **BrQMP** with peptide **S4** was taken under a basic condition. Different amount () of DIPEA or triethyl amine (TEA) were mixed with **Br-QMP** and **S4**, the coupling did not happen (monitored by RP-HPLC). The failure of DIPEA and TEA in coupling may be caused by competition between these bases with the free

amine group from the 5 terminus of peptide-PNA conjugate. Various solvents for the reaction such as water, acetonitrile and DMF did not facilitate the coupling.

Then 3-(N-morpholino)propanesulfonic acid (MOPS) and acetonitrile/DMF system was tried. 100 μ l peptides (5 mM) dissolved in MOPS (250 mM, pH= 7.5) was mixed with 100 μ l Br-QMP (7.5 mM) in acetonitrile/DMF (v/v = 2/1) and incubated for 8 hours at room temperature. RP-HPLC showed that coupling happened (**Figure 3.1**). In the MOPS and acetonitrile/DMF system, several reasons are supposed to contribute to the success of coupling: 1) DMF may decrease the aggregation of peptide; 2) basic condition rendered by MOPS buffer may help the existence of free amine group and then facilitate the coupling.

Once the condition for coupling between peptides and Br-QMP was determined, the same conditions for coupling peptide-PNA conjugates with Br-QMP were tested.

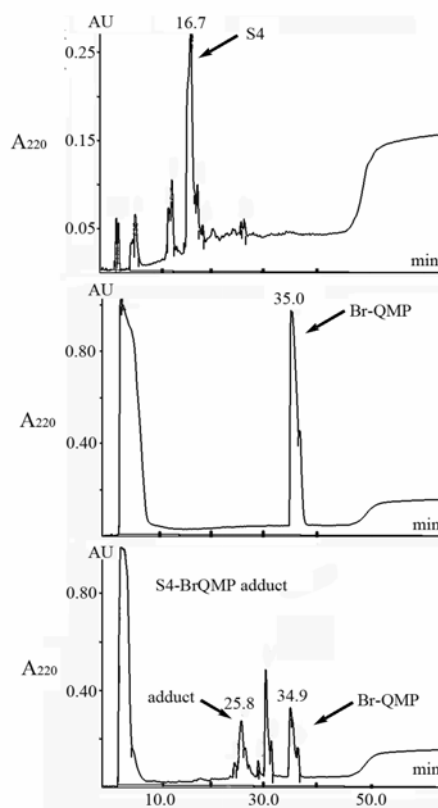
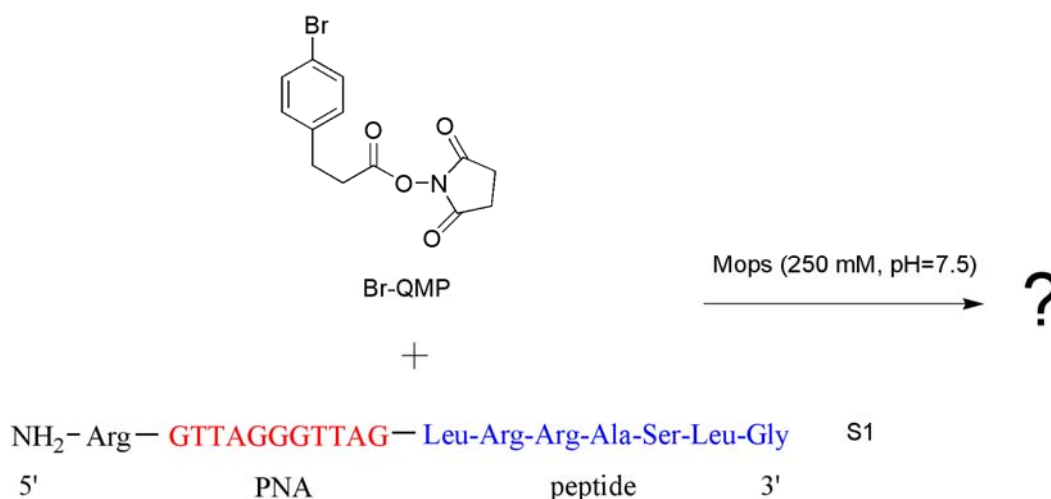


Figure 3.1 Formation of BrQMP-peptide conjugate, 25.8 min eluent is the conjugate

3.2.3 Conditions for coupling between Br-QMP, QMP1 and peptide-PNA

The same condition in 3.2.2 was used in coupling **Br-QMP** with peptide-PNA conjugate (**S1**) (**Scheme 3.4**).



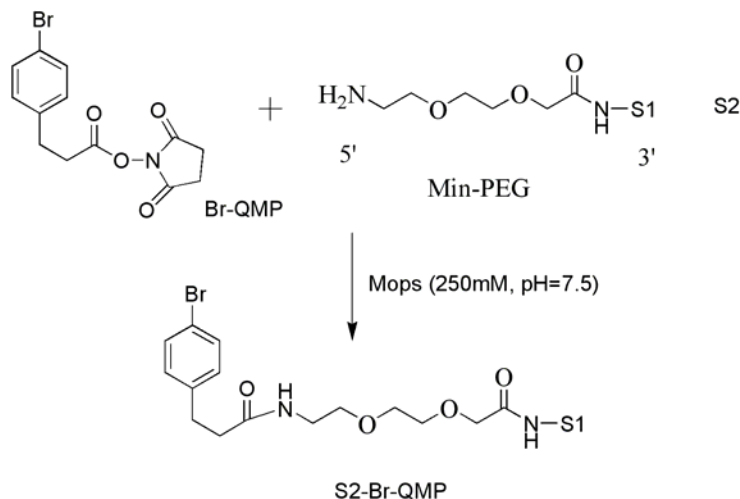
Scheme 3.4 Try to couple Br-QMP with peptide-PNA conjugate S1

100 μl **S1** (5mM) dissolved in Mops (250 mM, pH = 7.5) was mixed with 100 μl **Br-QMP** (7.5 mM) in acetonitrile/DMF (v/v = 2/1). After 24 hours, no reaction happened at either room temperature or 37°C (The RP-HPLC showed no change).

The failure of coupling may be that the peptide-PNA chain folds by itself and the amine group at the 5' position is hindered by the PNA chain. Several groups showed that Mini-PEG may elongate the peptide chain and keep its amine group far away from the PNA backbone.^{56,57} **S2** was synthesized and coupled with Br-QMP following the conditions in 3.2.1 (**Scheme 3.5**).

100 μl **S2** (1.0 mM) dissolved in MOPS (250mM, pH= 7.5) was mixed with 100 μl **Br-QMP** (3.5 mM) in acetonitrile/DMF (v/v = 2/1). HPLC showed that coupling happened after 10 hours at room temperature. Examined by MALDI-TOF, the found

peak is consistent with the coupling product **S2-Br-QMP**. Calculated mass = 4354.7, MALDI found mass: 4353.9 (**Figure 3.2**)



Scheme 3.5 Coupling between **Br-QMP** and conjugate **S2**

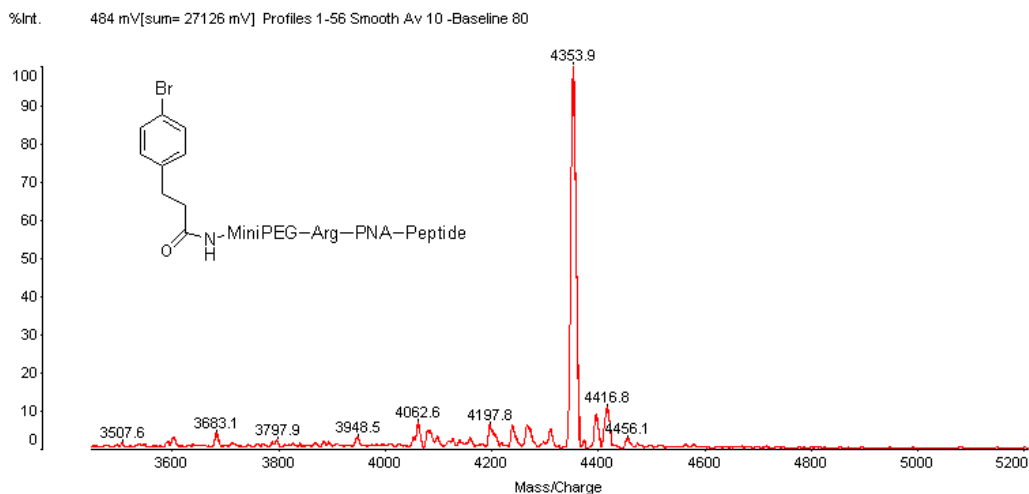


Figure 3.2 MALDI of **S2-BrQMP** (Br-QMP-MiniPEG-R-GTTAGGGTTAG-LRRASLG), Calculated mass: 4354.7, MALDI found: 4353.9

Then the same condition was applied to couple final **QMP1** with **S2**. 100 μ l **S2** (1.0 mM) dissolved in MOPS (250mM, pH= 7.5) was mixed with 100 μ l **QMP1** (3.5 mM) in acetonitrile/DMF (v/v = 2/1) and incubated at room temperature. 10 hours later, the mixture was purified by C18 RP-HPLC (**Figure 3.3**). The 32 minutes eluent

were collected and examined by MALDI. The calculated mass is 4478.9, MALDI found mass is 4477.6 (**Figure 3.4**).

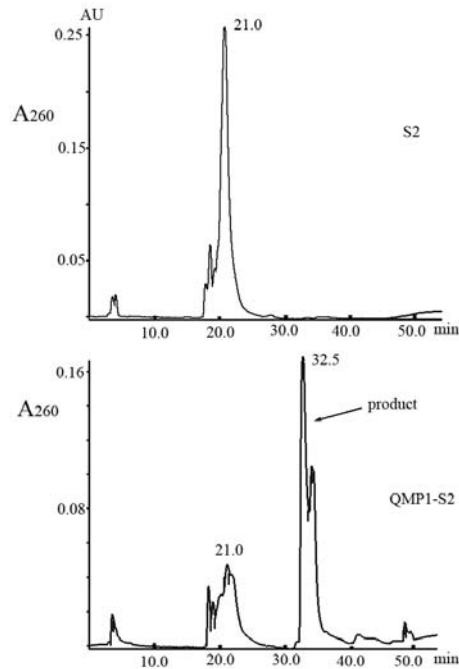


Figure 3.3 RP-HPLC of peptide-PNA-Arg-PEG-QMP conjugate, 32.5 minute eluent was collected and tested by MALDI

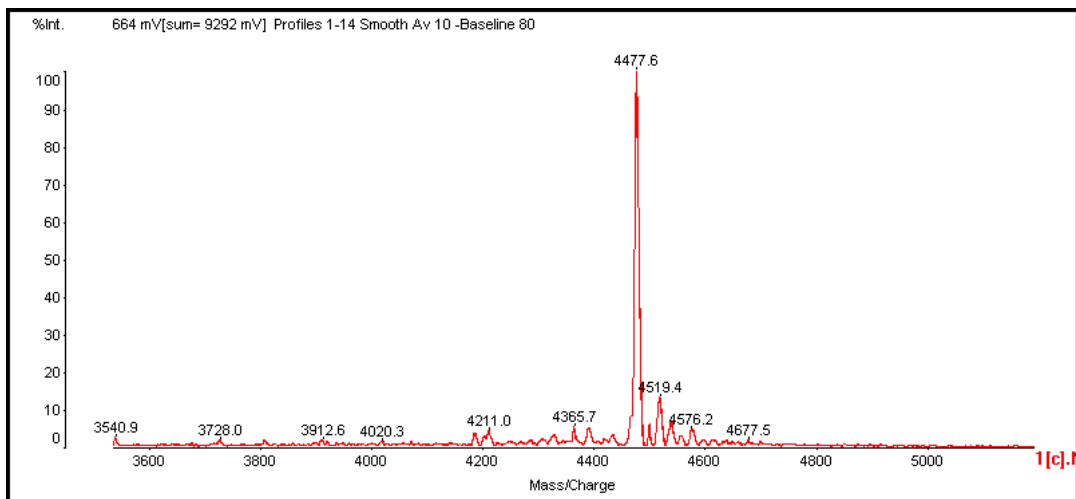


Figure 3.4 MALDI of peptide-PNA-Arg-PEG-QMP conjugate, Calculated mass: 4478.9, MALDI found: 4477.6

3.2.4 Formation of self-adduct

3.5 mM **QMP1** in MOPS (250 mM, pH = 7.5) was added to 1.0 mM **S2** in acetonitrile/DMF (v/v = 2/1) at room temperature. The resulting solution was purified by RP-HPLC after 24 hours. White solid was collect after lyophilization. The formation of self-adduct was accomplished using the protocol from previous paper.²⁷ 200 μ l KF (1.0 M) was added into 100 μ l 2-(N-morpholino)ethanesulfonic acid (MES) (20 mM, pH 7.0) that contains **S2-QMP1** conjugate (Ca. 20 μ M) at room temperature. The resulting solution was incubated at room temperature for 8 hours. Self-adduct of **S2-QMP1** was purified by RP-HPLC. The mass is confirmed by MALDI (**Figure 3.5**). Calculated mass is 4305.8, MALDI found mass is 4304.7.

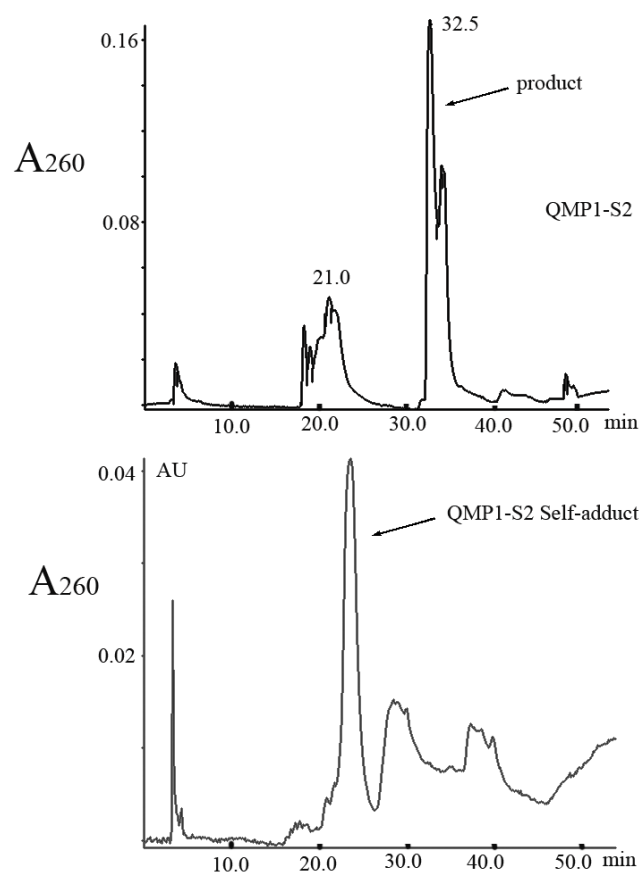


Figure 3.5 RP-HPLC of peptide-PNA-Arg-PEG-QM self-adduct

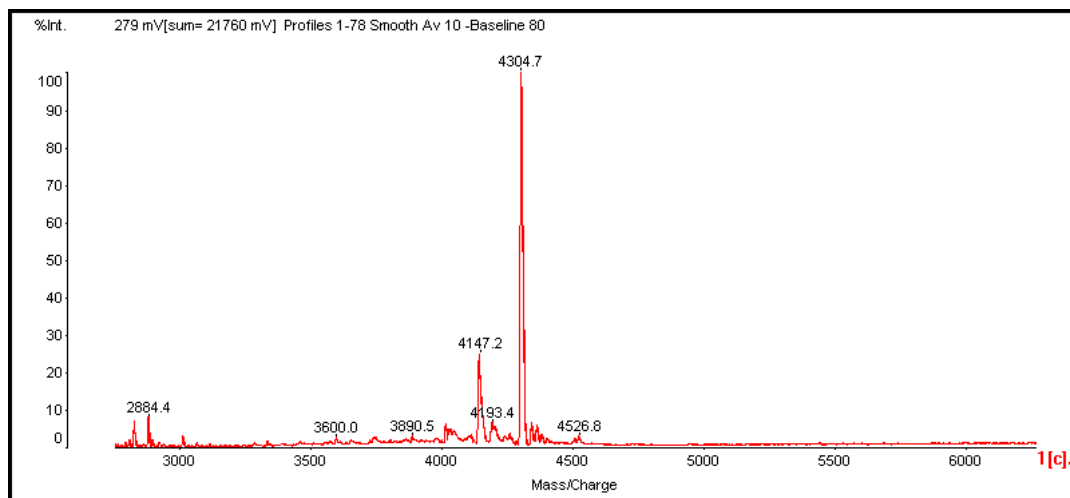


Figure 3.6 MALDI of peptide-PNA-Arg-PEG-QM self-adduct, calculated mass: 4305.8, MALDI found: 4304.7

3.2.5 Conclusion:

Succinimidyl activated QMP was coupled with peptides in Mops-acetonitrile-DMF system. The *tert*-butyloxycarbonyl-8-amino-3,6-dioxaoctanoic acid is essential in coupling QMP with peptide-PNA conjugates. RP-HPLC was successfully used to monitor the reaction and purify product. In the presence of KF, the **S2-QMP1** conjugate can be converted to its corresponding self-adduct. The self-adduct may alkylate complementary DNA sequence later.

3.3 Experimental

Synthesis of *N*-hydroxysuccinimidyl-3-(3-bromophenyl)propionate (Br-QMP)

1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDCI) (422 mg, 2.20 mmol) was added to a 50 ml round bottom flask containing 3-(3-bromophenyl)propionate acid (458 mg, 2.00 mmol) and *N*-hydroxysuccinimide (253 mg, 2.20 mmol) in 12 ml DMF at 0°C. The resulting yellow solution was then stirred for 20 hours at room temperature,

diluted with brine, and extracted with 30 ml ether 3 times. The organic phase were combined and washed with brine, dried over MgSO_4 , and concentrated under reduced pressure. The white precipitate appeared at the bottom of the flask. Ethyl ether (5 ml) was added to the flask to wash the white solid and the solid was collected by filter the solution. The solid was washed by additional 10 ml ether and dried under vacuum to yield Br-QMP as a white solid 380 mg. Yield: 58.5%

$^1\text{H NMR}$ (CDCl_3) δ 7.40 (d, $J= 8.4$, 2H), 7.08 (d, $J= 8.4$, 2H), 2.98 (t, $J = 7.6$, 2H), 2.86 (m, 2H), 2.82 (s, 4H).

3-[3-Hydroxymethyl-4-hydroxyphenyl]propionic acid (3.2)

Ca. 5 ml 10% NaOH aqueous solution was added to 3-(4-hydroxyphenyl) propionic acid (2.0 g, 12 mmol), and the pH was adjusted to 8 by NaOH solution. Formaldehyde (37%, 5 mL) was added to the resulting solution and stirred at 50°C for 17 hours. The resulting yellow solution was poured into 100 ml acetone. The orange oil was collected from the bottom of the flask. The oil was then mixed with 5 ml methanol and poured into 150 ml acetone to form a white precipitate. The solid was collected by filtration.

The solid was then dissolved in 10 ml water and 1 M HCl was added to adjust the pH to 3. The aqueous solution was extracted with 50 ml ether 3 times. After the removal of ether by rotoevaporator, the remaining liquid was purified by chromatotron (hexane : ethyl acetate = 2 : 1 and 0.5% HOAc) and yielded 3.2 as a faint yellow solid (500 mg, 21.2%).

$^1\text{H NMR}$ (D_2O) δ 7.04 (s, 1H), 6.96 (s, H), 6.72 (s, H), 4.48 (s, 2H), 2.72 (t, $J=8$, 2H), 2.52 (d, $J=8$, 2H).

The ^1H NMR is consistent with the literature²⁷

3-[3-*tert*-Butyldimethylsilyloxymethyl-4-*tert*-butyldimethylsilyloxyphenyl] propionic acid (3.3)⁶⁰

Imidazole (1.02 g, 15 mmol) was added to a solution of *tert*-butyldimethylsilyl chloride (TBDMSCl, 1.50g, 10 mmol) and 3.2 (500 mg, 2.55 mmol) in 12 ml of DMF. The mixture was stirred at room temperature for 15 hours, diluted with brine (100 mL), and extracted with 200 mL ether for 3 times. The organic phases were combined, dried over MgSO_4 , and concentrated under reduced pressure. The product was redissolved in 10 ml MeOH and 1.50 g of potassium carbonate was added to the methanol solution. The solution was stirred for 3 h and adjusted with 0.2 M HCl to pH = 3. The mixture was then diluted with water and extracted with 100 ml ether for 3 times. The organic phases were combined, washed with brine, dried over MgSO_4 , and concentrated under reduced pressure. The residue was purified by silica gel chromatotron (hexane: ethyl acetate= 19:1) to yield **3.3** as a colorless oil (400.5 mg, 36.9%).

^1H NMR (CDCl_3) δ 7.26 (s, 1H), 6.93 (d, J = 8.0, 1H), 6.65 (d, J = 8.0, 1H), 4.17 (s, 2H), 2.87 (t, J = 8.0, 2H), 2.63 (t, J = 8.0, 2H), 0.98 (s, 9H), 0.18 (s, 6H).

The ^1H NMR is consistent with the literature²⁷

3-[3-Acetoxymethyl-4-*tert*-butyldimethylsilyloxyphenyl] propionic acid (3.4)⁶⁰

Solid ferric chloride (10 mg, 0.62 mmol) was added to a solution of **3.3** (200.0 mg, 0.83 mmol) in acetic anhydride (3 mL) at 0 °C. The reaction mixture was stirred for 30 minutes and then diluted with 150 mL ether three times. All the organic phase was combined. The combined organic phases were washed with water and saturated

NaHCO₃, dried with MgSO₄, and concentrated under reduced pressure. The residue was subjected to silica gel flash chromatography (hexane: ethyl acetate= 10:1) and yielded **3.4** as a colorless liquid (44 mg, 27%).

¹H NMR (CDCl₃) δ 7.13 (s, 1H), 7.03 (d, *J*= 8.0, 1H), 6.74 (d, *J*= 8.0, 1H), 5.06 (s, 2H), 2.87 (t, *J*= 8.0, 2H), 2.63 (t, *J*= 8.0, 2H), 0.97 (s, 9H), 0.21 (s, 6H).

The ¹H NMR is consistent with the literature²⁷

***N*-Succinimidyl-3-(3-acetoxymethyl-4-*tert*-butyldimethylsilyloxyphenyl) propionate (QMP1)²⁷**

N-Hydroxysuccinimide (46 mg, 0.42 mmol) was added to a DMF solution (5.0 mL) of **3.4** (40 mg, 0.11 mmol). This mixture was cooled to 0 °C and combined with EDCI (80 mg, 0.42 mmol). The resulting yellow solution was then stirred for 20 hours from 4 °C to room temperature, diluted with brine, and extracted with 30 mL ether for 3 times. The organic phase was combined and washed with brine, dried over MgSO₄, and concentrated under reduced pressure. The resulting residue was subjected to silica gel flash chromatography (hexane: ethyl acetate, 3:1) to yield **QMP1** as a white solid (24 mg, 47.0%).

¹H NMR (CDCl₃) δ 7.14 (s, 1H), 7.05 (d, *J*= 8.0, 1H), 6.75 (d, *J*= 8.0, 1H), 5.07 (s, 2H), 2.97 (t, *J*= 8.0, 2H), 2.88 (t, *J*= 8.0, 2H), 2.82 (s, 4H), 2.08 (s, 3H), 0.97 (s, 9H), 0.21 (s, 6H)

¹H NMR is consistent with the literature²⁷

Coupling between QMP1 and S2 (NH₂-Mini PEG-Arg-PNA-peptide)

100 μl **QMP1** (3.5 mM) in CH₃CN/DMF (v/v = 2/1) was mixed with the appropriate 5'-amino peptide-PNA conjugate (100 μl, 1.0 mM) in MOPS buffer (250

mM, pH 7.5) and incubated for 24 hours at room temperature to generate **S2-QMP1** conjugate. The conjugates were purified by C18 RP-HPLC and monitored at 260 nm. The eluents were 0.1% TFA in water and 0.1% TFA in acetonitrile. The gradient was 10 - 55% in 30 minutes. The 31 - 35 minute eluents were collected and lyophilized. The product was confirmed by MALDI-TOF.

MALDI-TOF (*m/z*) calculated for the conjugate of **S2-QMP1** was 4478.9, and MALDI-TOF (*m/z*) found was 4477.6.

Self-Adduct of the S4-QMP1 conjugate

200 μ l KF (1.0 M) was added to MES (25 mM, pH 7.0) buffer that contains **S2-QMP1** conjugate (20 μ M) at room temperature. Self-adduct of **S2-QMP1** was generated after 8 hours. The conjugates were purified by C18 RP-HPLC at 260 nm. The eluents were 0.1% TFA in water and 0.1% TFA in acetonitrile. The gradient was 10 - 55% in 30 minutes. The 22 - 26 minute eluents were collected and lyophilized. The successful generation of self-adduct was confirmed by MALDI-TOF.

MALDI-TOF (*m/z*) calculated for the S2-QM self-adduct was 4305.8, and MALDI-TOF (*m/z*) found was 4304.7.

Chapter 4 Conclusion

Quinone methides (QMs) can alkylate DNA and thus have a potential to act as anti-cancer drugs. In order to improve the target specificity of QM, peptide nucleic acids (PNAs) were studied as a delivery agent. PNA oligomers are resistant to degradation by nucleases and proteases, bind with high sequence specificity to complementary DNA, and form PNA/DNA duplex with higher stability than DNA/DNA duplex. It is important to study the synthesis of PNAs and the ability of PNAs as QM delivering agents.

This thesis focused on the practical synthesis of various peptide-PNA conjugates by solid phase peptide synthesis (SPPS). From the synthesis of a 7mer peptide, coupling the peptides with a quinone methide precursor (QMP) could be optimized by reacting under different kinds of conditions such as reaction concentration, reaction time and optimal cleavage conditions. Because PNA monomers are more difficult to dissolve in DMF than amino acids, they were normally mixed with DMF and vortexed for several minutes to help the dissolution. PNA monomers also stick to the surface of glassware, and thus plastic tubes were used to hold all the PNA solution.

C18 Reverse-Phase HPLC (RP-HPLC) also showed the ability to purify both peptides and peptide-PNA conjugates at room temperature. The gradient of acetonitrile was from 2% to 35% in 30 minutes. Peptide-PNA and peptide-PNA-QMP conjugates were eluted out during 20% to 35% acetonitrile.

Since the peptide-PNA-QMP conjugate is a key compound in selective delivery of QMP, conditions for coupling peptide-PNA conjugates with QMP are evaluated. 8-

amino-3,6-dioxaoctanoic acid (mini-PEG) shows an essential linker in coupling QMP with PNA conjugates. Experiments also showed that peptide-PNA conjugated dissolved in MOPS buffer (pH = 7.5) and QMP in acetonitrile/DMF = 2/1 guaranteed the desired product. MES buffer works for generation of PNA-QM self-adduct by adding KF aqueous solution.

Appendices

Appendix 1 Vials for SPPS



Instructions for the following tables: The sheets can be printed and followed in reactions. These sheets mark each step of the sequence--giving the reagents used and the times required. Working down a column, each block can be checked off as that particular step is completed. When a column is finished, goes to the top of next column.

Appendix 2 Procedures for peptide synthesis

resin 100 mg (0.7 mmol/g, 70 μ mol) was swelled in DMF for three hours								
3-GlyLeuSerAlaArgArgLeu-PNA-Arg-QMP								
AA monomers	G	Capping	L	S	A	R	R	L
95% TFA/ m-cresol (2 \times 4 min)	×	×						
DCM wash (3 \times 0.5 min)	×	×						
Kaiser test		×						
DMF wash (1 \times 1 min)		×						
DCM wash (2 \times 0.5 min)		×						
Coupling (20 min)		Capping						
DMF wash (2 \times 1 min)								
DCM wash (2 \times 1 min)								
Kaiser test								
Next column								

Appendix 3 Procedures for PNA synthesis

Resin-kemptide 60 mg (0.7 mmol/g, 42 μ mol) was swelled in DMF for three hours														
3-GlyLeuSerAlaArgArgLeu-GATTGGGATTG-Arg-miniPEG-QMP														
PNA monomers	G	Capping	A	T	T	G	G	G	G	G	A	T	T	G
95% TFA/ m-cresol (2 \times 4 min)		\times						\times		\times				
DCM wash (2 \times 0.5 min)		\times						\times		\times				
NMP wash (1 \times 0.5 min)		\times						\times		\times				
DCM wash (2 \times 0.5 min)		\times						\times		\times				
Kaiser test		\times								\times				
DMF wash (1 \times 1 min)		\times						\times						
DCM wash (2 \times 0.5 min)		\times						\times		\times				
Coupling (20 min)		Capping												
DMF wash (2 \times 1 min)														
DCM wash (2 \times 1 min)														
Kaiser test	\times													
Next column														

Appendix 7 Amount of amino acids used in SPPS

resin 100 mg (0.7 mmol/g, 70 μ mol)								
	resin	G	L	S	A	R	R	L
M.W. (g/mol)		175	249	295	182	465	465	249
Eq. to resin		5	5	4	5	4	4	5
μ mol	70	350	350	280	350	280	280	350
mg	100	61.2	87.2	82.6	63.7	130.2	130.2	87.2
HBTU (mg)		170.6	170.6	136.4	170.6	136.4	136.4	170.6
DIEA (μ l)		57.8	57.8	46.2	57.8	46.2	46.2	57.8
DMF (ml)		2.5	2.5	2.0	2.5	2.0	2.0	2.5
Conc. (mM)		140	140	140	140	140	140	140

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