Received: 22 November 2017 | Revised: 28 January 2018 | Accepted: 29 January 2018

DOI: 10.1002/pep2.24063

ORIGINAL ARTICLE

WILEY PeptideScience

Converting polar cyclic peptides into membrane permeable molecules using *N*-methylation

Leo L. H. Lee | Laura K. Buckton | Shelli R. McAlpine

Chemistry, University of New South Wales, Sydney, New South Wales, Australia

Correspondence
Shelli R. McAlpine, Chemistry, University of New South Wales, Sydney, New South Wales, Australia.
Email: s.mcalpine@unsw.edu.au
and
Laura K. Buckton, Chemistry, University of New South Wales, Sydney, New South Wales, Australia.
Email: laura.buckton@unsw.edu.au

Funding information
National Health and Medical Research Council; Grant number: GNT1043561

Abstract

Described are the design, synthesis, and biological evaluation of 5 *N*-methylated analogs that are based on a lead drug structure **LB51**. **LB51** is a cyclic pentapeptide that inhibits heat shock protein 90 and although a potent inhibitor of the protein function, it has poor cell permeability. Introduction of an *N*-methyl moiety at each amino acid produces 5 analogs of **LB51**, where all 5 show significantly improved membrane permeability over the lead molecule despite the presence of 4 highly polar side chains.

KEYWORDS
cell permeability, cyclic peptides, peptide, N-methyl

1 | INTRODUCTION

Therapeutic peptides are predicted to go from a $21 billion to a $46.6 billion dollar industry by 2024.[1] The revenue increase is anticipated to come from the discovery of new peptide drugs. Cyclic peptides are a highly promising class of molecules, because they are stabile to proteases,[2] have well-defined 3-D structures,[3,4] and have restricted conformational movement. These properties provide energetic binding and steric advantages for cyclic peptides over small molecules and linear peptides inhibitors of protein–protein interactions.[5] For these reasons cyclic peptides are anticipated to be at forefront of new peptide drug development.[6]

One of the greatest challenges facing peptide drugs is cell permeability.[6] Predicting which strategies will improve cell permeability of any given molecule is impossible, and only the synthesis of molecules that incorporate individual strategies and experimental evaluation reveal which molecules penetrate cell walls. Given the unpredictability of developing cell permeable peptides, most research has focused on improving cell permeability without considering target-binding affinity.

There are 4 main strategies aimed at improving the cell permeability of cyclic peptides. These strategies include: *N*-methylation of the peptide backbone,[7–11] side-chain modifications,[4,12,13] inclusion of D-amino acids,[4,14–16] and incorporation of asparagine-like side chains.[17] All of these approaches are aimed at impacting the cyclic peptide's conformation, which correlates with the molecule's cell permeability. Controlling the cyclic peptide conformation is challenging and unpredictable,[4,16–19] with the 4 strategies described being used to rationally design modified cyclic peptides. A host of literature clearly demonstrates that although cell permeable cyclic peptides may be rationally designed using a series of strategies, the ability to accurately predict which of these rationally designed compounds will be membrane permeable is not possible,[4,7–19] and determining the apparent permeability ($P_{app} \times 10^{-6}$ cm/s) is only feasible after synthesis and evaluation of each compound.[20,21]

Previous literature reports that converting cyclic peptide scaffolds into cell permeable compounds ($P_{app} \geq 1$)[4,7] relies heavily on multiple *N*-methylations and using primarily hydrophobic amino acids and typically a maximum of 2 polar side chains. Figure 1 shows the most cell permeable cyclic peptides produced from other labs using these 4 strategies. Transforming peptide scaffolds that contain more than 3 polar amino acids (defined as the following: glutamine, asparagine, histidine, serine, threonine, tyrosine, cysteine, tryptophan, arginine, lysine, aspartic acid, or glutamic acid) into cell permeable molecules has not been reported. Kessler and Yudin both incorporated 3 *N*-methyl groups on structures with 3 polar side chains (**5** and **6**, Figure 1) in order to produce cell permeable molecules of P_{app} ~4. Since small molecules have a median P_{app} ~16, and the most commonly reported values are in the range of P_{app} ~3–4,[22] the goal for peptide drugs is to identify structures that have $P_{app} \geq 8$, as this would place them in the same range as small molecules.

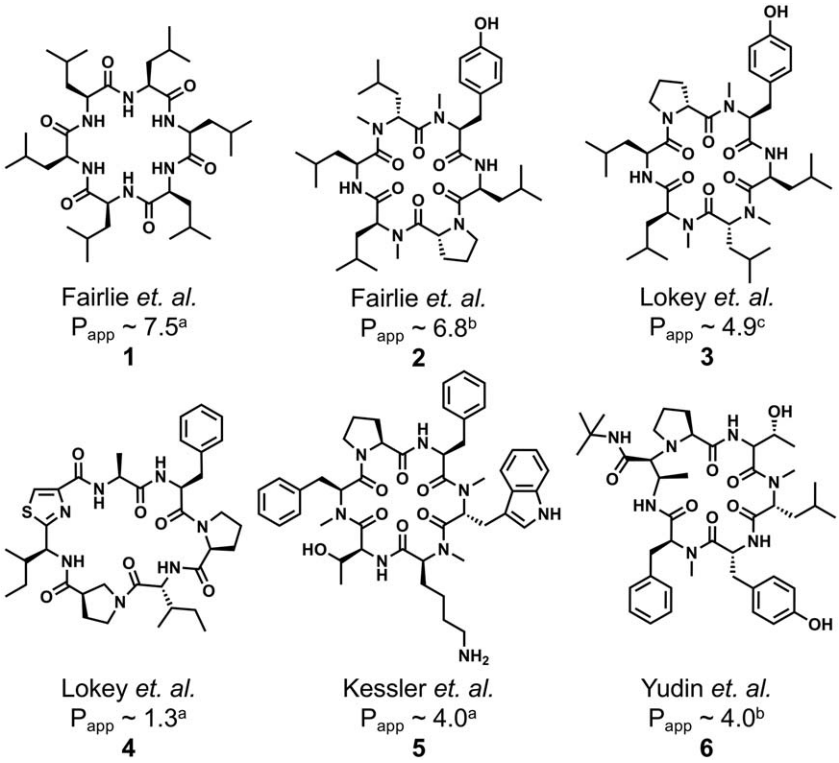


FIGURE 1 Structures and permeability values of macrocyclic peptides studied by leading groups in the field. Permeability is reported as $P_{app} \times 10^{-6}$ cm/s, where the superscript indicates values from A, Caco-2 cells; B, PAMPA; or C, RRCK cells

While backbone *N*-methylation is commonly used to modify pharmacokinetic properties of peptides such as membrane permeability and metabolic stability, it is well-established that *N*-methylation can also impact biological activity.[23,24] Introducing an *N*-methyl group to the peptide backbone often improves the pharmacokinetics by introducing lipophilicity and modulating the conformation. While these modifications are often favorable for cell permeability and metabolic stability, any change in the hydrophobicity or 3-D structure of a molecule is likely to impact target binding. Given that even the addition of a single *N*-methyl group can have long-range impacts across a molecule and conformational changes are sequence- and stereochemistry-dependent, it is challenging to predict the effects on target binding affinity, potency, and selectivity. However, despite the potential impacts on biological activity, backbone *N*-methylation provides a significant opportunity to improve peptide drug permeability, and therefore it is still a widely used strategy. As such, many groups have researched the impact of *N*-methylation on the bioactivity of peptide scaffolds. Patterson and co-workers reported the synthesis and biological activity of tubulysin analogs (Figure 2).[25] Tubulysins inhibit tubulin polymerization and induce apoptosis. However Tubulysin D, the most potent inhibitor, contains a labile *O*-acyl *N*,*O*-acetal moiety, which is a metabolic liability and a synthetic challenge. The authors replaced this moiety with an *N*-methyl group by developing an expedient synthesis, (compound **7**, Figure 2) and observed only a ~2-fold drop in activity.

Doedens and co-workers reported the synthesis and activity of *N*-methylated analogs of Melanotan-II (MT-II) (Figure 2).[26] MT-II is a potent but nonselective agonist for 4 of the 5 melanocortin receptor subtypes including MC_1, MC_3, MC_4, and MC_5. Melanocortin receptors are widely distributed in human tissues and have diverse biological functions. The goal of this work was to maintain the potency but improve receptor selectivity and pharmacokinetics by introducing *N*-methyl groups. Through the introduction of 4 backbone *N*-methyl groups, the authors achieved receptor selectivity for MC_1 although there was a ~4-fold drop in potency (4–14 nM). Given that both MT-II and compound **8** (Figure 2) both have low nanomolar potency, a 4-fold drop in potency is a small sacrifice to exchange for selectivity. Thus, backbone *N*-methylation offers exciting opportunities for improving both the pharmacokinetics and biological activity of peptides *via* modulation of their 3-D conformations.

Incorporating *N*-methylated amino acids into cyclic peptides can sometimes enhance biological activity and pharmacokinetic properties. Synthesis of molecules that include these amino acids has been limited by the relatively high cost of some *N*-methylated amino acids, versus the price of their non-*N*-methylated counterparts. Thus, numerous methods have been developed for producing *N*-methylated amino acids and peptides and conditions have been optimized for reactions in solution and on solid phase. Solution-phase syntheses are most useful when large quantities of *N*-methylated amino acid are required.[27] The Benoiton method[28] (Scheme 1) is one example and it utilizes amino acids, where the amine is protected with a base-stable protecting group such as Boc or Cbz. A strong base is used to deprotonate the amine, which is then methylated with methyl iodide. This method is limited

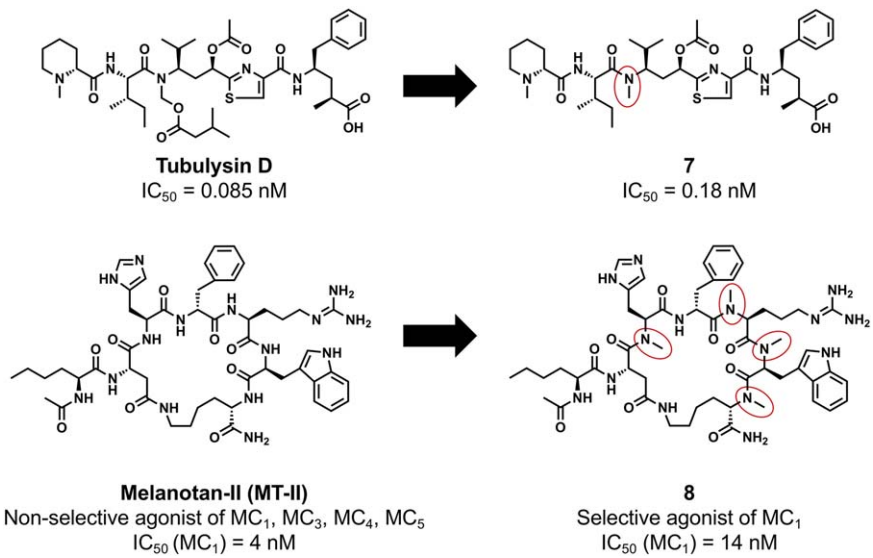
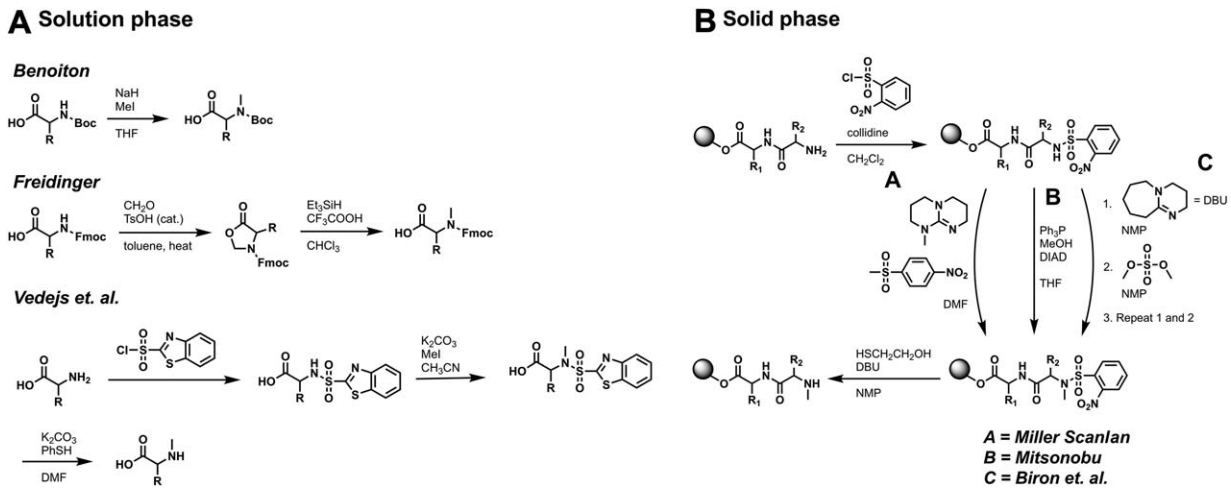


FIGURE 2 Structures of biologically active peptides and their *N*-methylated analogs. Compound **7** was developed by Patterson and co-workers[25] and exhibits similar potency to parent compound Tubulysin D. However, compound **7** had increased metabolic stability and a straightforward synthesis. Compound **8** was developed by Doedens and co-workers[26] and is based on the potent but nonselective drug candidate MT-II. Selectivity was achieved by introducing *N*-methyl groups and nanomolar potency was retained

because the Fmoc-protected amino acids are deprotected under these conditions. In order to address some of the limitations associated with the Benoiton method, Freidinger and co-workers developed a new method that maintained the Fmoc on the amino acids.[29] This method utilizes an Fmoc-protected amino acids and aldehyde, which undergo an acid-catalyzed condensation to produce the oxazolidinone, which can then be reduced to the *N*-methylated amino acid using triethylsilane and trifluoroacetic acid (Scheme 1). Diverse Fmoc-protected amino acids and aldehydes are commercially available, thus this approach is extremely versatile.

While these solution-phase syntheses are critical for the large-scale synthesis of *N*-methylated amino acids, when only small quantities of amino acid are required, e.g. mg of material, it is best to directly *N*-methylate amino acids on solid-phase.[27] The Miller Scanlan[30] approach involves addition of a nitrobenzenesulfonyl group (*O*-NBS) group to the free N-terminus. The amine is subsequently methylated using dimethyl sulfate and the hindered base 7-Methyl-1,5,7-triazabicyclo[4.4.0]dec-5-ene (MTBD). The *O*-NBS group is then removed with beta-mercaptoethanol and DBU (Scheme 1). This method is limited because it is not suitable for peptides that contain trityl-protected histidine residues because the trityl group is cleaved and the histidine side chain is also methylated, and MTBD is expensive. The Miller Scanlan method has been optimized by various groups in order to decrease reaction times and make the reaction economical. Utilizing the same *O*-NBS protection and deprotection conditions, Yang and co-workers adapted the methylation step of synthesis to use a Mitsonobu reaction (Scheme 1).[31] The resin-bound amino acid or peptide is methylated with triphenylphosphine, DIAD, and methanol. This synthesis has a shorter reaction time than the Miller Scanlan methylation and does not impact trityl-protected histidine residues. Biron and co-workers



SCHEME 1 Synthetic approaches to N-methylation of amino acids and peptides: A, solution-phase methods versus and B, solid-phase syntheses

reduced reaction times by utilizing NMP as a solvent, and decreased the cost per reaction by replacing the expensive base MTBD with the lower-cost alternative DBU (Scheme 1).[32] However, like the original Miller Scanlan method, peptides containing trityl-protected histidine are impacted, and thus the Mitsonubu remains the most efficient synthesis for peptides and amino acids with labile protecting groups on side chains. Vedejs and co-workers applied these methods to solution phase synthesis, replacing the *O*-NBS group with a benzothiazole-2-sulfonyl (Bts) group, which facilitated the synthesis of sterically hindered *N*-methylated and *N*-alkylated amino acids in high yields and utilized readily available and inexpensive reagents (Scheme 1).[33]

Herein we describe the synthesis, membrane permeability, and biological activity of 5 new *N*-methylated macrocyclic pentapeptides that are based on a therapeutically effective inhibitor **LB51**. Despite having 4 polar amino acids within its structure, **LB51** has a $P_{app} = 3.3$ and 3.0 when evaluated in Caco-2 cells and PAMPA assays, respectively. In this manuscript, we discuss our efforts to improve the permeability of this lead structure by incorporating a single *N*-methyl moiety at each position around the peptide (Figure 2, compounds **9–13**) and subsequently evaluate the impact of these modifications on biological activity.

2 | EXPERIMENTAL SECTION

2.1 | General remarks

All chemicals were purchased from commercial suppliers (Chem-Impex International, Sigma Aldrich, and Merck) and used without further purification. All moisture sensitive reactions were performed using anhydrous solvents under nitrogen gas. Removal of solvent was carried out under reduced pressure using a Buchi R-210 rotary evaporator.

2.2 | General procedure for synthesis of linear peptides

2.2.1 | Solid-phase peptide synthesis

Stepwise SPPS was performed in a polypropylene solid-phase extraction cartridge fitted with a 20 μm polyethylene frit purchased from Applied Separations (Allentown, PA).

2.2.2 | Resin loading

A 2-chlorotrityl chloride resin was weighed, transferred to the cartridge and swelled in CH_2Cl_2 for 30 min prior to the resin loading reaction. The appropriate Fmoc-protected amino acid was dissolved in the minimum amount of 0.4 M DIPEA in CH_2Cl_2. The swelled resin was then drained and the dissolved amino acid was added and the suspension was agitated for 4 h. The resin was then washed 3 times with CH_2Cl_2, 3 times with DMF, and 3 times with CH_2Cl_2. The resin was then dried *in vacuo* overnight. A ~5 mg sample of resin was used to determine the amino acid loading. A 20% piperidine in DMF was added to the sample to cleave the Fmoc protecting group. The resin was filtered away and the remaining solution diluted 1 in 20 and the UV absorbance measured at 301 nm using a Cary 50 Bio UV-Vis instrument. DMF was used as a blank and samples were measured in a 1 mL quartz cuvette. The resin loading was then determined using the following formula:

$$Resin\ loading = \frac{A_{301} \times V \times d}{\varepsilon \times W \times m}$$

where A_{301} = UV absorbance at 301 nm, V = cleavage volume (1 mL), d = dilution factor (20), ε = molar extinction coefficient of Fmoc group (7800 mL $mmol^{-1}$ cm^{-1}), W = cuvette width (1 cm), and m = resin mass in g.

2.2.3 | Coupling reaction

Couplings were performed in DMF at a concentration of 0.3 M. Fmoc-protected amino acid (2 equiv.) and HOBt (2 equiv.) were mixed with the resin. DIC (4 equiv.) was then added to activate the reaction. Coupling reaction was run for approximately 2 h while shaking (Labquake tube shaker, Thermo Fisher Scientific) at room temperature. A negative ninhydrin test was used to confirm reaction completion. Once completed, the reaction mixture was drained and the resin was subjected to Fmoc Removal. (Note: For particularly hindered coupling reactions, HOBt was replaced with HOAt.)

2.2.4 | Fmoc removal

The Fmoc protecting group was removed using the following washes: DMF (3 × 1 min), 20% piperidine in DMF (1 × 5 min), 20% piperidine in DMF (1 × 10 min), DMF (2 × 1 min), *i*PrOH (1 × 1 min), DMF (1 × 1 min), *i*PrOH (1 × 1 min), and DMF (3 × 1 min). The resin was then ready for the next coupling reaction.

2.3 | General procedure for on-resin methylation

2.3.1 | Addition of *O*-NBS activating group

The addition of *O*-NBS activating group to a resin bound peptide with a free amine was carried out using anhydrous CH_2Cl_2 at a concentration of 0.03 M *O*-NBS-Cl (4 equiv.) was dissolved separately in 60% of the final volume dry CH_2Cl_2. The solution was then added to the resin, with *sym*-collidine (10 equiv.) and the remaining 40% of anhydrous CH_2Cl_2 added subsequently to the reaction. The reaction mixture was stirred for a minimum of 5 h at room temperature. Once completed, the resin was washed with CH_2Cl_2 multiple times and a small sample of peptide was cleaved from the resin using trifluoroethanol (TFE) in CH_2Cl_2 (1:1 *v/v*) and analyzed *via* liquid chromatography/mass spectrometry (LC/MS). Once the reaction was complete, the resin-bound peptide was subjected to an *N*-methylation reaction under Mitsunobu conditions.

2.3.2 | *N*-methylation under mitsunobu conditions

Selective *N*-methylation was achieved using anhydrous solvents at a concentration of 0.03 M. Triphenylphosphine (5 equiv.) and dry MeOH (10 equiv.) were dissolved in 50% of the final volume of dry THF. The solution was then added to the resin-bound *O*-NBS protected peptide and stirred at room temperature for 10 minutes. Diisopropyl azodicarboxylate (DIAD) (5 equiv.) and the remaining dry THF was added to the reaction solution slowly and was stirred for a minimum of 4 h at room

temperature. The resin was then washed with DMF multiple times. A small sample of peptide was cleaved from the resin and reaction completion was confirmed by LC/MS. Once complete, the *O*-NBS group is removed.

2.3.3 | *O*-NBS removal

Removal of the *O*-NBS was achieved using the following reagents: DMF (0.03 M), mercaptoethanol (10 equiv.), DBU (5 equiv.). The reaction mixture was stirred for a minimum of 3 h and subsequently washed with DMF multiple times. A small sample of peptide was cleaved from the resin and reaction completion was confirmed by LC/MS. Once complete, additional coupling reactions were completed as described previously.

2.3.4 | Resin cleavage of linear peptide

Once the desired peptide was generated, the final Fmoc protecting group was removed following Fmoc Removal procedure with the following additional washes: DMF (3 × 1 min), *i*PrOH (3 × 1 min), and MeOH (3 × 1 min). The resin-bound peptide was then dried *in vacuo* overnight. The resin was then cleaved from the linear peptide using TFE and CH_2Cl_2 (1:1 v/v) at a concentration of 10 mL/g resin. The reaction mixture was stirred at room temperature for 24 h before filtering the resin. The filtrate was concentrated and washed at least 10 times with CH_2Cl_2 to remove residual entrapped TFE. The product was then dried *in vacuo* overnight to produce the linear peptide.

2.4 | General procedure for cyclization

Macrocyclization of the linear peptide was achieved using a cocktail of 3 coupling reagents: HATU (1 equiv.), TBTU (0.8 equiv.), and DMTMM (0.8 equiv.). The reaction was performed in dilute conditions using anhydrous CH_2Cl_2 at a concentration of 0.001 M. The linear peptide and coupling reagents were dissolved separately in CH_2Cl_2, where 20% of the final volume was used to dissolve the linear peptide and the other 80% dissolved the coupling reagents. DIPEA (4 equiv.) was added to each solution. The linear peptide solution was then added drop-wise to the coupling reagents solution *via* a syringe pump over approximately 2 h. The reaction was stirred overnight and monitored *via* LC/MS. (Note: if the reaction failed to reach completion after stirring overnight, additional HATU (1 equiv.) was added and the reaction was monitored using LC/MS.) Upon completion, the reaction mixture was evaporated and the dry solid was redissolved in CH_2Cl_2 and extracted 3 times with milli-Q water. The aqueous layers were combined and extracted 3 times with fresh CH_2Cl_2. All organic layers were combined and dried over Na_2SO_4, filtered, and evaporated under reduced pressure before the compound was dried *in vacuo* overnight.

2.5 | General procedure for the removal of side chain protecting groups

Amino acid side chain protecting groups were removed using trifluoroacetic acid (TFA) in CH_2Cl_2 (9:1 *v/v*) with anisole (2 equiv. per protecting group). Anisole was added to the peptide, whilst stirring, followed by the TFA solution at a concentration of 4 mL/g compound. The reaction was left stirring at room temperature for 4 h. The reaction was monitored using LC/MS and once complete the reaction solution was dried under a stream of nitrogen before redissolving in CH_2Cl_2 and evaporating multiple times to remove residual entrapped TFA. The product was triturated in diethyl ether with sonication, collected *via* centrifugation and lyophilized to produce the crude cyclic peptide, which then underwent high-performance liquid chromatography (HPLC) purification.

2.5.1 | Liquid chromatography/mass spectrometry

LC/MS analyses were performed using a Phenomenex Aeris XB-C18 column (3.6 μm, 2.1 × 100 mm) on either a Shimadzu LCMS 2020 or Shimadzu LCMS 8030. The mobile phase consisted of milli-Q water with 0.1% (v/v) formic acid (Mobile Phase A), and HPLC grade acetonitrile with 0.1% (v/v) formic acid (Mobile Phase B) at a flow rate of 0.2 mL/min, starting at 95% Mobile Phase A and 5% Mobile Phase B.

2.5.2 | High-performance liquid chromatography

Semi-preparative HPLC for purification was performed using a GRACE VisionHT C18 column (5 μm, 22 × 150 mm) or a Phenomenex Aeris XB-C18 column (5 μm, 21.2 × 150 mm) on a Shimadzu Prominence system. The mobile phase consisted of milli-Q water with 0.1% (v/v) formic acid (Mobile Phase A), and HPLC grade acetonitrile with 0.1% (v/v) formic acid (Mobile Phase B) at a flow rate of 5 mL/min, starting at 95% Mobile Phase A and 5% Mobile Phase B.

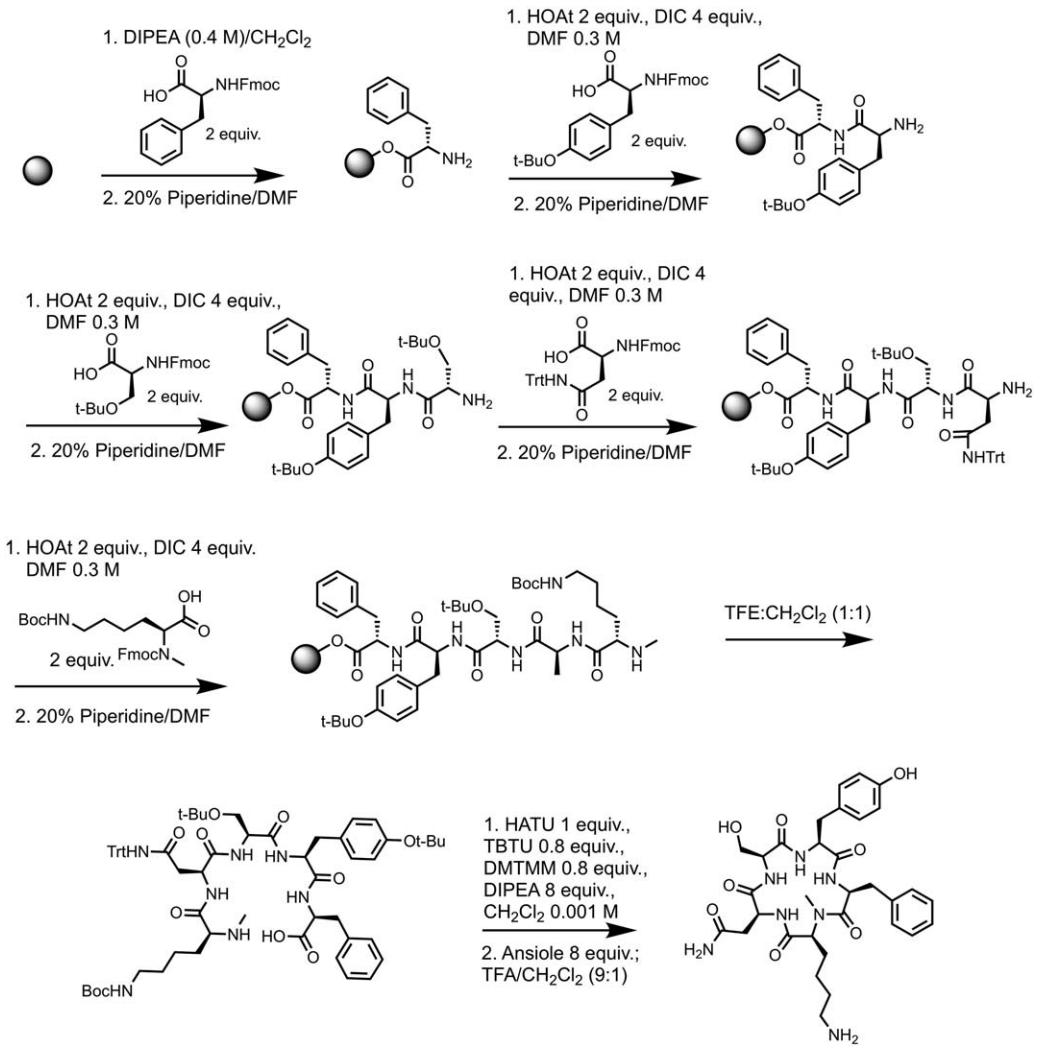
2.5.3 | NMR

^{1}H and ^{13}C NMR spectra were obtained on Bruker Avance III 600 MHz. Multiplicity of NMR signals were represented by the following abbreviations: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad, dd = doublet of doublet. Assignment of resonances for each residue was accomplished using ^{1}H, HSQC, HMBC, and COSY spectra.

2.6 | General procedures—biology

2.6.1 | Protein binding assay

The binding assays were performed using a HSP90β (C-terminal) Inhibitor Screening Kit (cat. 50314) purchased from BPS Bioscience. The assay was performed according to the manufacturers protocol and utilized AlphaLISA technology (PerkinElmer). The test compounds were dissolved in 100% DMSO and diluted with water to the desired concentration so that the final dilution was dissolved in 5% DMSO with water. A 2 μL of the dilution was added to a 10 μL reaction so that the final concentration of DMSO was 1% in all reactions. The reactions were conducted at room temperature for 30 min in a 10 μL mixture containing assay buffer, 6 ng (24 nM) of a C-terminal HSP90β (Uniprot P08238, a.a. 527–724), 40 ng (60 nM) Cyp40, and the test compound. After the 30 min incubation, 10 μL of buffer containing 20 μg/mL glutathione acceptor beads (Perkin Elmer) were added to the reaction mix and incubated for 30 min in the dark. About 10 μL of 40 μg/mL streptavidin donor beads (Perkin Elmer) were then added and the final 30 μL



SCHEME 2 The synthesis of compound **10** using solid phase and sequential amino acid couplings. Cyclization occurred after cleavage, and global deprotection and subsequent purification provided pure compound

mixture was incubated for 1 h the dark. The AlphaLISA signal was measured using a Tecan F200 Pro multimode plate reader.

2.6.2 | Parallel artificial membrane permeability assay (PAMPA)

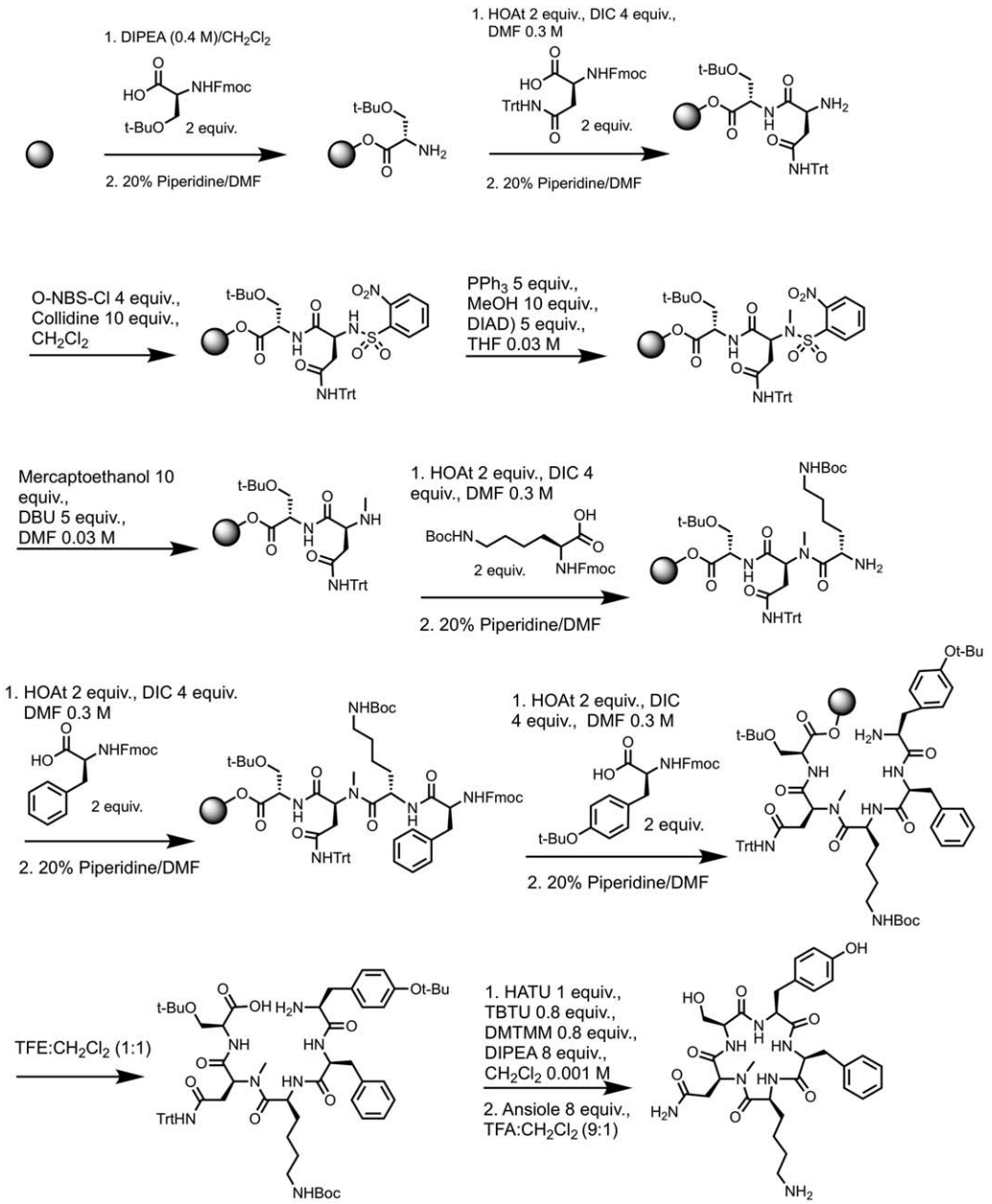
The 24-well Transwell plates with 0.4 μm polycarbonate membrane supports (Corning cat. # 3413) were used in the PAMPA. A 1.8% (*w/v*) solution of lecithin in dodecane was prepared fresh. The solution (15 μL) was applied to each membrane and allowed to adsorb for 15 minutes. The acceptor wells were prepared by adding 600 μL of PBS (pH 7.4) to each well. The donor wells were prepared by adding 200 μL of each compound (50 μM in 1% DMSO/PBS (pH 7.4)). The lid was placed on the plate and the system was incubated for 16 h in a sealed chamber with wet paper towels to prevent evaporation. Once the assay was complete, a sample from each donor well (150 μL) and acceptor well (400 μL) was collected. The samples were then analyzed using LC/MS in selected ion monitoring (SIM) mode to detect the parent masses and quantified using a standard curve of known compound concentrations. The permeability values ($P_{app} \times 10^{-6}$ cm/s) were calculated using the following equation:

$$P_{app} = \frac{V_B}{A \times C_i} \times \frac{C_B}{\Delta t}$$

where V_B = volume of acceptor well (0.6 cm^3), A = membrane area (0.33 cm^2), C_i = concentration added to donor well (50 μM), C_B = concentration determined by LC/MS in the acceptor well, and t = time (57 600 seconds). A minimum of 2 data points were collected for each compound.

3 | RESULTS AND DISCUSSION

Of the 5 analogs synthesized (**9–13**), 4 of the molecules (**10–13**) were produced using an almost identical approach, *via* Fmoc solid-phase peptide chemistry (Scheme 1). We purchased 4 of the *N*-methylated amino acids required for the synthesis of molecules **10-13** and because of the unavailability of 1 *N*-methylated amino acid, analog **9** (Scheme 3) was produced following the Miller Scanlan/Mitsonubu strategy using



SCHEME 3 The synthesis of **9** using on-resin *N*-methylation and subsequent sequential amino acid couplings. Cyclization occurred after cleavage, and global deprotection and purification provided pure compound

an on-resin methylation. The synthesis of 1 analog, **10**, is described as a representative example of the 4 molecules made using purchased amino acids. Phenylalanine (Phe) was loaded onto 2-chlorotrityl chloride resin. The resin-bound peptide underwent sequential coupling and Fmoc removal with Fmoc-protected tyrosine (*t*-Bu), serine (*t*-Bu), asparagine (Trt), and *N*-methyl lysine (Boc) amino acids, respectively. Once couplings were complete, the pentapeptide was cleaved from the resin under mildly acidic conditions (trifluroethanol, TFE). Crude linear pentapeptide was cyclized in dilute conditions (0.001 M) using a combination of 3 coupling agents (HATU, TBTU, and DMTMM).[34] Subsequent deprotection of the side-chains was accomplished using TFA with anisole as the carbocation scavenger. Reversed-phase HPLC purification produced pure compound **10** as a white solid (2 mg, 1% overall), where LC/MS, ^{1}H NMR, and 2D NMR were used to confirm the final structure and purity. Compounds **11–13** were produced, isolated as white solids, and characterized in a similar manner. The overall yields for each compound were as follows: compound **11** (6 mg, 2%), compound **12** (10 mg, 2%), and compound **13** (7 mg, 2%).

Compound **9** was synthesized using an on-resin *N*-methylation of asparagine. Serine (*t*-Bu) was loaded onto 2-chlorotrityl chloride resin, whereupon asparagine (Trt) was coupled to the serine residue (Scheme 2). The activating group *O*-nitrobenzyl sulfonyl chloride (*O*-NBS-Cl) and collidine were used to produce the nitrobenzyl sulfonated amino acid.[19] Methylation of the activated asparagine amine was accomplished *via* Mitsunobu conditions, and removal of the activating group produced the resin-bound *N*-methylated dipeptide. Subsequent

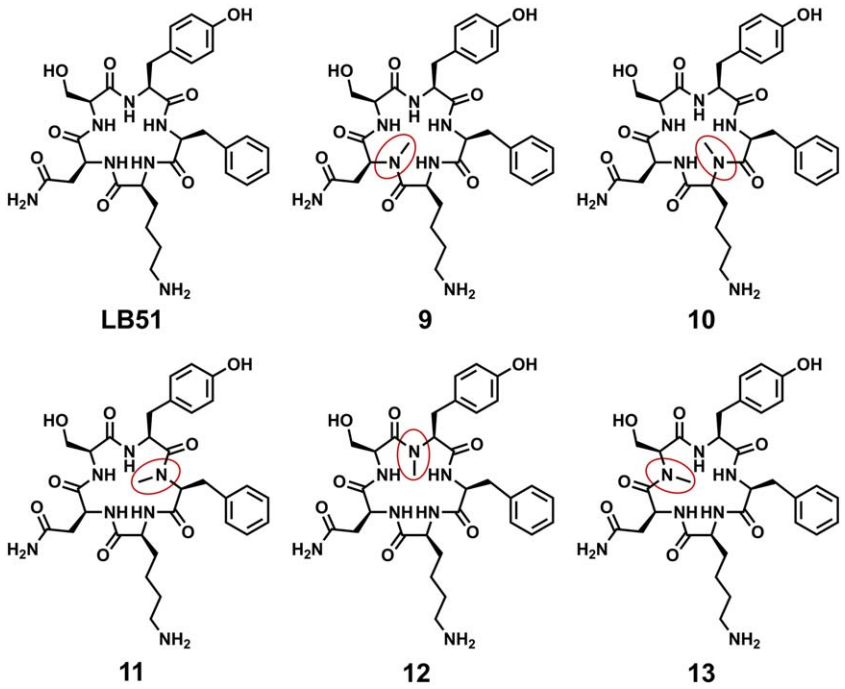
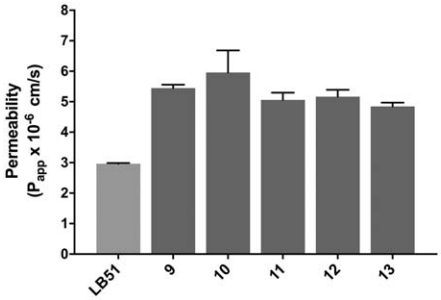


FIGURE 3 Structure of 5 molecules synthesized and evaluated for membrane permeability (**9–13**). All structures are *N*-methyl analogs of lead compound LB51

sequential coupling and Fmoc removal with lysine (Boc), phenylalanine, and tyrosine (*t*-Bu) amino acids respectively produced the linear peptide. Cleavage under mildly acidic conditions (TFE), and cyclization under dilute conditions (0.001 M) using a combination of 3 coupling agents (HATU, TBTU, and DMTMM) produced the compound **9**.[34] Subsequent deprotection of the side-chains was accomplished using TFA and reversed-phase HPLC purification produced pure compound **9** as a white solid (18 mg, 4% overall), where LC/MS, ^{1}H NMR, and 2D NMR were used to confirm the final structure and purity. Compound **9** was produced with a yield that was comparable to the 4 other molecules produced using commercially available *N*-methylated amino acids. This indicates that despite being a longer synthesis, on-resin *N*-methylation is a viable synthetic approach.

Upon completion of the synthesis, these compounds were evaluated in a parallel artificial membrane permeability assay (PAMPA). PAMPA measures passive diffusion of a molecule through an artificial lipid membrane. The artificial membrane was produced by soaking the porous filter of a Transwell plate (Corning cat # 3413) in a solution of lecithin in dodecane for 15 min. Compounds were dissolved in a solution of 1% DMSO in PBS and the whole system was incubated for 16 h in a humidified sealed chamber. After incubation, a sample from top and bottom wells was analyzed using LC/MS and quantified using a standard curve of known compound concentrations. The diffusion rate was then calculated and expressed as $P_{app} \times 10^{-6}$ cm/s.

Backbone *N*-methylation increased the membrane permeability of **LB51**, from P_{app} ~3 to between 5 and 6 (Figure 3). These data confirm previous reports that *N*-methylation is an effective strategy for improving membrane permeability. *N*-methylation of the peptide backbone changes the conformation and intramolecular hydrogen bonding pattern compared to the nonmethylated parent compound, and alters the solvent exposure of the backbone amides. In this series, the location of the *N*-methyl group appears unimportant and all 5 analogs increase passive diffusion by a similar factor. These data suggest that the intramolecular hydrogen bonding pattern and conformation of all molecules



Compound	*N*-methylated amino acid	P_{app} (x 10^{-6} cm/s)
LB51	-	3.0 ± 0.1
9	Asparagine	5.4 ± 0.1
10	Lysine	6.0 ± 0.7
11	Phenylalanine	5.1 ± 0.2
12	Tyrosine	5.2 ± 0.2
13	Serine	4.8 ± 0.1

FIGURE 4 Membrane permeability of all 5 *N*-methylated **LB51** analogs: compounds **9–13**, as measured in PAMPA. Graph of mean ± SEM, average of at least 2 experiments

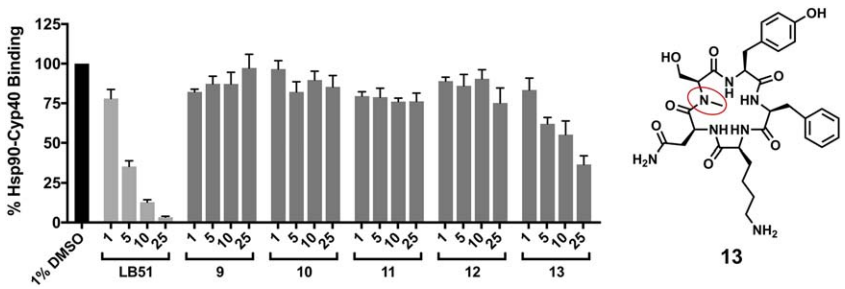


FIGURE 5 Impact of analogs on inhibiting the binding event between 2 proteins (Hsp90 and Cyp40). Graphs of mean ± SEM, average of at least 2 experiments

is similar for the entire series, and distinct from **LB51**, regardless of the *N*-methyl placement, which explains the comparable permeability values.

Balancing membrane permeability with biological target binding is challenging because these 2 properties often have opposing requirements. For cyclic peptides to be membrane permeable, they must be able to adopt and interchange between multiple conformations as they traverse biological membranes. A "polar" conformation is present when they are in extracellular and intracellular aqueous environments, and a "hydrophobic" conformation is present as they passively diffuse across lipid membranes. In the "polar" conformation, the polar side chains would be available for interacting with water or the biological target in order for cyclic peptides to bind tightly. *N*-methylation promotes or increases the "hydrophobic" conformation most likely by restricting available conformations and facilitating the intramolecular hydrogen bonding of polar groups, thereby increasing membrane permeability. Since goal is to produce a cell membrane permeable derivative of **LB51** while maintaining the biological activity, thus it was essential to investigate the impact of backbone *N*-methylation on the bioactivity of these analogs.

LB51 is an heat shock protein 90 (Hsp90) inhibitor, and is highly effective at blocking the interaction between Hsp90 and Cyp40.[5] It is well-established that Hsp90 facilitates tumor development by stabilizing oncogenic client proteins, and as such Hsp90 inhibitors are a promising class of anti-cancer molecules. Compounds **9–13** were evaluated for their ability to block the interaction between Hsp90 and Cyp40 using a commercially available AlphaLISA assay (Hsp90β C-terminal Inhibitor Screening Kit from BPS Biosciences, cat. # 50314) (Figure 5). Each compound was incubated at multiple concentrations, with the C-terminal fragment of Hsp90β (UniProt P08238, a.a. 527–724) and Cyp40 protein following the manufacturer's protocol. DMSO (1%) was used as a control, representing 100% binding between the C-terminus of Hsp90β and Cyp40. Based on the binding data (Figure 5), the only molecule that inhibited this interaction was **13**, which contains an *N*-methyl at the serine position. This molecule displays concentration-dependent inhibition of the protein-protein interaction with an IC_{50} of ~15 μM, which is 3-fold lower than that of **LB51**. The other 4 compounds all appear to have nonspecific hydrophobic interactions as their activity shows no concentration dependence. These data clearly show that while inclusion of an *N*-methyl group is an excellent strategy for improving membrane permeability, the resulting conformational changes have significant impacts on biological activity.

4 | CONCLUSION

Converting polar cyclic peptides into cell permeable molecules is a significant challenge, and the difficulty is increased by the need to balance membrane permeability and bioactivity. As such, many reports focus only on improving permeability without considering target binding. We report the synthesis of 5 new compounds, their membrane permeability values, and their biological activity. All 5 compounds showed improved permeability over the lead **LB51**, with P_{app} values on par with successful examples produced by other labs. However, this increase in permeability resulted a reduction in binding affinity, likely because of changes in the molecules' conformation. Previously reported compounds included 3 or fewer polar groups, and this polarity is offset by the inclusion of multiple backbone *N*-methyl groups. In contrast, our molecules contained 4 polar groups and a single *N*-methyl exemplifying that the strategy can be successful with a minimum number of *N*-methyl moieties. We have also recently reported a prodrug strategy where including masked polar side chains, with no N-methylation of the backbone produces relatively permeable molecules, with 4–5 fold decrease in potency.[18] Studies aimed at maintaining activity while examining the synergistic effects of *N*-methylation, inverting stereochemistry, and modification of side chains will be reported in due course.

ORCID

Shelli R. McAlpine http://orcid.org/0000-0003-1058-5252

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SUPPORTING INFORMATION

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How to cite this article: Lee LLH, Buckton LK, McAlpine SR. Converting polar cyclic peptides into membrane permeable molecules using *N*-methylation. *Peptide Science*. 2018;e24063. https://doi.org/10.1002/pep2.24063