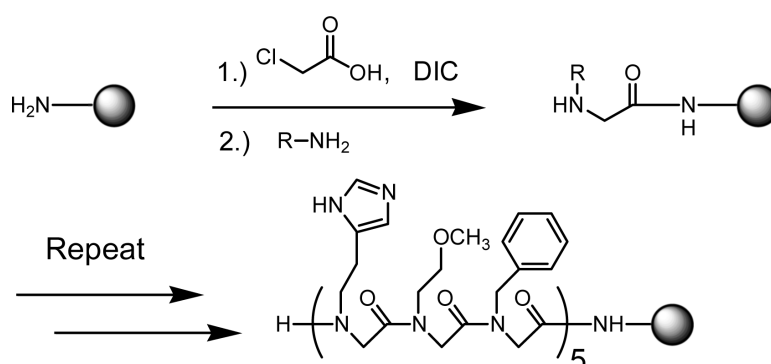Article

Incorporation of Unprotected Heterocyclic Side Chains into Peptoid Oligomers via Solid-Phase Submonomer Synthesis

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Incorporation of Unprotected Heterocyclic Side Chains into Peptoid Oligomers via Solid-Phase Submonomer Synthesis

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Abstract: Peptoids (N-substituted glycines) are an important class of biomimetic oligomers that have made a significant impact in the areas of combinatorial drug discovery, gene therapy, drug delivery, and biopolymer folding in recent years. Sequence-specific peptoid oligomers are easily assembled from primary amines by the solid-phase submonomer method. However, most amines that contain heterocyclic nitrogens in the side chain do not incorporate efficiently. We present here a straightforward revision of the submonomer method that allows efficient incorporation of unprotected imidazoles, pyridines, pyrazines, indoles, and quinolines into oligomers as long as 15 monomers in length. This improved method uses chloroacetic acid instead of bromoacetic acid in the acylation step of the monomer addition cycle, and allows for the incorporation of new side chains that should enable the synthesis of peptoids with entirely new properties.

Introduction

Unnatural sequence-specific heteropolymers are a new class of bioinspired materials that are beginning to mimic some of the structural and functional properties of proteins.[1] These synthetic materials must be made in a stepwise manner by the addition of one monomer unit at a time with extremely high coupling efficiencies and with a reasonable diversity of side chain functionalities. Chemists have developed a wide variety of such oligomeric systems, including β-peptides,[2] γ-peptides,[3] peptoids,[4] @-tides,[5] azatides,[6] oligopyrrolinones,[7] oligoureas,[8] oligocarbamates,[9] oligoanthranilamides,[10] and oligosulfones.[11] These oligomers all vary in their backbone composition, linking chemistry, mode of synthesis (solution-phase vs solid-phase), side chain chemical diversity, ease of monomer synthesis, and efficiency of monomer coupling. Yet, they are all unique structural solutions to the problem of peptide and protein mimicry with an unnatural oligomeric backbone. As a group, these materials have shown utility in crossing membranes, specifically killing bacteria, binding receptors, disrupting protein−protein interactions, forming defined secondary and tertiary structures, and resisting protease degradation.

N-Substituted glycines (peptoids) have emerged as a particularly versatile and synthetically accessible heteropolymer. Peptoids with chiral side chains can fold into helices[12] and assemble into discrete multimers exhibiting tertiary structure.[13] Peptoid oligomers containing cationic groups can efficiently cross cellular membranes,[14] can condense and deliver DNA to cells with excellent efficiency and low cellular toxicity,[15] and can specifically target RNA−protein interactions.[16] Short pep-

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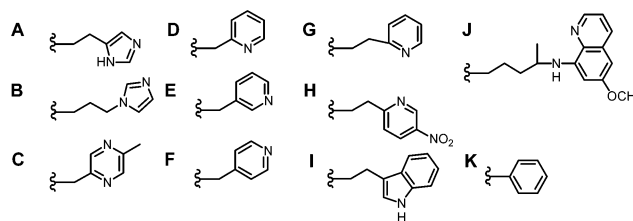
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toid oligomers have been shown to exhibit antimicrobial activity in cells,[17] and bind a variety of cellular receptors with high affinity.[18] Peptoid oligomers also resist protease degradation,[19] but can be readily sequenced by Edman degradation or by mass spectrometry.[13,20] Peptoid−peptide hybrids have been shown to adopt collagen-like structures,[21] increase the potency and selectivity of peptidic ligands,[22] and increase the membrane permeability of peptides.[23]

X = Br or Cl; R = peptoid side chain.
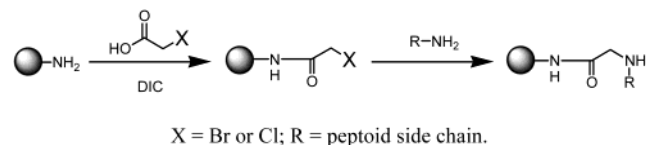
Peptoids can be synthesized using a solid-phase submonomer synthesis protocol that is based on a two-step monomer addition cycle.[4a] The method is unique in that each monomer is assembled on the solid support from two simple submonomers, without the need of main chain protecting groups. The first step is the acylation of the resin-bound amine with bromoacetic acid (where X = Br) activated in situ with *N,N′*-diisopropylcarbodiimide (DIC). The second is the displacement of the bromide with a primary amine. This highly efficient cycle has been repeated up to 48 times.[15a] Hundreds of monomers can be readily incorporated into peptoids using the solid-phase submonomer method that span a wide variety of side chain functionalities. However, some very important and pharmaceutically relevant

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Chart 1. Heterocyclic Side Chains[a]

[a] Side chains derived from the following commercially available amine submonomers: (**A**) histamine, (**B**) 1-(3-aminopropyl)imidazole, (**C**) 2-aminomethyl-5-methylpyrazine, (**D**) 2-(2-aminomethyl)pyridine, (**E**) 3-(2-aminomethyl)pyridine, (**F**) 4-(2-aminomethyl)pyridine, (**G**) 3-(2-aminoethyl)pyridine, (**H**) 2-(2-aminoethyl)-5-nitropyridine, (**I**) tryptamine, (**J**) (*R,S*)-primaquine, (**K**) aniline.

heterocyclic side chains (e.g., imidazole, pyridine, pyrazine, and quinoline side chains) have been difficult to incorporate efficiently. When an unprotected heterocyclic nitrogen-containing side chain is present within the peptoid, product yields can drop precipitously, and the solid support often appears dark purple in color. Here, we present a simple modification to the standard submonomer synthesis conditions that significantly reduces the extent of side product formation and increases the overall synthetic yield.
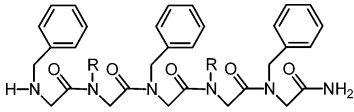
Results and Discussion

Acylation Step. Initial efforts to incorporate heterocyclic side chains focused on the optimization of the acylation step. Tryptamine has previously been incorporated into peptoid oligomers, but at lower yields than other nonheterocyclic side chains. Heterocycle-containing primary amine submonomers have also been successfully incorporated at the N-terminal position of a peptoid,[24] where no further bromoacetylation steps were performed. However, under standard conditions,[12a] attempts to incorporate other amine submonomers with significantly more nucleophilic side chain nitrogens (Chart 1) in the interior of a peptoid oligomer were not successful. The crude oligomer product was an uncharacterizable complex mixture, and the overall yields were unacceptable.

Bromoacetic acid activated with DIC is a potent acylating agent. In the presence of peptoids containing unprotected heterocyclic side chains, activated bromoacetic acid would be expected to temporarily acylate most side chain aromatic nitrogens. However, as in the case of using unprotected histidine in peptide synthesis,[25] these reactions are readily reversed in the presence of base. Because the second step of the submonomer cycle involves treatment with a high concentration of amine, this unwanted acylation was not thought to be the problem. Instead, efforts were focused on reducing the unwanted alkylation of the aromatic nitrogen by the activated bromoacetic acid. Normally, during submononomer synthesis, this alkylation side reaction is not observed, because acylation of an N-terminal secondary amine is not reversible and is approximately 1000 times faster than alkylation. We hypothesized that the reversibility of the heterocycle acylation may allow the irreversible alkylation side products to slowly accumulate during the acylation step. It is known that the relative reaction rate of a chloride leaving group is approximately 40 times less than that

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Table 1. Comparison of Bromoacetic Acid with Chloroacetic Acid for 5-mers with Two Test Side Chains

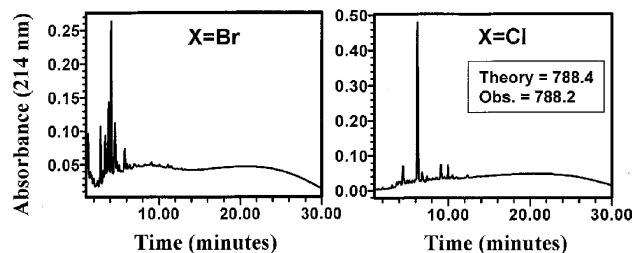|			bromoacetic acid			chloroacetic acid		
compound	R	molecular weight	purity (%)	yield (%)	mol. wt. obs.	purity (%)	yield (%)	mol. wt. obs.
1	A	760.2	34	74	760.4	89	81	760.4
2	B	788.4	38	76	788.2	82	84	788.2
3	C	785.2	83	81	784.4	83	83	784.4
4	D	754.2	<10	43	754.0	78	79	754.4
5	E	754.2	13	65	754.4	92	76	754.4
6	F	754.2	<10	58	754.6	87	71	754.4
7	G	782.4	36	50	782.0	86	79	782.2
8	H	902.0	74	72	902.4	92	82	902.4
9	I	858.4	44	81	858.4	82	83	858.4
10	J	1056.7	83	93	1056.7	95	92	1056.7
11[a]	K	724.2	86	79	724.1	< 5	53	724.2
11[b]	K	724.2	87	77	724.1	87	86	724.2

[a] 2 M aniline in DMF. *[b]* 2 M aniline in DMF with 1 M KI.

of a bromide.[26] Therefore, we further hypothesized that by switching to the less labile halo-acid, chloroacetic acid (where X = Cl), the selectivity between the acylation step and the unwanted alkylation would be increased, resulting in reduced irreversible heterocycle alkylation with potentially little effect on submonomer synthesis efficiency.

To investigate the extent of alkylation during oligomer synthesis, test sequences containing the relatively nucleophilic *N*-propyl imidazole side chain were synthesized with chloroacetic acid. Interestingly, as the per cycle acylation time was reduced, the product yield and purity increased. A similar trend was observed when the concentration of the acylating reagent was reduced, consistent with increased selectivity for the acylation reaction. With a reduction in the time from 40 min to 5 min and a reduction in the concentration of the acylating reagent from 1.2 to 0.4 M, the percent yield was increased from ∼20% to ∼70% (data not shown). No deletion products resulting from incomplete acylation steps were observed. Typically, solid-phase synthesis reagents are used in large excess for an extended period of time to ensure reaction completion. In contrast, this situation requires a reactivity balance between acylation and alkylation.

Using these optimal acylation conditions, we compared the crude product purities of a series of 5-mers containing a variety of different heterocyclic side chains, synthesized with either chloroacetic or bromoacetic acid (Table 1). In several of the cases, less than a 30% purity of the desired product and a significant amount of byproducts formed when bromoacetic acid was used, whereas excellent purities were obtained when chloroacetic acid was used. Sequences containing the pyridyl group exhibited the highest levels of impurities, yielding <10% of the pure product (Figure 1). The pyrazine (**C**), indole (**I**), and quinoline (**J**) side chains appeared to be well tolerated under both conditions.

Displacement Step. Although the reduced reactivity of the chloride during the acylation step slows the unwanted alkylation side reactions, it has an unfavorable effect on the displacement
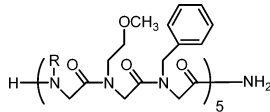
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Figure 1. HPLC traces comparing the crude synthesis products from bromoacetic acid and chloroacetic acid synthesis conditions for compound **4** (reversed-phase HPLC C$_4$, 5−95% acetonitrile/water + 0.1% TFA, linear gradient for 30 min, flow = 0.8 mL/min).

Table 2. Comparison of Halogen Exchange Conditions for the Synthesis of Compound **11**

| reaction conditions	halide exchange step	displacement step	purity (%)
a	none	2.0 M amine in NMP	<5
b	1.0 M tetrabutylammonium bromide in NMP	2.0 M amine in NMP	31
c	1.0 M tetrabutylammonium iodide in NMP	2.0 M amine in NMP	23
d	1.0 M KI in DMF	2.0 M amine in NMP	92
e	1.0 M KBr in DMF	2.0 M amine in NMP	90
f	0.6 M CsI in DMF	2.0 M amine in NMP	70
g	1.0 M KBr with 1 equiv of 18-crown-6 in NMP	2.0 M amine in NMP	40
h	1.0 M LiBr with 1 equiv of 12-crown-4 in NMP	2.0 M amine in NMP	44
i	none	2.0 M amine with 1.0 M KI in DMF	87

step. To offset the lower reactivity of the chloride, the amine concentration was increased to 2 M in NMP and the reaction time was extended to 1 h. Submonomer amines with poor nucleophilic character such as aniline (**K**) were difficult to couple under these conditions (compound **11**, method **a**). A series of deletion products were observed, with the desired product accounting for only 5% of the total yield. For these weakly nucleophilic amine submonomers, additional measures were taken. To improve the halogen leaving group ability, the chloride of the resin-bound chloroacetamide was exchanged with a more reactive halogen, either bromide or iodide.[27] Prior to the displacement step, a series of different reagents and reaction conditions were investigated (Table 2). A separate halogen exchange step was introduced into the synthesis protocol for this set of monomers. It was clear that the displacement efficiency was improved for conditions **d** and **e**, but at the expense of total synthesis time. The optimal conditions were found to be the in situ halogen exchange with iodide, where the KI is present with the 2.0 M amine in the DMF solvent (1 M KI in DMF was used to dissolve the amine). Presumably, the iodide exchange with the chloroacetamide is the rate-limiting step before its displacement with the amine. During the displacement step, the temperature was increased to 45 °C and the reaction time was extended to 180 min. These changes improved the aniline 5-mer yield from ∼5% to over 85% (compound **11**, method **i**). These modifications need only be employed during monomer addition cycles containing members of this subset of amines.

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Table 3. Comparison of Bromoacetic Acid with Chloroacetic Acid for 15-mers with Five Test Side Chains

compound	R	molecular weight	bromoacetic acid			chloroacetic acid		
			purity (%)	yield (%)	mol. wt. obs.	purity (%)	yield (%)	mol. wt. obs.
12	**A**	2082.5	<10	32	N.D.	81	80	2083.5
13	**B**	2152.9	<10	27	N.D.	68	81	2154.0
14	**C**	2145.0	<10	26	N.D.	78	92	2143.5
15	**D**	2067.5	<10	34	N.D.	86	78	2068.5
16	**I**	2328.0	33	69	2328.0	74	82	2328.0
17	**J**	2823.8	48	74	2823.8	94	91	2823.8
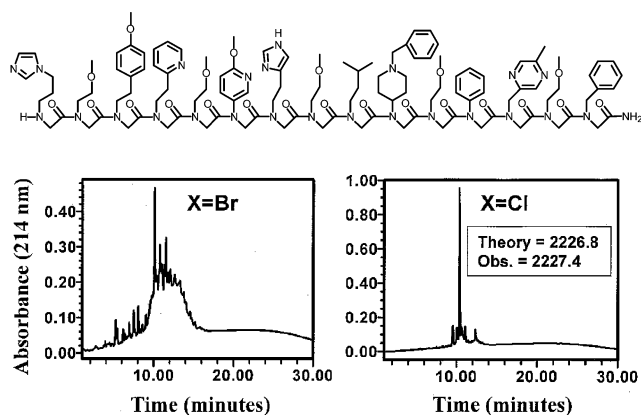
Figure 2. HPLC traces comparing the crude products from the synthesis of a diverse 15-mer peptoid using bromoacetic acid versus chloroacetic acid.

The changes in the synthesis conditions allowed for the incorporation of most of the previously accessible monomer side chains (in addition to the new class of side chains) at sequence lengths of up to 15-mers (Table 3). The trends observed in the 5-mers were even more pronounced when analogous 15-mers were prepared. After 15 bromoacetylation steps, the desired products were almost undetectable, and the resin was black in color. Both pyrazine (**C**)- and quinoline (**J**)-containing peptoids, which were relatively pure as 5-mers (compounds **3** and **10**), were very impure as 15-mers (compounds **14** and **17**). In contrast, the peptoids prepared with chloroacetic acid under identical reaction conditions had an average purity of >80% desired product, and the resin was only slightly yellow.

Diverse 15-mer. The optimized reaction conditions for each class of monomers were then used to make a 15-mer sequence encompassing the diversity of side chains that can now be included. The sequence contained six different side chains that have unprotected heterocyclic nitrogens and two aniline-like side chains. A final direct comparison between the products of a chloroacetic acid versus a bromoacetic acid synthesis was made (Figure 2). The 15-mer prepared with chloroacetic acid was 82% pure and exhibited the correct molecular weight by mass spectrometry. By contrast, the product produced with bromoacetic acid was almost undetectable.

Conclusion

In conclusion, the use of chloroacetic acid during peptoid synthesis allows for the efficient incorporation of an entirely

new set of heterocyclic side chains that were previously inaccessible under prior submonomer conditions. This method is easily automated and provides a less laborious alternative to a full monomer synthesis[28] or to the preparation of protected heterocyclic submonomers. Side chains that are important for their ability to chelate metals, catalyze molecular transformations, exhibit unique spectroscopic properties, and mimic "drug-like" moieties are now readily accessible, thus expanding significantly the peptoid side chain toolbox.

Experimental Section

General Methods. Histamine free base was purchased from Calbiochem (San Diego, CA). 1-(3-Aminopropyl)imidazole, 2-(2-aminomethyl)pyridine, 4-(2-aminomethyl)pyridine, 3-(2-aminoethyl)-pyridine, 2-(2-aminoethyl)-5-nitropyridine, benzylamine, tryptamine, 4-amino-1-benzyl piperidine, 5-amino-2-methoxypyridine, bromoacetic acid, chloroacetic acid, piperidine, and trifluoroacetic acid were purchased from Aldrich (Milwaukee, WI). (*R,S*)-Primaquine was purchased from ICN Biomedicals (Aurora, OH). Aniline and 2-(4-methoxyphenyl)ethylamine were purchased from Alfa Aesar (Ward Hill, MA). 2-Aminomethyl-5-methylpyrazine, 3-(2-aminomethyl)pyridine, isoamylamine, and 2-methoxyethylamine were purchased from TCI (Portland, OR). *N,N′*-Diisopropylcarbodiimide was purchased from Chem-Impex International (Wood Dale, IL). Solvents were purchased from Mallinckrodt Baker (Phillipsburg, NJ). All reagents and solvents were used without further purification. The free base of primaquine was prepared from the diphosphate salt using 1.0 equiv of NaOH as described.[29] Fmoc-protected Rink amide polystyrene resin (Novabiochem, San Diego) with a substitution level of 0.57 mmol/g was used for all oligomer syntheses.

Peptoid Synthesis. The peptoid oligomers were synthesized on an automated peptoid/peptide synthesizer.[30] Each peptoid sequence was prepared following this general procedure:

Fmoc Deprotection. For each oligomer synthesis, 88 mg of resin (50 μmol) was deprotected with 2.0 mL of 20% piperidine in *N,N′*-dimethylformamide (DMF) for 20 min, followed by draining and then washing with DMF (5 × 2.0 mL).

Two-Step Monomer Addition Cycle. Step 1: Acylation. To the resin-bound amine at 35 °C was added a 0.4 M solution of the haloacetic acid (either bromoacetic or chloroacetic acid) (850 μL, 0.34 mmol) in DMF followed by 2.0 M *N,N′*-diisopropylcarbodiimide (DIC) (200 μL, 0.40 mmol) in DMF. The resin was mixed for 5 min, drained, and then washed with DMF (5 × 2.0 mL).

Step 2: Displacement. The resin-bound halogen was then displaced with the primary amine submonomer. To the resin-bound halogen at 35 °C was added a 2.0 M solution of the primary amine (0.85 mL, 1.7 mmol) in *N*-methylpyrrolidinone (NMP), and this was allowed to react for 60 min. The displacement step for cycle #1 was extended to 180 min due to incomplete displacement at 60 min. The aromatic amine submonomers (aniline and 5-amino-2-methoxypyridine) were dissolved at a concentration of 2.0 M in DMF containing 1.0 M potassium iodide (KI). The time of the displacement step was extended to 180 min, and the temperature was increased to 45 °C for these monomers. In all cases, after the displacement reaction, the resin was drained and then washed with DMF (6 × 2.0 mL).

The monomer addition cycle was repeated until the desired oligomer length was achieved.

Cleavage Protocol. The peptoid product was cleaved from the resin in a cleavage cocktail of 49:49:2 trifluoroacetic acid/dichloroethane/

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water (v/v) (6.0 mL) for 60 min at room temperature with gentle stirring. The cleavage solution was filtered to remove the resin beads, and the filtrate was evaporated under a stream of nitrogen, resuspended in 1:1 acetonitrile/water (v/v), and then lyophilized twice. The crude percent yield was calculated on the basis of the weight of the lyophilized product plus the appropriate counterions.

Analytical HPLC. The synthetic purity was evaluated by reversed-phase analytical HPLC on a Waters 2690 system with a photodiode array detector. First, 10 μL of the cleavage solution was evaporated in a 96-well plate and resuspended in 100 μL of 1:1 acetonitrile/water (v/v). From this HPLC sample, 10 μL was injected on to a C4 Column (Duragel G, 3 μm, 300 A, 0.2 × 5.0 cm) using a linear gradient from 5% to 95% of solvent B in solvent A (solvent A = water + 0.1% TFA (v/v), solvent B = acetonitrile + 0.1% TFA (v/v)) at 0.8 mL/min and a column temperature of 60 °C over 30 min.

HPLC/MS. Molecular weights were verified by liquid chromatography/mass spectrometry (LC/MS) using a Hewlett-Packard 1100 MSD series electrospray system in the positive mode with a 125 V cone voltage and a mass range from 400 to 2000 amu. First, 15 μL of the HPLC sample was injected onto a C4 Column (Zorbax, 5 μm, 0.5 × 2.1 cm) using a linear gradient from 5% to 95% of solvent B in solvent A (solvent A = water + 0.05% TFA (v/v), solvent B = acetonitrile + 0.05% TFA (v/v)) at 0.8 mL/min and a column temperature of 60 °C over 3.5 min.

JA0352101