

Figure 2. Cysteine-based and cysteine-free native chemical ligation.

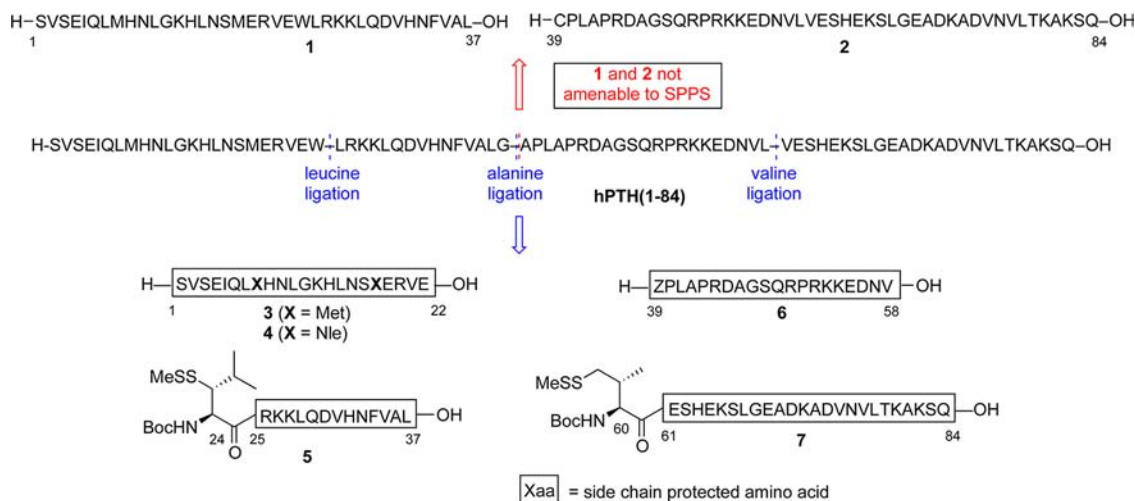


Figure 3. PTH(1–84) target amino acid sequence and synthetic strategy.

likely depend on the incorporation of non-encoded amino acid modifications through synthetic means.

A growing focus of our laboratory<sup>9</sup> has been the development of strategies to enable the controlled total synthesis of complex biologic targets, including peptides,<sup>9a</sup> carbohydrates,<sup>10</sup> and glycopeptides.<sup>11</sup> Through major advances in *de novo* chemical synthesis, we have gained the capacity to assemble challenging “biologic” level target systems, with standards of homogeneity and purity not always achievable through biosynthetic or recombinant approaches. In the context of the research described below,<sup>12</sup> we envisioned applying the synthetic advances developed in our laboratory to the preparation of a series of PTH-derived compounds for biological evaluation, with the ultimate goal of identifying therapeutically viable analogues with enhanced shelf- and biostability.

In designing our target analogue structures, we took note of the finding that degradative oxidation of the two methionine residues (Met8 and Met18) appears to generate products with decreased adenylate cyclase activity.<sup>13</sup> We postulated that replacement of such methionine residues with sulfur-free, closely isosteric norleucine (Nle) residues might well mitigate oxidative degradation of the biologic agent, thereby enhancing peptide stability, both under storage conditions and in *in vivo* settings. An analogue of the hPTH fragment incorporating unnatural Nle residues at positions 8 and 18 had been previously prepared,<sup>14</sup> although no full-length Nle-containing analogues have been reported. We thus sought to prepare, through chemical synthesis, hPTH(1–84) as well as the full-length [Nle<sup>8,18</sup>]hPTH(1–84) analogue. For comparison

purposes, we would also synthesize the hPTH(1–37) and [Nle<sup>8,18</sup>]hPTH(1–37) fragments. We describe herein the synthesis and preliminary biological evaluations of these PTH-derived peptides. We believe that the approaches described are of interest in terms of the PTH field itself but also are suggestive of future directions at a more general level.

Central to our proposed program was the perception that chemical synthesis has reached the stage where it can be seen as a realistic possibility for gaining access to proteins, and even glycoproteins, previously seen as “biologics” to be synthesized by biological means (i.e., by natural or naturally inspired expression synthesis). Clearly, a major leap forward in this regard was the discovery and development of solid phase peptide synthesis (SPPS). Another major advance was the advent of native chemical ligation (NCL), pioneered by Kent and associates (Figure 2a).<sup>15,16</sup> NCL provided a means of covalently joining substantially sized unprotected synthetic peptidic segments. In this way, one could assemble polypeptides of sizes that exceed the capacity of SPPS to produce adducts of required purity.

While NCL has indeed proven to be a powerful tool, in its initial form it carried with it a significant limitation, i.e., that there had to emerge a cysteine at the N-terminus of the proposed ligation site. The possibility of using NCL to achieve ligation at what would become an alanine residue was well recognized by Dawson and was reduced to practice through postligation Raney Ni reduction of the erstwhile cysteine thiol function.<sup>17a</sup> While providing a very clever insight, this method lacked broad generality for two main reasons. First, the protocol was not well translated to the aqueous-type conditions

in which the NCL had been conducted. Moreover, the metal-induced technology was not shown to be compatible with common functionalities in glycopeptide synthesis, such as thiazolidine (Thz) moieties or carbohydrate domains.

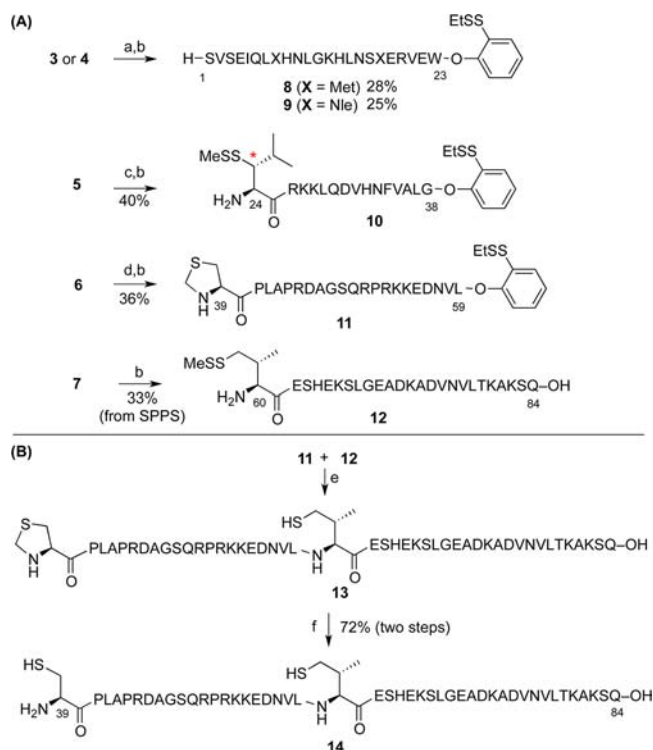
The next large advance was occasioned by the discovery of metal free dethylation (MFD).<sup>18</sup> The method employed involved application of the Walling–Rabinowitz dethylation logic,<sup>19</sup> discovered in a totally different context as a curiosity in physical organic chemistry. Applicable in aqueous medium, the Walling–Rabinowitz reaction suggested that an alanine residue could be crafted at a specific point in the primary structure, even in the presence of protected cysteines destined to eventually emerge as cysteine residues (Figure 2b). More recently, our laboratory and then others have expanded the logic of NCL to encompass a menu of non-cysteine amino acids, including phenylalanine,<sup>17b,20</sup> leucine,<sup>21</sup> lysine,<sup>22</sup> threonine,<sup>23</sup> valine,<sup>24</sup> and proline<sup>25</sup> residues, using MFD. As will be shown, application of this powerful new technology was to prove crucial to the successful total synthesis of PTH and its analogues.

## RESULTS AND DISCUSSION

**Synthetic Design.** Previous approaches to full-length hPTH have required either coupling of fully protected peptide segments<sup>26</sup> or linear assembly through solid phase peptide synthesis (SPPS).<sup>27</sup> However, these strategies suffer from significant practical limitations arising from synthetic inefficiency and difficulties in peptide purification. In contrast, we envisioned a convergent, ligation-based approach to hPTH and our selected analogues. We first attempted to prepare hPTH(1–84) from two segments, Ser1–Gly38 and Ala39–Gln84 (Figure 3, in red). However, our efforts to synthesize the requisite peptides, **1** and **2**, through standard Fmoc-based methods resulted in significant quantities of byproducts, arising from amino acid deletions and aspartimide formation. Thus, even at this writing, the power of SPPS can be quite sequence-sensitive.

In order to maximize product purity and synthetic efficiency, we next devised a modified route, wherein the peptide would be dissected into four fragments (Figure 3, in blue). Through iterative leucine, alanine, and valine ligations, the individual peptides would be merged to generate the full PTH sequence. Notably, the oxidation-prone Met8 and Met18 residues are both contained within fragment 3. Thus, this proposed disconnection approach offers high modularity for the assembly of our proposed Nle-containing analogues (see peptide 4). In the event, peptide fragments 3–7 were synthesized through standard Fmoc-based SPPS protocols in good yield and purity. Interestingly, segment 6 was found to be prone to formation of aspartimide byproducts during SPPS. This problem could be eliminated through the use of Fmoc-Asp(ODie)-OH in place of the Fmoc-Asp(OtBu)-OH amino acid.<sup>28</sup> As outlined in Scheme 1A, the ligation substrates (**8**–**12**) were prepared from the SPPS precursors through established protocols. We note that, in the case of the thio-leucine amino acid surrogate (cf. **10**), we had previously observed a significant rate differential, dependent on the stereochemical configuration of the  $\beta$ -thiol functionality (see asterisk). Thus, the thio-leucine isomer depicted in structure **10** is significantly more reactive than is its  $\beta$ -thio epimer.<sup>21b</sup> Notably, (2-ethylthiolphenyl)ester, developed in our laboratory, was installed at the C-termini of segments **8**–**11**.<sup>29</sup> We were now prepared to attempt three NCL-inspired ligations followed by appropriate MFD, to co-

## Scheme 1. Preparation of Peptide Segments for Ligation<sup>a</sup>

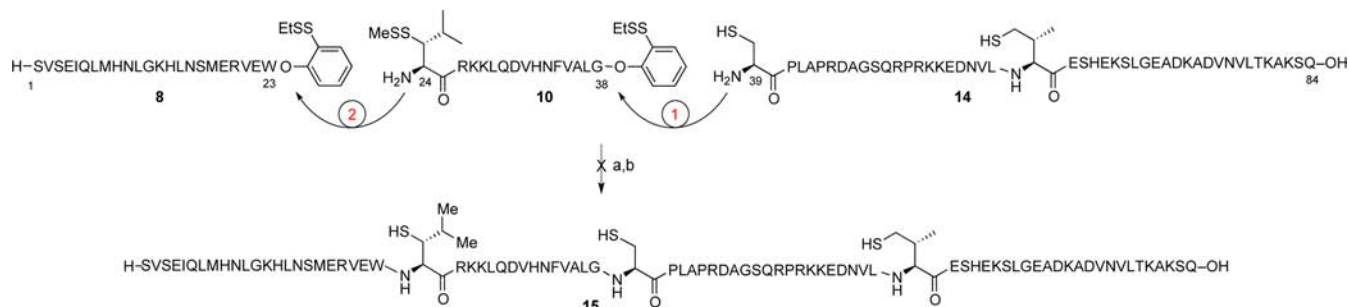


<sup>a</sup>Reaction conditions: (a) HCl·H-Trp-O(EtSS)Ph, EDC, HOObt, TFE/CHCl<sub>3</sub> (1:3), 4 h; (b) TFA/TIS/H<sub>2</sub>O (95:2.5:2.5), 45 min; (c) HCl·H-Gly-O(EtSS)Ph, EDC, HOObt, TFE/CHCl<sub>3</sub> (1:3), 4 h; (d) HCl·H-Leu-O(EtSS)Ph, EDC, HOObt, TFE/CHCl<sub>3</sub> (1:3), 4 h; (e) 6 M Gn·HCl, 100 mM Na<sub>2</sub>HPO<sub>4</sub>, and 50 mM TCEP, pH 7.5, 15 h; (f) MeO-NH<sub>2</sub>·HCl, pH 4, 3 h. EDC = *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide; HOObt = hydroxy-3,4-dihydro-4-oxo-1,2,3-benzotriazine; TFE = trifluoroethanol; TIS = triisopropylsilane; Gn = guanidine; TCEP = tris(2-carboxyethyl)phosphine.

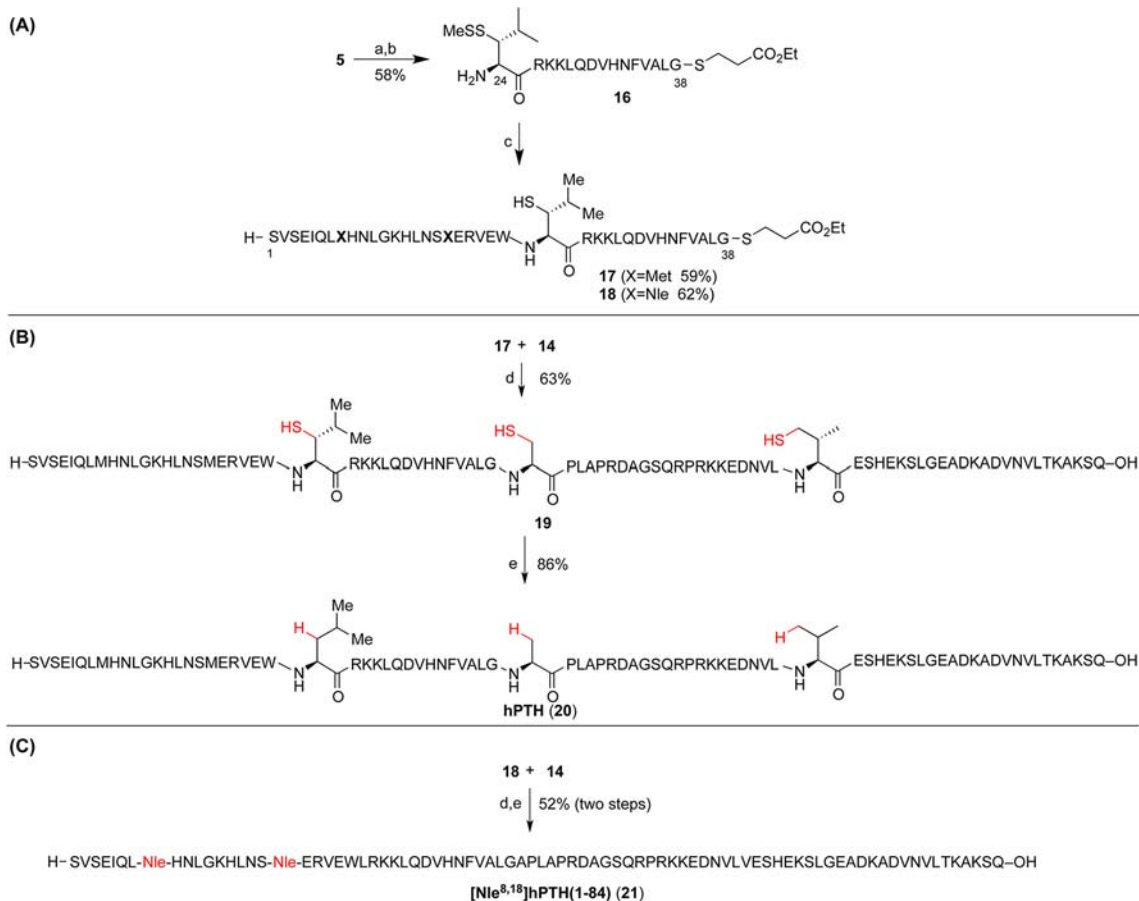
assemble the four peptide components en route to full-length hPTH. As shown in Scheme 1B, segments **11** and **12** readily underwent “thio-valine ligation”,<sup>24b</sup> followed by thiazolidine ring opening,<sup>30</sup> to afford peptide **14**, encompassing the PTH(39–84) region. The newly unveiled N-terminal cysteine residue was envisioned to serve as an implement for subsequent formal alanine ligation.

We next explored the feasibility of a one-flask iterative ligation protocol (Scheme 2). We had previously observed a significant rate differential between cysteine- and thio-leucine based NCL.<sup>21b</sup> It was hoped to exploit this reactivity ordering by first exposing **10** and **14** to ligation conditions and then adding peptide **8** to the reaction flask, without isolation of the ligated peptide intermediate. The thought was that **10** and **14** would first undergo the more facile cysteine ligation, and the resultant peptide would then merge with **8** in a subsequent leucine ligation, to afford full length PTH bearing three extraneous thiol groups. However, in practice, no coupled product was obtained from the first ligation of segments **10** and **14**. Rather, LC–MS analysis indicated formation of a cyclic peptide, presumably arising from cyclization of **10**. This result suggests that the reduced steric demands of intramolecular thioester formation significantly override the enhanced ligation reactivity of cysteine compared with thio-leucine.

We next investigated the merger of the PTH(24–38) fragment with peptide **8** through kinetically controlled ligation

Scheme 2. Attempted One-Pot Sequential Ligations<sup>a</sup>

<sup>a</sup>Reaction conditions: (a) **10**, **14**, 6 M Gn·HCl, 100 mM Na<sub>2</sub>HPO<sub>4</sub>, 50 mM TCEP, pH 7.2, 2 h; (b) **8**, 6 M Gn·HCl, 100 mM Na<sub>2</sub>HPO<sub>4</sub>, 50 mM TCEP, pH 7.2.

Scheme 3. Synthesis of hPTH(1–84) and [Nle<sup>8,18</sup>]hPTH(1–84) Using Cysteine-Free NCL and Metal-Free Desulfurization<sup>a</sup>

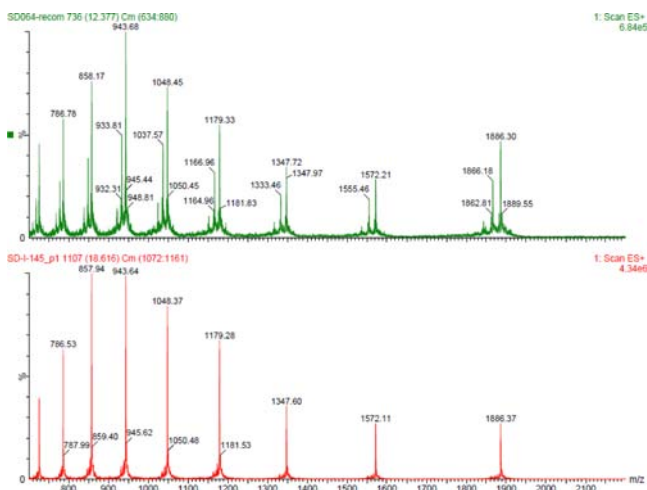
<sup>a</sup>Reaction conditions: (a) HCl·H-Gly-SCH<sub>2</sub>CH<sub>2</sub>COOEt, EDC, HOObt, TFE/CHCl<sub>3</sub> (1:3), 4 h; (b) TFA/TIS/H<sub>2</sub>O (95:2.5:2.5), 45 min; (c) **8** or **9**, 6 M Gn·HCl, 100 mM Na<sub>2</sub>HPO<sub>4</sub>, and 50 mM TCEP, pH 7.2, 3 h; (d) 6 M Gn·HCl, 300 mM Na<sub>2</sub>HPO<sub>4</sub>, 200 mM MPAA, and 20 mM TCEP, pH 7.3, 2 h; (e) VA-044, *t*Bu-SH, TCEP, H<sub>2</sub>O, MeCN, 37 °C, 2 h. VA-044 = 2,2'-azobis[2-(2-imidazolin-2-yl)propane]dihydrochloride.

(KCL).<sup>31</sup> Thus, peptide **5** was converted to **16**, bearing the less reactive alkyl thioester at the C-terminus, in place of (2-ethylthiophenyl)ester (see **16** vs **10**). As shown in Scheme 3A, peptide **16** underwent the hoped-for leucine ligation with peptides **8** [PTH(1–23)] or **9** [[Nle<sup>8,18</sup>]PTH(1–23)]. Notably, the alkyl thioester remained inert under these conditions, and only small amounts of cyclic peptide side product (ca. 10%) were observed. Adduct **17** was subsequently merged with peptide **14** under NCL conditions in the presence of MPAA, to afford full length hPTH(1–84) (**19**), bearing three extraneous thiol groups. Under standard metal-free desulfurization

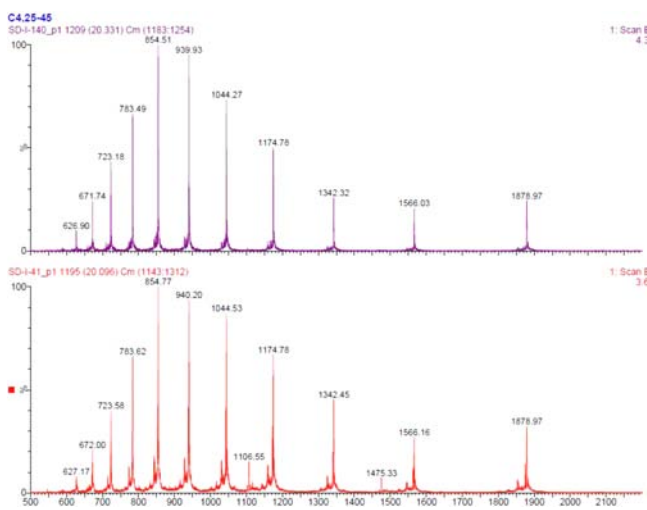
conditions, all three thiol groups were smoothly removed to reveal full-length hPTH protein in good overall yield (Scheme 3B). Comparative HPLC–MS analysis of a commercial sample (from Bachem) and our synthetic material (**20**) revealed the latter to possess significantly higher purity (Figure 4).

The [Nle<sup>8,18</sup>]hPTH(1–84) analogue (**21**) was prepared from **18** and **14** in an analogous manner (Scheme 3C). HPLC–MS analysis reveals that the product was obtained in high purity (Figure 5), particularly in comparison to a synthetic sample obtained through a different route (see Supporting Information), and CD spectroscopy data strongly indicate that the





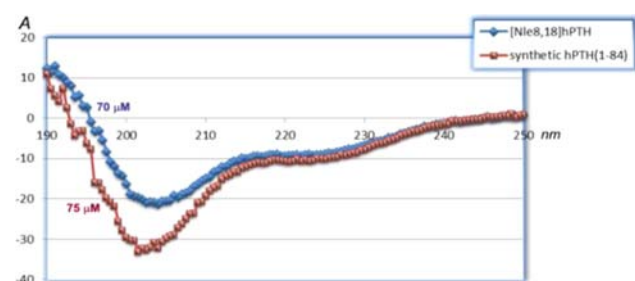
**Figure 4.** MS data of commercial hPTH(1–84) and synthetic hPTH(1–84). Top: commercial sample. Bottom: synthetic polypeptide **20**.



**Figure 5.** MS data of [Nle<sup>8,18</sup>]hPTH(1–84) prepared from two routes. Top: four segments (**9**, **16**, **11**, and **12**) synthesis. Bottom: three segments (**9**, **16**, and **2**) synthesis.

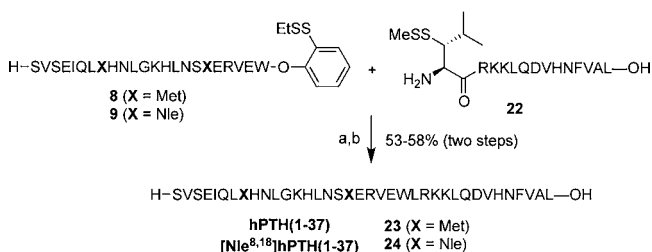
secondary structure of the bis-norleucine analogue, **21**, is similar to that of the native sequence (Figure 6).

Finally, the truncated fragments, PTH(1–37) (**23**) and [Nle<sup>8,18</sup>]PTH(1–37) (**24**), were prepared through ligation of peptides **8** and **9** with peptide **22**, bearing an N-terminal thioleucine amino acid surrogate (Scheme 4). Ligation, followed by metal-free dethylation, delivered **23** and **24** in 53% and 58% yields, respectively.



**Figure 6.** CD spectra of hPTH(1–84) and [Nle<sup>8,18</sup>]hPTH(1–84).

#### Scheme 4. Synthesis of Analogues PTH(1–37) and [Nle<sup>8,18</sup>]PTH(1–37)<sup>a</sup>



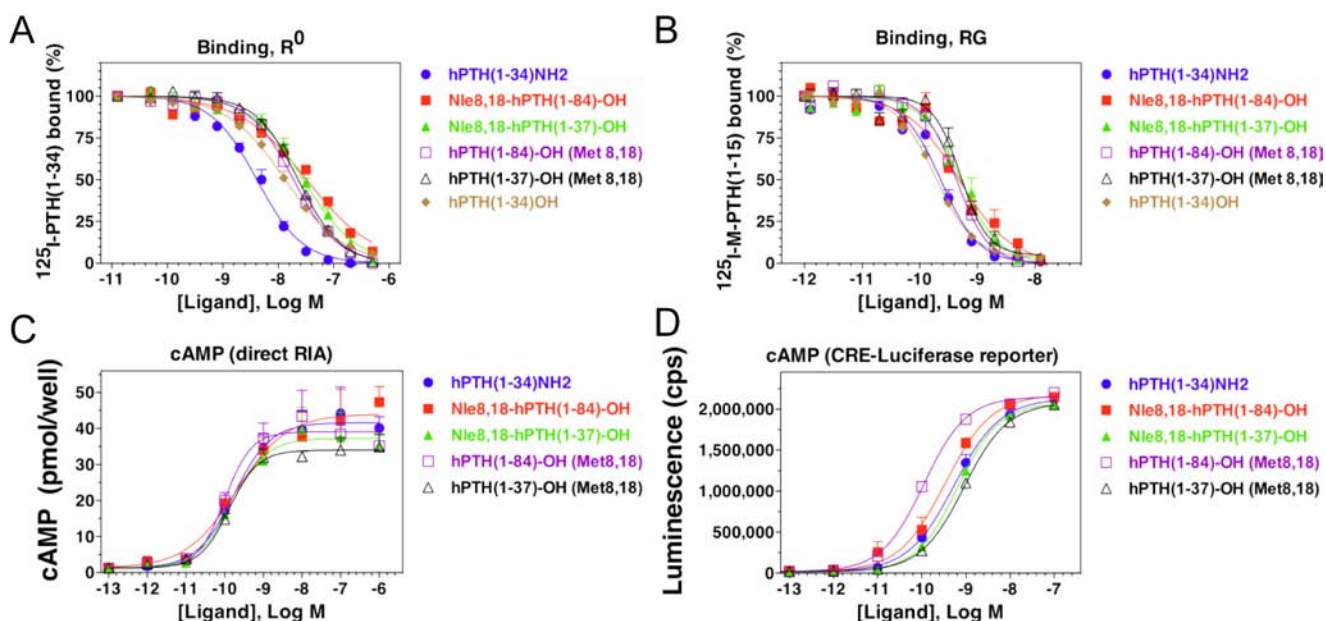
<sup>a</sup>Reaction conditions: (a) 6 M Gn-HCl, 100 mM Na<sub>2</sub>HPO<sub>4</sub>, and 50 mM TCEP, pH 7.2, 2 h; (b) VA-044, tBu-SH, TCEP, H<sub>2</sub>O, MeCN, 37 °C, 2 h.

**Evaluation of Storage Stability of hPTH and Analogues.** Oxygen- or light-induced methionine oxidation is known to be one of the major degradation pathways of a number of pharmaceutically used proteins, including hPTH,<sup>32</sup> interleukin-2,<sup>33</sup> and human growth hormone.<sup>34</sup> A more stable variant would offer significant advantages for the synthesis, purification, formulation, manufacturing, and storage of these therapeutically important biomedicines.<sup>35</sup> With this consideration in mind, we first evaluated the shelf life of our synthetic PTH analogues.

For synthetic hPTH (**20**) and analogues **21**, **23**, and **24**, degradation of the pure samples was monitored using HPLC–MS over a period of time (see Supporting Information). Under ambient conditions (room temperature, air, water solution, and neutral pH), the analytical results suggested that natural PTH(1–84) decomposed significantly over the time, and after 7 days, greater than 90% (estimated on the basis of UV signal) of PTH degraded to fragments or other byproducts. In contrast, analogue [Nle<sup>8,18</sup>]hPTH(1–84) showed far greater stability under the same conditions, with less than 10% degradation observed after 7 days. By contrast, the truncated analogues, hPTH(1–37) and [Nle<sup>8,18</sup>]hPTH(1–37), showed similar levels of shelf stability, and the analytical results suggested approximately 70% decomposition after 7 days in both cases.

Interestingly, in the case of natural 84-mer, the major decomposition products observed were not simple oxidized products, but fragments with significant mass loss in comparison to the original peptide. It may be tentatively surmised that oxidation of the Met residues could have permutated the secondary and tertiary structures of this protein, thus initiating or accelerating further degradation pathways.<sup>36</sup> However, it could also be possible that the introduction of Nle residues on [Nle<sup>8,18</sup>]PTH(1–84) may alter the physicochemical characteristics of the peptide, such as folding and subunit association, leading to better sustainability under light- or oxygen-induced oxidative conditions. It should also be taken into account that other amino acid residues, including His and Trp, are also susceptible to oxidation. Such oxidative mechanisms may be responsible for the serious shelf life instability of the two 37-mer analogues, [PTH(1–37) and [Nle<sup>8,18</sup>]PTH(1–37)]. Presumably, in these more vulnerable systems, other common instabilities tend to mask the incremental benefits of the synthetically accomplished methionine → Nle mutations.<sup>37</sup>

**Biological Evaluation of Synthetic hPTH(1–84) and Analogue.** Parathyroid hormone (PTH), via its receptor, the



**Figure 7.** *In vitro* biological data. The capacities of the PTH analogues to bind to the PTHR in a G protein-independent conformation, R<sup>0</sup>, and a G protein-dependent conformation, RG, were assessed in membrane-based competition assays. Assays for R<sup>0</sup> were performed using <sup>125</sup>I-PTH(1–34) tracer radioligand and in the presence of excess GTPγS (A), and assays for RG binding were performed using <sup>125</sup>I-M-PTH(1–15) tracer radioligand and membranes from cells expressing a high affinity *Gαs* mutant (B). The cAMP signaling properties of the analogues were assessed using intact HEK-293 cells transfected to express the human PTHR; PTH-stimulated cAMP formation was assessed by direct radioimmunoassay performed 30 min after ligand application in the presence of IBMX (C), as well as by using cells stably transfected to express the PTHR together with a luciferase reporter gene under transcriptional control of a cAMP-response element-containing promoter, and measuring luciferase activity, as luminescence, 4 h after ligand application (D).

**Table 1.** Ligand Binding and cAMP-Signaling Properties on the hPTHRI<sup>a</sup>

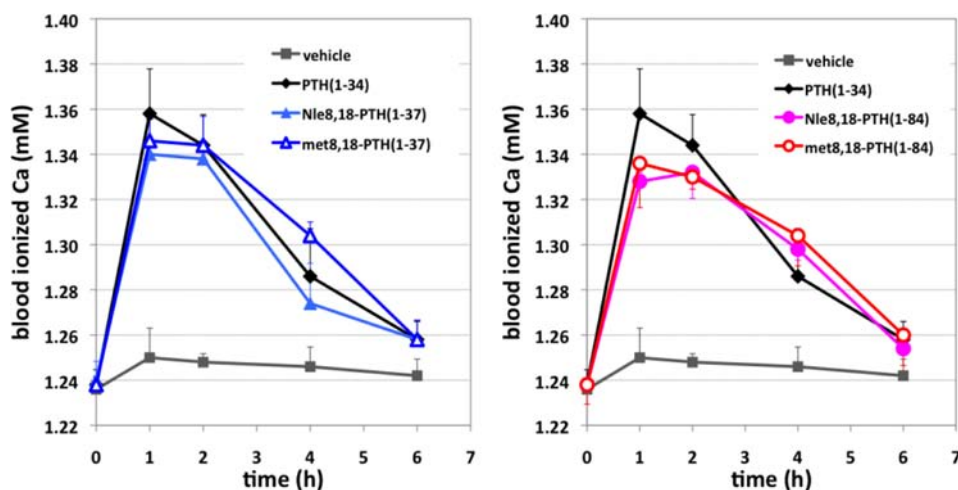
peptide	binding COS-7/hPTHRI cell membranes pIC <sub>50</sub>		cAMP HEK-293/hPTHRI cells pEC <sub>50</sub>	
	R <sup>0</sup>	RG	direct RIA	Cre-Luc
hPTH(1–34)NH <sub>2</sub>	8.38 ± 0.11	9.70 ± 0.05	9.74 ± 0.14	9.27 ± 0.18
	<i>4.14</i>	<i>0.20</i>	<i>0.18</i>	<i>0.54</i>
Nle <sup>8,18</sup> -hPTH(1–84)-OH	7.55 ± 0.14	9.08 ± 0.30	9.62 ± 0.16	9.57 ± 0.03
	<i>28.3</i>	<i>0.84</i>	<i>0.24</i>	<i>0.27</i>
Nle <sup>8,18</sup> -hPTH(1–37)-OH	7.56 ± 0.09	9.29 ± 0.17	9.68 ± 0.16	9.17 ± 0.09
	<i>27.7</i>	<i>0.51</i>	<i>0.21</i>	<i>0.68</i>
hPTH(1–84)-OH (Met 8,18)	7.73 ± 0.11	9.35 ± 0.05	9.88 ± 0.32	9.90 ± 0.06
	<i>18.7</i>	<i>0.44</i>	<i>0.13</i>	<i>0.13</i>
hPTH(1–37)-OH (Met 8,18)	7.64 ± 0.04	9.31 ± 0.09	9.58 ± 0.22	9.03 ± 0.09
	<i>22.7</i>	<i>0.49</i>	<i>0.27</i>	<i>0.93</i>
hPTH(1–34)OH	7.92 ± 0.14	9.74 ± 0.12	ND	ND
	<i>11.9</i>	<i>0.18</i>		

<sup>a</sup>Data are means ± SEM of three experiments, each performed in duplicate. Values are shown as the negative log concentration (with SEM) and as the corresponding nM value (italic). ND = not determined.

PTH (or PTHR1), plays a critical role in maintaining normal blood concentrations of ionized calcium (Ca<sup>2+</sup>) and inorganic phosphate (Pi). Thus, in rapid response to a decrease in the blood Ca<sup>2+</sup> concentration, PTH is secreted from the parathyroid glands and acts on bone to promote resorption of the mineralized matrix and on kidney to promote reabsorption of Ca<sup>2+</sup> from the glomerular filtrate. These coordinated actions in bone and kidney serve to maintain blood and fluid Ca<sup>2+</sup> levels within a narrow range (~1.2 mM ± 10%). The PTHR is a class B G protein-coupled receptor that signals mainly via the *Gαs*/cAMP/PKA second messenger pathways. Recent studies show that the PTHR can form two pharmacologically distinct conformations: a G protein-dependent conformation, called RG, and a G protein-independent conformation, called R<sup>0</sup>, and a

high relative capacity of a ligand to bind to R<sup>0</sup> is associated with a prolonged signaling response induced by that ligand *in vitro* and *in vivo*.<sup>38,39</sup>

The capacities of the analogues to bind to the PTHR in a G protein-independent conformation, R<sup>0</sup>, and a G protein-dependent conformation, RG, were assessed in membrane-based competition assays.<sup>38</sup> Assays for R<sup>0</sup> were performed using <sup>125</sup>I-PTH(1–34) tracer radioligand and in the presence of excess GTPγS. Under these R<sup>0</sup> conditions, each analogue bound with an affinity in the low- to midnanomolar range (IC<sub>50</sub>'s = 4–30 nM; Figure 7A). Assays for RG binding were performed using <sup>125</sup>I-M-PTH(1–15) tracer radioligand and membranes from cells expressing a high affinity *Gαs* mutant. Under these RG conditions, each analogue bound with an



**Figure 8.** Effects of PTH analogues on blood  $\text{Ca}^{2+}$  levels in mice. Nine-week-old male mice, strain C57BL/6, were injected subcutaneously with vehicle or vehicle containing a PTH analogue at a dose of 20 nmol/kg body weight, and blood was withdrawn at times thereafter, as well as just prior to injection ( $t = 0$ ), and assessed for ionized calcium.

affinity in the subnanomolar range ( $\text{IC}_{50}$ 's = 0.13–0.27 nM; Figure 7B, Table 1).

**cAMP Assays.** The signaling properties of the analogues were assessed using intact HEK-293 cells transfected to express the human PTHR. Cells transiently transfected with the PTHR were treated with ligand for 30 min in the presence of IBMX, and the intracellular cAMP levels were measured directly by radioimmunoassay (RIA).<sup>36</sup> The analogues were also assayed using cells stably transfected to express the PTHR together with a luciferase reporter gene under transcriptional control of a cAMP-response element-containing promoter. In these assays, the analogues exhibited potencies in the low-nanomolar range ( $\text{EC}_{50}$ 's =  $\sim$ 0.1–0.9 nM; Figure 7C and D, Table 1).

**Effects of PTH Analogues on Blood  $\text{Ca}^{2+}$  Levels in Mice.** The capacities of the analogues to stimulate increases in blood  $\text{Ca}^{2+}$  were assessed in normal 9-week-old, male, C57BL/6 mice. Prior to injection, the blood  $\text{Ca}^{2+}$  concentrations in the wild-type mice were  $\sim$ 1.24 mM (Figure 8). Following injection of the PTH analogues, blood  $\text{Ca}^{2+}$  levels increased robustly and reached a peak of  $\sim$ 1.36 mM by 1 h postinjection. Blood  $\text{Ca}^{2+}$  levels then returned to vehicle-control levels by 6 h with each analogue tested. Experiment results indicated that all four synthetic peptides increased the  $\text{Ca}^{2+}$  concentration in mice. However, no significant difference was observed in comparison to PTH(1–34), with respect to peak [ $\text{Ca}^{2+}$ ] and half-life.

## CONCLUSION

Utilizing three non-cysteine-based native chemical ligations, with subsequent MFD, developed in our laboratory, we have synthesized the desired full length parathyroid hormone and a number of analogues with high levels of purity. That the four new PTH analogues presented here showed *in vitro* and *in vivo* potencies comparable to those of the PTH(1–34) control peptide confirms that a chemical synthesis-driven approach offers a plausible means for the molecular editing of therapeutic level proteins. In the case at hand, the methods and overall logic have been demonstrated to be effective, not only for the generation of full length PTH(1–84) but also for incorporating chemical modifications, such as non-coding amino acids,<sup>6,7</sup> into the peptide chain for the purposes of improving action on the receptor, conformational stability, and ultimately *in vivo* activity. PTH analogues with improved activity could offer new

therapeutic approaches for the diseases of osteoporosis<sup>40</sup> and hypoparathyroidism.<sup>41</sup> In the former case, analogues that can stimulate new bone formation without inducing hypercalcemia, as can occur with native PTH peptides,<sup>42</sup> would offer great advantage to current therapies. For hypoparathyroidism, long-acting analogues that can maintain normal blood  $\text{Ca}^{2+}$  levels for prolonged periods after administration would offer clear benefit over native PTH and the more conventional treatment of vitamin D and Ca supplementation.<sup>43</sup> Possibilities for integrating more closely the capacities of chemical synthesis and the needs of the biology–medicine interface are likely to expand.

## ASSOCIATED CONTENT

### Supporting Information

General experimental procedures, including spectroscopic and analytical data for new compounds. This material is available free of charge via the Internet at <http://pubs.acs.org>.

## AUTHOR INFORMATION

### Corresponding Author

s-danishesky@ski.mskcc.org

### Notes

The authors declare no competing financial interest.

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