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SOLID PHASE REDUCTIVE ALKYLATION TECHNIQUES IN ANALOGUE PEPTIDE BOND AND SIDE-CHAIN MODIFICATION

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ABSTRACT. The presence of a reduced peptide bond in a biologically active peptide usually has profound effects on conformation and potency. The CH_2NH group may be a useful tool for delineating important hydrogen-bonding points and may also offer a general strategy for the production of enzyme resistance and potent competitive antagonists containing minimal structural alteration. The introduction of the group by solid phase reductive alkylation with a protected amino acid aldehyde offers a rapid route to peptides containing one or even many bond replacements. Potential racemization problems have been investigated and appear to be mostly related to the $LiAlH_4$ reduction of amino acid dimethylhydroxamates to the aldehydes. Conditions are presented which prevent this, however, prolonged storage of amino acid aldehydes does appear to result in substantial racemization. The stability of commonly used side chain protecting groups to synthetic conditions was also investigated. The reactivity of the CH_2NH group to subsequent coupling reactions is surprisingly low. Its utility as an additional derivatization point in analogue design is examined. Some biological effects of peptide bond replacement and also solid-phase N-alkylation of several types of peptide hormones are discussed.

Modification of the peptide bond CONH group is one of the most direct approaches to altered peptide conformation and perhaps the only one which does not effect side chain structure. Additionally, modified bonds become advantageously resistant to enzymatic hydrolysis.

(A) Boc-Trp-Leu-Asp-Phe-NH_2

(B) Boc-Trp-Leu-Asp-ψ[CH_2NH]-Phe-NH_2

(C) Boc-Trp-Leu-ψ[CH_2NH]-Asp-Phe-NH_2

Figure 1: Tetragastrin (A) and two pseudodipeptide antagonist analogues (B and C) shown in increasing order of potency.

Numerous structural mimics of the bond have been proposed and used (1), but the simplest isostere is the reduced peptide bond or CH_2NH group. This has recently been used in the design of potent ACE inhibitors (2) and, in the peptide hormone area, in the production of potent gastrin antagonists (3) such as the tetragastrin analogues shown in Figure 1.

The preferred method for the introduction of the reduced peptide bond unit is by reductive alkylation of an α-amino group by the reaction of a protected amino acid aldehyde in the presence of sodium cyanoborohydride (4). In a successful attempt to significantly increase the

speed of this type of synthesis, we recently reported (5) the adaptation of this method to the solid phase technique. In acidified DMF, the alkylation reaction proceeds rapidly and cleanly. Importantly, with the exception of Gly, little evidence was found for subsequent derivatization of the secondary NH group during carbodiimide couplings using large excesses of Boc-amino acids. The side reaction with Gly could be avoided if it was coupled as the p-nitrophenyl ester (5).

A complete series of somatostatin octapeptide analogues was then prepared in quite normal yields containing the reduced peptide bond at each consecutive position (6) in turn. The biological data obtained from this series of peptides appeared to correlate well with conformational predictions from 500 Mhz NMR studies (7) in that CH_2 for CO replacements at intramolecular H bonding points resulted in the greatest losses of GH release inhibiting activity. It is hoped, therefore, that this peptide bond replacement strategy will be a useful general addition to conformation prediction approaches.

Modification of peptide and protein side chain amino groups by reductive alkylation has been recognized for some time to be a useful semi-synthetic technique [for a review see Feeney (8)]. It occurred both to this group and that of Chorev (9) that the N-alkylation of amino groups using alkyl aldehydes and ketones might significantly extend the utility of the solid phase method. We found (10,11), for example, that epsilon amino groups present on Lys residues of potent LH-RH antagonists could be readily alkylated with a great variety of carbonyl compounds and $NaBH_3CN$ whilst the peptides were still attached to the resin, resulting in analogues with reduced histamine releasing side-effects. Ron et al. (9) recently described methods for linear and branched N-alkylation of α-amino groups using the C-terminal hexapeptide of substance P as the model. Excellent results were obtained and the method seems to be by far the most convenient route to complex N^{α}-higher-alkyl peptides, although problems with subsequent acylation of the sterically hindered NHR groups need to be addressed.

Despite the obvious usefulness of solid phase alkylation, we felt that several important questions remained to be answered regarding potential pitfalls, particularly in peptide bond replacement. For instance, the possibility for amino acid racemization during aldehyde generation, storage, and reductive alkylation never appeared to have been thoroughly investigated in the literature. General stability of the common side chain protecting groups during aldehyde formation with $LiAlH_4$ likewise needed to be investigated. Subsequent reactivity of the reduced peptide bond NH group was also of interest to us not only as a possible source of impurities but also from a novel analogue design viewpoint since it could potentially be used as a new derivatization site, for the introduction of additional conformational restraint, or even as a new type of cyclization point. These questions are examined in the present paper.

METHODS

Boc-amino acid aldehydes These were prepared by the method of Fehrentz and Castro (12) via the $LiAlH_4$ reduction of the Boc-amino acid O,N-dimethylhydroxamates. Conditions for DCC-mediated hydroxamate formation and mild reduction to the aldehydes were investigated. Aldehyde formation and side chain protecting group stability was assessed by TLC. Except in model experiments, no attempt was made to purify the generated aldehyde prior to solid phase synthesis. Products usually contained unreacted hydroxamate as the primary contaminant.

Solid phase synthesis For the introduction of the CH_2NH bond, the amino acid aldehyde (3 equiv.) was added to the resin in DMF containing 1 % AcOH. $NaBH_3CN$ (3 equiv.) was then added in DMF solution followed by shaking and monitoring of the coupling by the Kaiser ninhydrin test (13). Reactions were usually complete within 1 h, although the secondary NH group does appear to produce a weak coloration upon prolonged heating.

Racemization The optical purity of Boc-L-Phe and Boc-D-Phe was examined by solid phase

preparation and HF cleavage of L–Phe–L–Leu–NH_2 and D-Phe-L-Leu–NH_2. These two peptides eluted at 9.5 and 24.5 min, respectively, on Vydac C_{18} (30 cm, 0-16% 0.1 M TEAP (pH 3)/CH3CN, 30 min, 1.5 ml/min). Less than1% of the contaminating isomer could be found in either preparation. The same Boc amino acids were then used for the preparation of the aldehydes followed by the model pseudodipeptides, L-Phe-ψ[CH_2NH]-L-Leu–NH_2 and D–Phe–ψ[CH_2NH]-L-Leu–NH_2 which eluted at 5.7 and 6.6 min respectively under 5-40% TEAP/acetonitrile gradient conditions. The extent of racemization found under a variety of hydroxamate and aldehyde synthesis conditions and solid-phase reductive alkylation is shown in Table 1.

Table 1 Racemization During Preparation and Solid-Phase Reductive Alkylation with Boc-Amino Acid Aldehydes

Conditions for synthesis of L(D)-Phe-ψ[CH2NH]-L-Leu-NH_2	% of Isomer D	L
(a) Boc-Phe hydroxamate formation with 0.1% DMAP added. 3 M xs of $LiAlH_4$		
Couple with $NaBH_3CN$ and:		
Boc-D-Phe-CHO	94	6
Boc-L-Phe-CHO	20	80
(b) Same as (a), no DMAP		
Boc-L-Phe-CHO	11	89
(c) Same as (b), small xs of $LiAlH_4$, reduce at 0° Alkylate 1 h	n.d.	100
(d) Same as (c), alkylate overnight	n.d.	100
(e) Same as (c), aldehyde stored as oil at 4° for 6 months	24	76

Protecting group stability During the course of the synthesis of many pseudopeptide analogues of several peptide hormones, several of the common α-amino protecting groups and many side-chain protecting groups were examined for stability by tlc during $LiAlH_4$-generation of the aldehydes. The results are summarized in Table 2.

Reactivity of the reduced peptide bond Boc-Pheψ[CH_2NH]–Leu-BHA resin (0.5 mmole), prepared for the racemization study, was coupled with the symmetrical anhydride of Boc-Gly (1.5 mmole) in the presence of a catalytic amount of dimethylaminopyridine (DMAP) (1 h). The extent of the reaction was measured by HPLC of the HF cleaved peptide under the same elution conditions described earlier. L–Phe–ψ[CH_2NH]–Leu–NH_2 (38%), D-Phe–ψ[CH_2NH]–Leu–NH_2 (27%), and a new, later emerging, single peak at 13.0 min (36%) were found.

Table 2. Protecting Group Stability During Amino Acid Aldehyde Generation

Stable	Unstable
α- and ε-Boc	N^g-Tos-Arg
α- and ε-FMOC	
Thr(Bzl)	
Ser(Bzl)	
Glu(Bzl)	
Asp(Bzl)	
Tyr(Br-Z)	
His(Z)	
Lys(Cl-Z)	

RESULTS AND DISCUSSION

As we have reported previously, the reductive alkylation of free amino groups to amino acid aldehydes proceeds smoothly and usually quite rapidly in the solid phase using acidified DMF as solvent. However, several important questions regarding potential racemization and side reactions remained to be answered. Although Fehrentz and Castro reported (12) that their dimethylhydroxamate-intermediate synthesis of amino acid aldehydes is essentially free from racemization, we felt it was advisable to examine this route in more detail, also, little work appeared to have been carried out on subsequent racemization problems during reductive alkylation with cyanoborohydride. The model dipeptide-resin system, Boc–Phe–ψ[CH_2NH]–Leu-BHA, was chosen in view of the enhanced racemization potential of Phe. Complete resolution of the HF-cleaved dipeptide diastereomers could then be achieved by hplc. As can be seen in Table 1, even though optically pure Boc-L and D-Phe were used as starting materials, an initial experiment using DMAP catalyzed formation of amino acid hydroxamate followed by reduction to the aldehyde with a large excess of $LiAlH_4$, substantial racemization was present in the final peptides. It was then determined that hydroxamates could be formed quite readily in the absence of DMAP, however, again a high degree of racemization was detected. Only when a small excess of $LiAlH_4$ was used at 0^o was racemization reduced to undetectable levels. In practical terms, it is preferable to follow the reduction reaction by TLC with the addition of aliquots of $LiAlH_4$ until acceptable conversion is obtained. Optically pure Boc-Phe-CHO was again checked for racemization after storage in the refrigerator for 6 months and was found to then contain 24% of the D-isomer. It therefore seems essential that aldehydes are generated and used immediately.

We have now had an opportunity to observe the stability of several α-amino and most of the common side chain protecting groups during $LiAlH_4$ reduction (Table 2). With the exception of the tosyl group on Arg, all are compatible with the necessary chemistry. It is assumed that Arg(NO_2) would also present problems so that Arg aldehydes probably should be generated by an alternate route not involving reduction.

The ability of the CH_2NH group to undergo subsequent side reactions during peptide assembly was addressed in an earlier paper (5). In a model tripeptide system, there appeared to be few problems due to its reaction with other Boc-amino acids during diisopropylcarbodiimide (DIC) couplings with the exception of Gly. Problems with Gly could be avoided by coupling with its p-nitrophenyl ester. We were, in fact, interested in utilizing any reactivity residing in the NH

group for derivatization purposes. This could re-introduce considerable backbone rigidity into the bond and also might be of use, for instance, as a novel cyclization point to mimic hydrogen bonding. The reactivity of the NH group in the Boc-Phe–ψ[CH_2NH]–Leu-BHA system was examined by coupling with Boc-Gly using a combination of normal DIC conditions followed by symmetrical anhydride in the presence of DMAP. Under these conditions only 36% reaction took place, whereas, in a previous Boc-Gly-Val–ψ[CH_2NH]–Ala-resin system, complete reaction took place (5) using regular DIC coupling alone. There are thus, perhaps not surprisingly, considerable sequence-dependent differences in reactivity. We have also examined the possibility of acetylating the CH_2NH group in several peptides. The Boc-D-Lys(Cl-Z)–ψ[CH_2NH]–Phe bond in a LH-RH antagonists was 30% acylated in the presence of a 2 M excess of acetyl chloride (10 min). In contrast, acetylation with acetic anhydride gave only about 1% reaction. A series of N-C terminal cyclized somatostatin hexapeptide ψ analogues (Table 3) were synthesized by the azide cyclization procedure. No evidence for side reactions involving acylation of the reduced peptide bond was detected.

The rapidity of ψ analogue synthesis by the solid phase procedure has enabled us to prepare quite a large number of peptide hormone analogues and to begin to gauge the types of influences on biological activity. One of our most exciting observations has been the generation of potent bombesin receptor antagonists (EC_{50} ca. 10^{-9} M) by replacement of the 9-10 and 13-14 peptide bonds (14). This, together with the report (3) of Rodriguez et al. on ψ gastrin antagonists, leads us to suspect that this might be a general approach to competitive antagonist design in linear peptides.

Although there is increased flexibility and rotation about the CH_2NH bond, perhaps the primary mechanism for the groups potential effects on peptide conformation lies in the destruction of hydrogen bonding to CO groups. Effects of peptide bond replacement on biological activity might thus be indicative of primary H-bonding sites. To examine this question, we chose the highly constrained, cyclic somatostatin analogue systems (15,16) for which much solution NMR data has accumulated (see Wynants et al. elsewhere in this volume). Octapeptide reduced peptide bond analogue potencies (Table 3) (6) generally agreed with current concepts of a type II' β-bend from residues 3 to 6 stabilized by hydrogen bonds between residues 1-8 and 3-6 in that the lowest biological activities resulted when the relevant CO groups were replaced. It is more difficult to correlate biological activities with hydrogen bonding in analogues based on the hexapeptide series (17) since these are far more rigid molecules containing two β-bends, one also present in the octapeptides and the second centered around the N-Me-Ala residue. Nevertheless, maximum potencies were found with the analogous peptide bond replacements (1-2 and 4-5). The Tyr-D-Trp bond replacement analogue proved to be very difficult to cyclize and was not obtained in sufficient yield to characterize.

Finally, the solid-phase reductive alkylation reaction has now also been used extensively to prepare analogues of several peptide hormones alkylated at either N^{α}-amino groups or N^{ε}-amino groups on Lys residues. [N^{α}-ethyl-Tyr^1]-GRF(1-29)NH_2 was readily prepared by $NaBH_3CN$ reduction in the presence of acetaldehyde and was found to be 5 times more potent in stimulating GH release in the rat. The N-Me analogue was only 42% active in the same assay system. In the preparation of the LH-RH antagonist derivative, [N–Et–D–Nal^1,D–$Phe^{2,3}$,D-Arg^6,Phe^7,D-Ala^{10}]-LH-RH (where Nal = β–naphthylalanine), the major product after alkylation on the resin was found to be the N,N-diethyl derivative indicating that caution must be exercised in the excess of carbonyl component and reagents used and reaction time.

Also in the LH-RH antagonist area, the method was used (10,11) to prepare a series of Ne-alkylated derivatives of [Ac–D–Nal^1,D–$Phe^{2,3}$,D–Lys^6,Phe^7,Lys^8,D-Ala^{10}]-LH-RH in an examination of factors effecting undesirable histamine releasing properties. The peptides were prepared using initial N^{ε}-FMOC protection on the Lys residues which was removed by piperidine treatment, followed by reductive alkylation with a number of aldehydes and ketones. Several analogues were found to have much improved antiovulatory:histamine releasing activity ratios.

Table 3. Effect of Peptide Bond Replacement with CH_2-NH on the Biological Activity of Hexapeptide and Octapeptide SRIF Analogues in Vivo and in Vitro In the Rat

	Potencies (SRIF=1)	
	in vivo	in vitro
D-Phe-Cys-Tyr-D-Trp-Lys-Val-Cys-Thr-NH_2	79	3.6
1 - 2	1.6	0.01
2 - 3	20	0.17
3 - 4	0.2	0.007
4 - 5	0.002	0.0003
5 - 6	4	0.67
6 - 7	0.002	0.0002
7 - 8	0.73	0.004
c[N-Me-Ala-Tyr-D-Trp-Lys-Val-Phe]	80	20
1 - 2	60	1
3 - 4	0.2	0.006
4 - 5	40	1-3
5 - 6	0.5	0.01

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