Amino Acids (2014) 46:1297–1303
DOI 10.1007/s00726-014-1691-5

ORIGINAL ARTICLE

Solid-phase synthesis of amidine-substituted phenylbenzimidazoles and incorporation of this DNA binding and recognition motif into amino acid and peptide conjugates

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Received: 22 November 2013 / Accepted: 31 January 2014 / Published online: 23 February 2014

Abstract Amidine-substituted phenylbenzimidazoles are well-established DNA-binding structural motifs that have contributed to the development of diverse classes of DNA-targeted agents; this ring system not only assists in increasing the overall DNA affinity of an agent, but can also influence its site selectivity. Seeking a means to conveniently exploit these attributes, a protocol for the on-resin synthesis of amino acid- and peptide-phenylbenzimidazole-amidine conjugates was developed to facilitate installation of phenylbenzimidazole-amidines into peptide chains during the course of standard solid-phase syntheses. Building from a resin-bound amino acid or peptide on Rink amide resin, 4-formyl benzoic acid was coupled to the resin-bound free amine followed by introduction of 3,4-diamino-*N*′-hydroxybenzimidamide (in the presence of 1,4-benzoquinone) to construct the benzimidazole heterocycle. Finally, the resin-bound *N*′-hydroxybenzimidamide functionality was reduced to an amidine via 1 M $SnCl_2 \cdot 2H_2O$ in DMF prior to resin cleavage to release final product. This procedure permits the straightforward synthesis of amino acids or peptides that are N-terminally capped by a phenylbenzimidazole-amidine ring system. Employing this protocol, a series of amino acid–phenylbenzimidazole-amidine (Xaa-R) conjugates was synthesized as well as dipeptide conjugates of the general form Xaa-Gly-R (where R is the phenylbenzimidazole-amidine and Xaa is any amino acid).

Keywords DNA binding · Benzimidazole · Amidine · Amino acids · Solid-phase synthesis

Introduction

The development and investigation of biologically active compounds that target DNA through equilibrium binding continue to be active areas of pursuit (Soeiro et al. 2013; Wei et al. 2013; Yang et al. 2013; Glass et al. 2010; Nguyen et al. 2009; Long et al. 2009; Tse and Boger 2004; Dervan and Edelson 2003; Neidle 2001). Among organic structural elements that have assisted in the development of such agents, the benzimidazole heterocycle has proven to be a versatile DNA recognition and anchoring moiety (Viger and Dervan 2006; Ismail et al. 2005; Briehn et al. 2003). These attributes are due to the overall size, shape, and heteroatom positioning of a benzimidazole ring that promote its steric compatibility with DNA minor groove binding and hydrogen bonding (Briehn et al. 2003; Neidle 2001). Indeed, Hoechst 33258, a well-studied agent that contains a bis-benzimidazole core, targets the DNA minor groove and has provided an important paradigm for the understanding of benzimidazole–DNA interactions as well as small molecule recognition of the DNA minor groove in general (Neidle 2001).

Given the above, benzimidazoles have added significantly to the repertoire of heterocycles investigated in the development of DNA-binding polyamides, peptides, and other agents (Briehn et al. 2003; Viger and Dervan 2006; Behrens et al. 2001; Farahat et al. 2011; Ismail et al. 2005; Goodwin et al. 2006; Tanious et al. 2007). In particular,

Electronic supplementary material The online version of this article (doi:10.1007/s00726-014-1691-5) contains supplementary material, which is available to authorized users.

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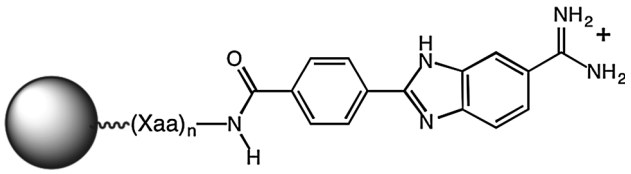

Fig. 1 General structure of a resin-bound amino acid (Xaa, $n = 1$) or peptide ($n > 1$) N-terminally capped with an amidine-substituted phenylbenzimidazole

phenylbenzimidazole-amidines, cationic species under physiological conditions, have led to a host of biologically active antiparasitic agents that target the DNA minor groove (Soeiro et al. 2013; Goodwin et al. 2006; Tanious et al. 2007). Indeed, aromatic diamidines in general containing the phenylbenzimidazole-amidine structural unit have attracted considerable interest due to their antimicrobial activities that are believed derived from DNA binding (Soeiro et al. 2013). The combined effect of the benzimidazole ring and its capacity for minor groove hydrogen bonding, along with the cationic amidine moiety, makes these structural units interesting candidates for the further development of DNA minor groove binding agents based on peptide chemistry.

Given the attributes of phenylbenzimidazole-amidines and with an eye towards the expedited generation of DNA-targeted agents through combinatorial strategies, we sought to develop a convenient on-resin route through which to modify natural or unnatural amino acids and peptides with this structural element or to N-terminally "cap" peptides to augment their DNA-binding activities (Fig. 1). These goals complement prior reported efforts in which: (1) the entire aryl bis-benzimidazole "core" element of Hoechst 33285 was redesigned for inclusion in solid-phase peptide syntheses (Behrens et al. 2001; Bunkenborg et al. 2002), and (2) strategies for the synthesis of unnatural amino acids and compound libraries bearing derivatized benzimidazole side chains (Koprowska-Ratajska et al. 2009; Vourloumis et al. 2003). Differing from these efforts, however, we have developed a solid-phase protocol for installing benzimidazoles that includes an amidine moiety of crucial importance to effective DNA binding and biological activity (Soeiro et al. 2013). Additionally, this strategy focuses on inclusion of single benzimidazole rings. A single ring would be expected to influence to a far less extent the DNA recognition and binding properties of a final product, perhaps working synergistically with other structural elements present in the total structure. Indeed, a complete Hoechst-like bis-benzimidazole structure appears to dominate the DNA recognition of peptides in which it has been included (Behrens et al. 2001).

Herein, we describe a solid-phase protocol that permits the on-resin assembly of phenylbenzimidazole-amidines and the straightforward generation of amino acids or peptides that are N-terminally capped with this moiety. Accordingly, we demonstrate the on-resin synthesis of the above and the syntheses of model end-capped amino acids and dipeptides. While developed and demonstrated here for amino acid and peptide modifications, these same procedures should permit the end capping of any reactive termini compatible with similar solid-phase syntheses.

Materials and methods

All chemicals and organic solvents were of reagent grade, purchased from Sigma-Aldrich or Fisher, and used without further purification. Protected amino acids were purchased from Bachem, and Rink amide resins (loading: 66 mmol/g) were purchased from EMD Millipore. ^{1}H NMR spectra were obtained using a 500-MHz Bruker Avance III NMR spectrometer with a 5-mm broadband probe. LC/MS analyses were performed using an Agilent 1100 series HPLC with a binary pump, auto-sampler, and diode array detector outfitted with a C18 column (150 × 4.6 mm; 5-micron particle size); the MS component was an Agilent 1946D mass spectrometry detector equipped with electrospray ionization.

General solid-phase synthetic procedures

All ambient temperature solid-phase syntheses were performed manually in fritted SPE column reservoirs (15 mL) outfitted with Teflon stopcocks and septa and agitated on an orbital shaker. Manual solid-phase syntheses carried out at elevated temperatures were performed in glass scintillation vials (40 mL) containing a stir bar and placed in a reaction block fitted to a hot plate stirrer with temperature control. Weighed portions of Rink amide resin were distributed to each reaction vessel and swollen in 1:1 DCM:DMF for 30 min to 1 h. Fmoc deprotection was performed by shaking resin, or resin-amino acids and resin-peptides, in 1:4 (v/v) piperidine: DMF for 30 min at room temperature; deprotections were monitored via Kaiser test (Stewart and Young 1984). Amino acid couplings to deprotected portions of Rink amide resin and the generation of resin-bound Gly-Xaa dipeptides prior to phenylbenzimidazole-amidine assembly were carried out using standard manual peptide synthesis protocols (0.25 g Rink amide resin, 0.825 mmol protected amino acid, and equimolar equivalents of HOBt and DIC in 15 mL DMF) (Stewart and Young 1984; Chan and White 2000). Couplings were carried out at room temperature for 1.5 h and verified by Kaiser test. After completion of each coupling step, reactants were drained from reaction vessels and resins or resin-peptides were washed with DMF

(3 × 5 mL), methanol (3 × 5 mL), DCM (3 × 5 mL), and diethyl ether (3 × 5 mL). Solvents were removed by filtration using a vacuum system. All reactions were performed under nitrogen.

Synthesis of 3,4-diamino-*N*′-hydroxybenzimidamide (**1**)

3,4-Diaminobenzonitrile (7.5 mmol, 1.0 g) in ethanol (20 mL) was mixed with 20 equivalents of 50 % aqueous hydroxylamine (150 mmol, 4.95 g) and refluxed for 5 h. Upon completion, the ethanol was evaporated under reduced pressure. The product was purified via column chromatography [EtOAc:MeOH (90:10)] and confirmed via LC/MS and ^{1}H NMR. (Yield: 1.06 g, 85 %); ^{1}H NMR (DMSO-d_6); δ 4.45 (s, 2H), 4.63 (s, 2H), 5.38 (s, 2H), 6.46 (d, $J = 8$ Hz, 1H), 6.71 (dd, $J = 8.5$ Hz, 1H), 6.85 (s, 1H), 9.11 (s, 1H); LC/MS calcd. for $[M + H]^+$: $C_7H_{10}N_4O$ 167.1, found 167.3.

Solid-phase syntheses of phenylbenzimidazole-amidines and amino acid/peptide conjugates

Portions of deprotected Rink amide resin (0.25 g, 0.66 mmol/g) [or equivalent amounts of Fmoc deprotected resin-bound amino acid (Xaa) or Gly-Xaa dipeptide] were coupled to 4-carboxybenzaldehyde (0.8250 mmol, 123.8 mg) in the presence of HOBt (0.8250 mmol, 111.5 mg), and DIC (0.8250 mmol, 104.1 mg) in 15 mL DMF (15 mL). The resulting mixtures were agitated on an orbital shaker at room temperature for 1.5 h and completion of each coupling was confirmed by Kaiser test. Upon resin washing with DMF (3 × 5 mL), methanol (3 × 5 mL), DCM (3 × 5 mL), and diethyl ether (3 × 5 mL), 3,4-diamino-N′-hydroxybenzimidamide, **1**, (1.650 mmol, 274.2 mg) and 1,4-benzoquinone (0.165 mmol, 17.8 mg) were dissolved in DMF (15 mL) and coupled to the resin-bound aldehyde for 5 h at 60 °C using an IKA-Werke magnetic stirrer/heat block. The above procedures led to a resin- or amino acid/peptide-bound equivalent of phenyl-benzimidazole containing an N′-hydroxybenzimidamide substituent. To effect final conversion of the resin-bound N′-hydroxybenzimidamide moiety to an amidine, $SnCl_2{\cdot}2H_2O$ (2.475 mmol, 558.5 mg) was dissolved in DMF (2.5 mL), introduced into the reaction vessel, and allowed to react. Reaction conditions were maintained using an IKA-Werke magnetic stirrer/heat block at 80 °C over a period of 40 h with fresh additions of $SnCl_2$ solution (500 mg in 2.5 mL DMF) being added to the reaction mixture every 4 h (excluding overnight hours). Final products were cleaved from resin supports via shaking in 1:1 solutions of TFA:DCM for 1.5 h. Resins were removed by filtration and rinsed with DCM. Filtrates containing products were collected in weighed vials, and evaporated with a stream of nitrogen until a small volume remained, which was then precipitated into cold ether. The resulting solid precipitate was collected by filtration, dissolved in methanol and flushed through a silica column with methanol (Biotage flash column cartridge containing 1 g silica gel) to remove bulk impurities. Products were purified by HPLC using C18 reverse-phase chromatography (A: H_2O, 0.1 % TFA; B: ACN, 0.1 % TFA. Gradient: 0 min, 80 % A; 2 min, 80 % A; 20 min, 20 % A; 25 min, 0 % A; 30 min, 0 % A; 32 min, 80 % A; 35 min, 80 % A). All products were confirmed via MS and ^{1}H NMR (Online Resource 1).

The generalized procedure described above led to **2** when 4-carboxybenzaldehyde was coupled directly to Rink amide resin; and compound series **3a–p** and series **4a–t** when a single, C-terminal amino acid, Xaa, or Gly-Xaa dipeptide, respectively, was present on the resin prior to the coupling of 4-carboxybenzaldehyde. At the synthesis scale employed throughout these studies (0.25 g Rink amide resin; 0.66 mmol/g), ~50–100 mg of final product was obtained per reaction (see Online Resource 1 for individual gram yields); at this time, there is no reason to suspect that difficulties will be encountered upon scale-up.

Results and discussion

The goal of the work described here was to develop a straightforward means to incorporate an N-terminal phenylbenzimidazole-amidine moiety into resin-bound amino acids or peptides. While reports exist in the literature that describe the on-resin generation of simple benzimidazoles and benzimidazole derivatives (Sun and Yan 1998; Koprowska-Ratajska et al. 2009; Vourloumis et al. 2003), these examples lead to the generation of benzimidazoles, unnatural amino acids bearing benzimidazole side chains, or independent, low-molecular weight species not intended for further conjugation. Thus, to the best of our knowledge, this is the first report of the assembly of the phenylbenzimidazole-amidine structural unit of established DNA-binding ability directly to a resin or resin-bound amino acid or peptide via a main-chain peptide linkage. As noted earlier, the main efforts of this work are directed towards the eventual generation of DNA-targeted agents given the promising biological activities demonstrated by compounds containing phenylbenzimidazole-amidines. However, the widespread use of benzimidazole heterocycles in medicinal chemistry (Bansal and Silakari 2012) makes it clear that this work could similarly impact the development of compounds for other biological purposes and targets.

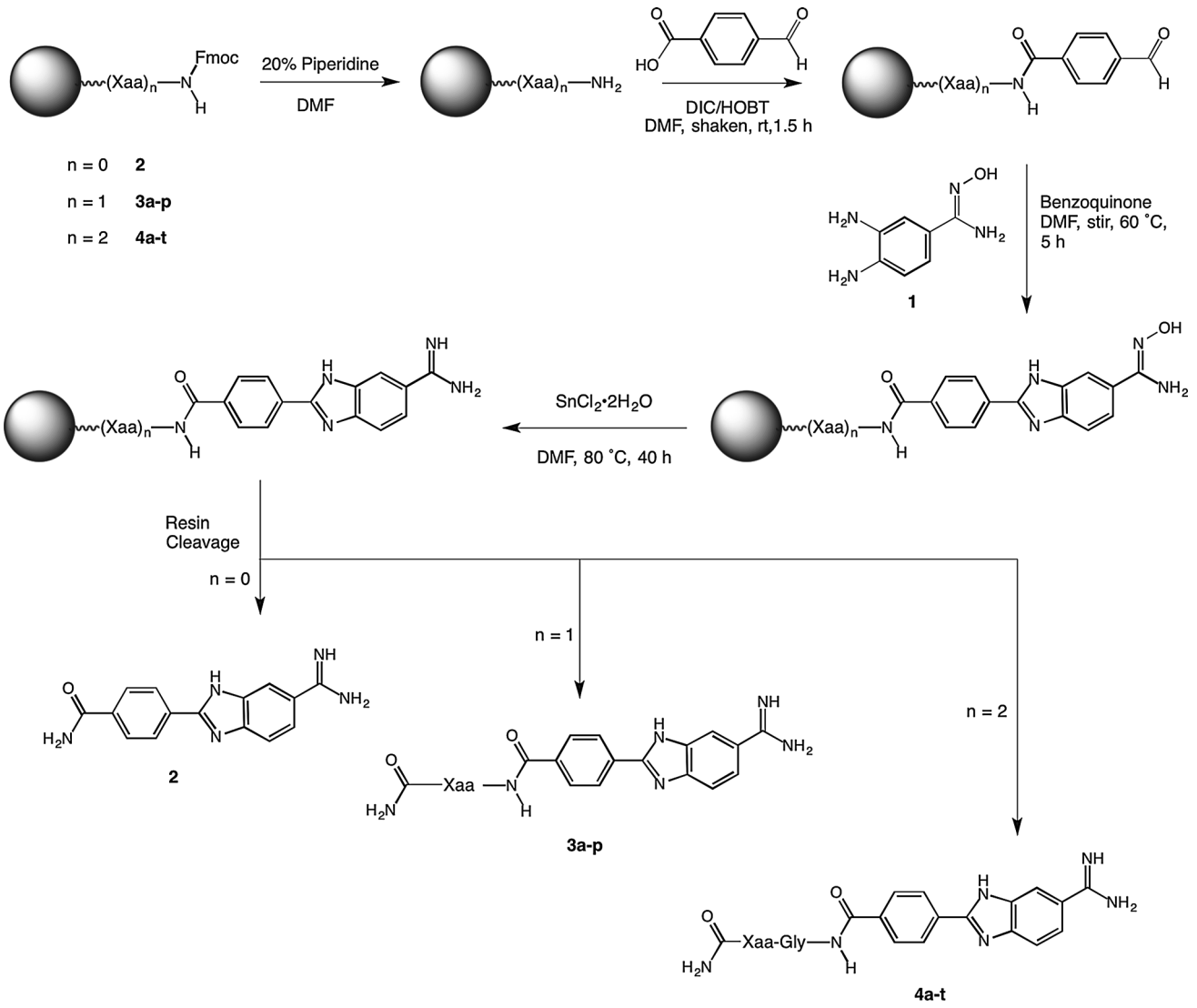

Fig. 2 Generalized scheme for the on-resin syntheses of amidine-substituted phenylbenzimidazole **2** or amino acids (**3a–p**) or peptides (**4a–t**) N-terminally capped with an amidine-substituted phenylbenzimidazole (Xaa_n represents any amino acid of *n* number of residues)

Solid-phase synthesis of phenylbenzimidazole-amidines

The initial target in our development of a solid-phase synthesis protocol for phenylbenzimidazole-amidines was **2** (Fig. 2). In this simplest case, the phenylbenzimidazole-amidine was assembled directly on resin and permitted development of the synthesis procedure in the absence of a C-terminal amino acid or peptide link to the solid phase. Throughout these syntheses, Rink amide resins were employed due to the nature of the ultimate intended purpose of the final compounds: DNA binding. Upon final product cleavage from the solid support, Rink amide resins release C-terminal amide functionalities rather than carboxylic acids. The presence of a C-terminal amide within the final compound thus eliminates the negative charge that would otherwise occur with a C-terminal carboxylate at physiological pH. The presence of a negative charge would diminish the DNA-binding capacity of a final product due to electrostatic repulsion.

To initiate the synthesis of **2**, 1,4-carboxybenzaldehyde was coupled directly to deprotected Rink amide resin using conditions typical for the coupling of amino acids to solid supports. It was determined that the most efficient means to drive this coupling to completion involved initiating a second coupling after an initial 1.5 h of reaction time; longer initial coupling times and increased amounts of initially added carboxybenzaldehyde failed to decrease the reaction times necessary for complete coupling.

Upon verification of complete coupling of the carboxybenzaldehyde to the solid support, the assembly of the benzimidazole ring was achieved through the introduction

of 3,4-diamino-*N*′-hydroxybenzimidamide (**1**). In our hands, reactions with this intermediate came to completion readily when 10 equivalents were allowed to react with the resin-linked aldehyde for 5 h at 60 °C. Using fewer equivalents of **1** led to incomplete coupling within this timeframe and room temperature reactions required up to 24 h to come to completion.

Having now formed a resin-bound benzimidazole bearing an N′-hydroxylated benzimidamide, the final step involved reduction of this *N*-hydroxylated substituent to the final desired amidine. This reduction was achieved through treatment of the resin with $SnCl_2{\cdot}2H_2O$ in DMF at 80 °C for 40 h (Dörwald 2002). To increase yields, aliquots of fresh $SnCl_2$ were added to the reaction mixture every 4 h. Initial attempts at this conversion using a single addition of $SnCl_2$ led to thickening of the reaction mixture and no desired product. Similarly, increasing the concentration and/or volume of the initially added $SnCl_2$ did not alleviate this problem and usually resulted in <25 % yield of desired product. An exploration of shortened reaction times also led to incomplete conversion to the amidine. Thus, 40 h was determined to be optimal. Eventually, additional aliquots added at 4-h intervals throughout the day, as described above, were discovered to prevent resin thickening and led to the desired product upon resin cleavage. Final resin cleavage generated typical product yields 76 %.

It is noteworthy that during the development of the above procedure attempts were made to incorporate amidines into resin-bound benzimidazoles via: (1) the Pinner conversion of an on-resin nitrile substituent (see Parrish et al. 1978 for an example); (2) the catalytic hydrogenation of an on-resin *N*-hydroxy benzimidamide via Pd on charcoal (Ismail et al. 2004, 2005); and (3) the conversion of an on-resin nitrile to an *N*-hydroxybenzimidamide via hydroxylamine and subsequent treatment with $SnCl_2{\cdot}2H_2O$ (Dörwald 2002). However, none of these procedures proved to be successful. In particular, in the latter case, we found that the on-resin benzimidazole decomposed during resin treatment with hydroxylamine. Thus, in light of reports indicating that $SnCl_2{\cdot}2H_2O$ reacted to convert aromatic nitro compounds to amines on solid support (Dörwald 2002), the use of $SnCl_2{\cdot}2H_2O$ was employed successfully in conjunction with **1** prepared in solution.

Solid-phase syntheses of amino acid and peptide conjugates of phenylbenzimidazole-amidine

Having developed an on-resin protocol for the generation of phenylbenzimidzole-amidines, the ability to react with resin-bound amino acids and peptides was explored. Two series of amino acid-bearing phenylbenzimidazole-amidines were synthesized: one series bearing a single C-terminal naturally occurring amino acid (Xaa), **3**, and another, **4**, bearing a C-terminal peptide containing a variable amino acid connected via a Gly linker (Gly-Xaa) to the phenylbenzimidazole-amidine moiety. This latter model system was explored not only as a peptide mimic, but also to determine if varied amino acid side chains influenced the coupling of 4-carboxybenzaldehyde to resin-bound peptide.

Table 1 Series **3** Xaa-phenylbenzimidazole-amidine conjugates and yields

Compound	R (Xaa)	Yield (%)	Compound	R (Xaa)	Yield (%)
3a	Gly	88	**3k**	Gln	73
3b	Ala	74	**3l**	Tyr	61
3c	Val	81	**3 m**	Lys	64
3d	Leu	76	**3n**	Arg	58
3e	Ile	73	**3o**	Asp	78
3f	Met	73	**3p**	Glu	72
3g	Pro	79	–	Cys	0[a]
3h	Phe	64	–	His	0[a]
3i	Thr	77	–	Ser	0[a]
3j	Asn	79	–	Trp	0[a]

[a] Final product not observed

Synthesis of series **3** followed the same route described for the synthesis of **2** upon coupling of the 4-carboxybenzaldehyde to a Rink amide resin bearing a deprotected amino acid. As shown in Table 1, acceptable yields for 16 out of the 20 naturally occurring amino acids were obtained. However, despite numerous attempts, final desired products derived from Rink-amide bearing Cys, His, Ser, and Trp were not obtained and appeared to fail during the course of the $SnCl_2{\cdot}2H_2O$ reduction step; all steps involving these amino acids prior to $SnCl_2{\cdot}2H_2O$ reduction were completed and analyses of resin-bound intermediates by LC/MS showed the expected masses confirming the presence of the initial resin-amino acids and their respective 4-carboxybenzaldehyde and *N*′-hydroxylated diaminobenzimidamide products (Fig. 2). The reason for the lack of reactivity for these four amino acids is not clear. However, based on the results described below for Xaa-Gly dipeptides leading to **4** that permitted inclusion of all 20 naturally occurring amino acids, it is likely that sterics and not chemical reactivity influenced the outcome.

Following the generation of the series **3** single amino acid bearing phenylbenzimidazole-amidines, a second dipeptide-based series (**4**) of conjugates was pursued (Table 2). In this series of reactions, we sought to determine the impact of the presence of a resin-bound dipeptide equipped uniformly with a flexible Gly linker on the generation of peptides N-capped with the phenylbenzimidazole-amidine. Synthesis of the dipeptide conjugates proceeded through the same protocol employed in the

Table 2 Series **4** Xaa-Gly-phenylbenzimidazole-amidine conjugates and yields

Compound	Xaa	Yield (%)	Compound	Xaa	Yield (%)
4a	Gly	93	**4k**	Thr	89
4b	Ala	82	**4l**	Asn	69
4c	Val	87	**4m**	Gln	73
4d	Leu	85	**4n**	Tyr	65
4e	Ile	91	**4o**	Cys	44
4f	Met	90	**4p**	Lys	94
4g	Pro	91	**4q**	Arg	78
4h	Phe	77	**4r**	His	61
4i	Trp	74	**4s**	Asp	85
4j	Ser	59	**4t**	Glu	71

generation of **2** and **3**. After the initial coupling of all 20 naturally occurring amino acids to the Rink amide resin, capping with Gly, and deprotection, 4-carboxybenzaldehyde was coupled to each terminal Gly residue. Subsequently, the phenyl-benzimidazole core was assembled along with reduction of the resin-bound *N*′-hydroxylated benzimidamide to an amidine. With each dipeptide resin, the N-terminal capping procedure leading to Xaa-Gly-phenylbenzimidazole-amidine proceeded smoothly to final product (Table 2). All 20 naturally occurring amino acid–Gly dipeptides in this instance led to final products (**4**). This outcome reinforces the notion that the inability to directly produce some of the amino acid (Cys, His, Ser, and Trp) conjugates in series **3** is likely due to inhibition of the reduction step through some means other than the direct reactivity with these particular protected side chains.

The results described above indicate that the procedures employed here can lead to the straightforward on-resin generation of amino acids and dipeptides that are N-terminally capped with a phenylbenzimidazole-amidine moiety. Aside from the difficulties encountered upon attempts to directly cap the amino acids Cys, His, Ser, and Trp, which can be circumvented through the use of a Gly spacer, all other amino acids appear amenable to the on-resin assembly of phenylbenzimidazole-amidine conjugates as described. In addition to the single amino acid and dipeptide conjugates described here, it is worthy to note that, to date, using these same procedures we have routinely accomplished the synthesis of tripeptide conjugates in similar yields (Wang 2010).

Conclusions

The foregoing work has described a straightforward means to incorporate an established DNA binding motif into on-resin amino acid and peptide syntheses. It was determined that the complete phenylbenzimidazole-amidine structural unit can be assembled on resin and also as an amino acid or peptide conjugate. In the latter case, uniform coupling to a resin-peptide terminating with a Gly "linker" leading to a phenylbenzimidazole-amidine derivative proceeded without difficulty. Thus, this work enables the inclusion of a unique structural unit into established peptide synthesis protocols and could permit the expedited or combinatorial generation of phenylbenzimidazole-amidine derivatives for biological testing. While beyond the scope and intention of the work described herein, we plan to report the impact of the incorporation of this moiety into DNA binding agents in due course.[1]

Acknowledgments We gratefully acknowledge financial support from an IUPUI Research Support Funds Grant (to ECL). The authors would also like to thank Prof. Marty O'Donnell (IUPUI) for comments on the manuscript during its preparation.

Conflict of interest The authors declare that they have no conflict of interest.

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[1] While the model compounds synthesized during the course of this work (**2**, **3**, and **4**) were not intended or designed purposefully with the targeting of DNA in mind, we nonetheless carried out preliminary tests of their DNA binding—these studies indicated that systems possessing the phenylbenzimidazole-amidine moiety exhibited significantly enhanced DNA binding. A full account of these activities within rationally designed peptide sequences will be reported in due course.

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