Combination of Allyl Protection and HYCRAM™-linker Technology for the Synthesis of Peptides with Problematical Amino Acids and Sequences [1])

Rita Gothe, Lydia Seyfarth, Christina Schumann, Inge Agricola, and **Siegmund Reissmann***

Jena, Friedrich-Schiller-University, Institute of Biochemistry and Biophysics

Axel Lifferth and **Christian Birr**

Heidelberg, ORPEGEN Pharma, Gesellschaft für biotechnologische Forschung, Entwicklung und Produktion m.b.H.

Magarita Pavlovna Filatova and **Alexander Kritsky**

Moscow (Russia), Institute of Biological and Medicinal Chemistry, Russian Academy of Medicinal Sciences

Vladimir Kibirev

Kiev (Ukraine), Institute of Bioorganic Chemistry and Petrochemistry of the Ukrainian National Academy of Sciences

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Abstract. Analogues of both the nonapeptides, bradykinin and bradykinin potentiating nonapeptide $BPP_{9\alpha}$, were synthesized using HYCRAM™-technique. The bradykinin analogues were assembled by the Boc-, Ddz- and Fmoc-strategy starting with Boc-Arg(Aloc)$_2$-OCr–OH, Ddz-Arg(Mtr)-OCr–OH and Fmoc–Arg(Mtr)-OCr–OH. While Boc- and Ddz-strategy provide peptides in good yield and purity, the Fmoc-strategy leads to a loss of peptide from resin. For simultaneous cleavage from HYCRAM™-resin and removal of Aloc-side chain protection optimized conditions for catalytic cleavage with Pd° were developed. As shown by the synthesis of $BPP_{9\alpha}$ analogues the HYCRAM™-linker and the chlorotrityl resin allow the assembly of peptides with the C-terminal sequence Pro-Pro by preventing dioxopiperazine formation. Since the $BPP_{9\alpha}$ sequence contains the tripeptide Trp-X-Arg an intramolecular migration of the N^G-protecting group to the indole ring under conditions used for its removal had to be avoided. By the use of HYCRAM™-linker in combination with Aloc protection for the guanidino group and Ddz for N^α no modification of Trp occurred. HYCRAM™-technology in combination with Boc-, Ddz- or Aloc/All-protecting groups facilitates the synthesis of peptides with such very labile amino acids like *cis*-4-hydroxyproline.

Solid phase syntheses of peptides with sensitive amino acids or sensitive pseudopeptide bonds require special linkers, which are stable under the conditions of coupling and deprotection and very smoothly to cleave without strong acidic or basic conditions. With the development of the hydroxycrotonoyl amino methyl linker (HYCRAM™) by Kunz *et al*. [1, 2, 3] and Birr [4, 5] a linker was found which fulfills these requirements in a good manner. The assembled peptides can be removed from the resin by treatment with Pd[P(C_6H_5)$_3$]$_4$ as catalyst under nearly neutral conditions. Catalysts, scavengers and conditions for the removal of allylic protecting groups through catalytic palladium π-allyl methodology are very recently reviewed by Guibé [6]. Avoiding the use of strong acids as CF_3SO_3H (TFMSA) or CF_3COOH (TFA) the used conditions are very convenient, especially for the synthesis of glycopeptides. Thus, Kunz *et al*. demonstrated the advantage of this anchor in the synthesis of such compounds as Lewis antigen type structures [7] and HIV envelope glycoprotein peptide T [8]. To improve the chemical properties of the linker Seitz and Kunz [9] developed the HYCRON-

[1]) Other abbreviations include: *o*-Aba; *m*-Aba, *ortho, meta* amino benzoic acid; Aloc, allyl oxycarbonyl; BK, bradykinin; Boc, *tert*-butyloxycarbonyl; $BPP_{9\alpha}$, bradykinin potentiating peptide 9α (Pyr-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro); BOP, benzotriazole-1-yl-oxy-tris (dimethylamino) phosphonium hexafluorophosphate; Bu_3SnH, tributyltin hydride; Bu^t, *tert* butyl, *cis* Hyp, *cis* hydroxy proline; DCM, dichloromethane; Dde, *N*-(1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl; Ddz, α,α-dimethyl-3,5-dimethoxy-benzyloxycarbonyl; DIEA, diisopropylethylamine; DIC, diisopropylcarbodiimide; DMF, *N*,*N*-dimethylformamide; DMSO, dimethylsulfoxide; DTE, dithioerithritol; Fmoc, 9-fluorenylmethyl oxycarbonyl; HOAt, 1-hydroxy-7-azabenzotriazole; HATU, O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3 tetramethyluronium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole; HOCr, hydroxycrotonic acid; HYCRAM, Hydroxycrotonyl amidomethyl linker; Mtr, methoxytrimethylbenzene sulphonyl; NMM, *N*-methylmorpholine; PAM, phenylacetamido-methyl; Pd[P(C_6H_5)$_3$]$_4$, palladium *tetrakis* triphenylphosphine; PyBop, benzotriazole-1-yl-oxy-tris-pyrrolidinophosphonium hexafluorophosphate; TBTU, 2-(1*H*-benzotriazol-1-yl)1,1,3,3-tetramethyluronium tetrafluoroborate; TFA, trifluoroacetic acid; TFFH, tetramethylfluoroformamidinium hexafluorophosphate; THF, tetrahydrofuran; Trt, triphenylmethyl.

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linker, characterized by a higher stability and increased clearability.

We were interested to use the HYCRAM™-technology for syntheses of sensitive analogues of bradykinin (BK: Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) with amino acid replacements mainly at positions 2 and 7 and analogues of the bradykinin potentiating peptide ($BPP_{9\alpha}$: Pyr-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro).

The pleiotrophic tissue hormone bradykinin is involved in the peripheral blood pressure regulation and evokes different inflammation processes under pathophysiological conditions [10]. Therefore bradykinin antagonists are of great therapeutical interest. We assume that there exist three structural types of bradykinin B_2 receptor antagonists derived by amino acid replacements at position 2 (type-2-antagonists) [11, 12] at position 5 (type-5-antagonists) [13] and mainly at position 7 (type-7-antagonists) [10, 14]. We were interested to replace proline at position 2 and 7 by conformationally restricted amino acids like *ortho*- as well as *meta* amino benzoic acid (*o*-Aba, *m*-Aba) and *cis*-4- hydroxyproline (*cis*-Hyp). The aim of these studies was to understand structural and conformational requirements for both the type 2 and type 7 antagonists as a prerequisite to develop more potent antagonists.

BK was the first peptide synthesized by the solid phase method [15] and seems to be easy to assemble on the solid support. In our preceding studies on linear and cyclic bradykinin analogues we used *tert*-butyloxycarbonyl (Boc-) and 9-fluorenylmethyl oxycarbonyl- (Fmoc-) strategy for the synthesis on 4-hydroxymethyl-phenoxymethyl (Wang)- and phenylacetamido-methyl (PAM)-resin. Especially, the synthesis of analogues with side chain [16] or backbone cyclization [17, 18] required strong acidic conditions for removal from resin and deprotection. To avoid destruction of labile peptide bonds such as *N*-alkyl amide bonds or anilide like amides, side chains or intrachainar bridges we were interested in a method without strong acidic conditions.

The bradykinin potentiating nonapeptide $BPP_{9\alpha}$ was firstly isolated from the venom of the snake *Bothrops jaraca*. It potentiates the action of bradykinin on smooth muscles and on blood pressure [19]. The biological background for our studies in this field was to differentiate between the potentiation of the bradykinin action and the inhibition of the bradykinin degradation by angiotensin converting enzyme. Thus, we hope to find a second action mechanism for this class of biological active peptides. Contrary to bradykinin itself, $BPP_{9\alpha}$ is very difficult to assemble on solid phase [20, 21]. On the one hand the C-terminal sequence Pro-Pro leads to dioxopiperazine formation [20] and thus to the cleavage from the resin, and on the other hand Trp is sensitive to strong acids and alkylating groups, as generated by the removal of protecting groups [22]. We replaced in the native sequence pyroglutamic acid by proline to get analogues with enhanced potentiating activity and to avoid the formation of glutamic acid by ring opening either under conditions used for peptide coupling or by deprotection and removal from the resin [23].

With our approaches to use the HYCRAM™-technology we pursued different aims. For the convenient synthesis of bradykinin analogues with sensitive amino acids we were interested to test different protecting groups in combination with the HYCRAM™-anchor. Thus, we would like to study advantages and limitations of combinations of N^{α}-Fmoc- with *tert*-butyl esters(O-Bu^t), N^G-methoxytrimethylbenzene sulphonyl (Mtr) or of N^{α}-α,α-dimethyl-3,5-dimethoxy-benzyloxycarbonyl (Ddz) with *O*-Bu^t and of N^{α}-Boc- with allyloxycarbonylester (*O*-Aloc) and N^G-allyloxycarbonyl (Aloc) (Schemes 1 and 2). In the case of the combination with N^G-Alloc and *O*-Aloc side chain protection we made an attempt to develop optimized conditions for simultaneous cleavage of the HYCRAM™-anchor and deprotection of all functional groups.

The synthesis of bradykinin analogues with amino acid replacements at positions 2 and 7 by *ortho*- or *meta*-aminobenzoic acid or *cis*-hydroxyproline should allow to check and to compare the different protecting group combinations with regard to yield and purity of the final products described above. The nonproteinogenic amino acid *cis*-4-hydroxyproline requires especially mild conditions for activation, peptide assembly and deprotection because of the tendency to form a lacton bridge between the carboxy group and the γ-hydroxyl group [24]. On the other hand incorporation of *cis*-Hyp in place of Pro into biologically active peptides can change the physiological behavior compared to that of parent compounds. To check the effect of *cis*-Hyp on the activity of bradykinin type II antagonists we were interested to incorporate it into position 2.

In the field of the $BPP_{9\alpha}$ analogues we were interested to compare the HYCRAM™-anchor with other linkers, like chlorotrityl- or 4-hydroxymethyl-phenoxymethyl-linker (Wang) in view of peptide cleavage from the resin through dioxopiperazine formation and of intactness of the sensitive amino acid Trp (Scheme 1).

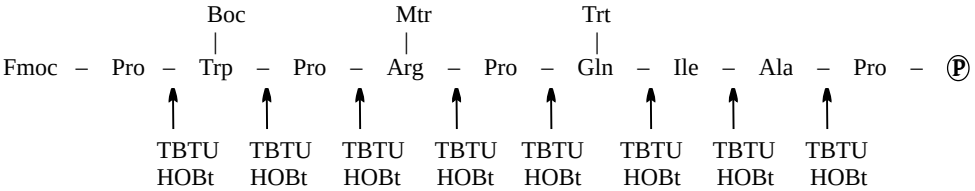
$BPP_{9\alpha}$ contains the sequence Trp-X-Arg, a sequence for that Stierandová *et al.* [22] has shown the intramolecular migration of the N^G-2,2,5,7,8-pentamethylchroman sulphonyl (Pmc)- group from Arg to Trp under usually applied conditions for acidic deprotection. Since this intramolecular migration is difficult to suppress we studied the intactness of the Trp residue after cleavage of N^G-Aloc protection and hydroxycrotonoyl-linker as well as of N^{α}-Ddz-protection.

Results and Discussion

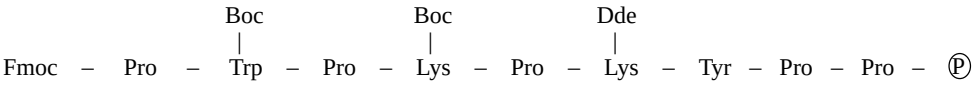
C-terminal Amino Acid Derivatives and Their Esters with Hydroxycrotonic Acid

For the possibility to choose between different resins *e.g.* aminomethyl polystyrene resin or aminomethyl Tentagel as well as resins with high or low loading we synthesized esters of the *C*-terminal amino acids with hydroxycrotonic acid. The general procedure and the analytical characterization for the most derivatives were described elsewhere [6]. Coupling to the aminomethyl resins occurs either directly or by a spacer, β-alanine. The spacer should give better results by the cleavage of the peptide from the resin and can be used as an internal standard [25]. In most cases the esters of the *C*-terminal amino acids with hydroxycrotonic acid were coupled to the resins using 2-(1*H*-benzotriazol-1-yl) 1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU).

1. Wang-resin: Fmoc-strategy

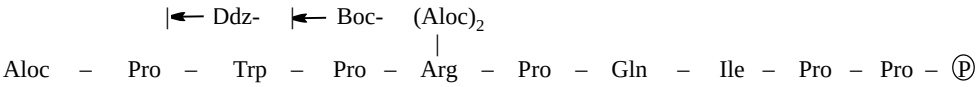


2. Chlorotrityl-resin: Fmoc-strategy



double couplings with TBTU/HOBt

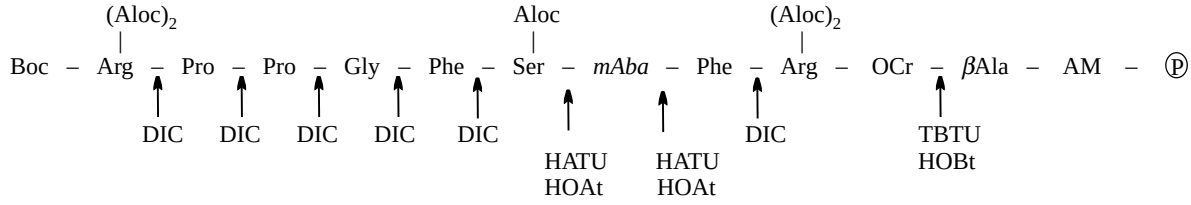
3. HYCRAM-resin: Boc-, Ddz-strategy

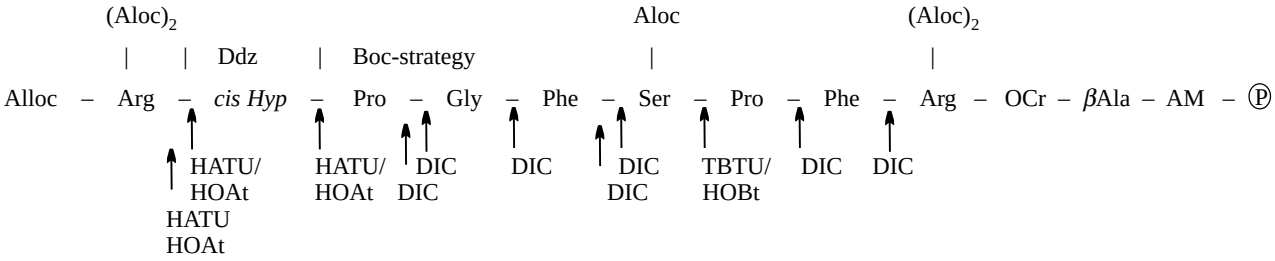


double couplings with HBTU/HOBt

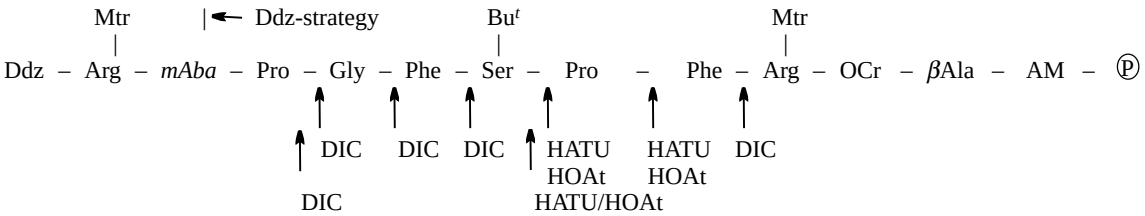
Scheme 1 Different strategies for syntheses of $BPP_{9\alpha}$-analogues

Boc-strategy





Ddz-strategy



Scheme 2 Different strategies for syntheses of Bradykinin analogues

Assembly of the Peptides

Despite peptides with *m*-Aba at positions 2 and 7 are obtained in good yield and purity in the case of *o*-Aba under the same conditions difficulties were observed. Instead of (*o*-Aba)^7-BK only a heptapeptide (Arg-Pro-Pro-Gly-Phe-Phe-Arg) lacking Ser^6-*o*-Aba^7 was achieved. The reason for that fact seems to be the elimination of the dipeptide Ser-*o*-Aba as a seven-membered cycle from the N^{α}-unprotected pentapeptide. Thus the MALDI-mass spectrum corresponds with the heptapeptide, and also the amino acid analysis shows correct relations for all amino acids except *o*-Aba and Ser.

Biological Activities

The biological activities of the different bradykinin analogues containing *m*-Aba and *cis*-Hyp and also of the $BPP_{9\alpha}$-analogues will be published separately. The activities of the bradykinin analogues will be compared with other type-2 and type-7 antagonists assembled by other strategies [26]. The potentiating activity on the bradykinin induced smooth muscle contraction and on the inhibition of the isolated angiotensin-converting enzyme will be discussed together with a set of more than 20 other analogues of the bradykinin potentiating peptide $BPP_{9\alpha}$ [27].

Comparison of HYCRAM™-resin with Other Resins by the Synthesis of the Peptides with the *C*-terminal Sequence Pro-Pro

The *C*-terminal sequence Pro-Pro is prone to dioxopiperazine formation. Therefore, the solid phase synthesis of peptides containing the *C*-terminal sequence Pro-Pro is difficult and provides only a very low yield of peptide. This difficulty could be the reason why there exists only a small number of references about the solid phase synthesis of $BPP_{9\alpha}$-analogues [20, 21] despite of the high number of synthesized compounds. We compared three different linkers for their ability to prevent dioxopiperazine formation by the synthesis of peptides with the *C*-terminal sequence Pro-Pro: the Wang-linker, the chlorotrityl linker and the HYCRAM™-anchor (Scheme 1). While the Wang-linker in combination with the Fmoc-strategy leads to a nearly complete loss of peptide from the resin, the chlorotrityl gives good yields of nonapeptides. Because the HYCRAM™-technology allows the acidolytic removal of N^{α}-Boc or N^{α}-Ddz-protection the analogues of $BPP_{9\alpha}$ could obtained in yields between 75 to 80% (Table 2). Thus, the loss of peptide by dioxopiperazine formation is very low despite of the activation of the *C*-terminal amino acid by formation of an allylic ester bond with hydroxycrotonoic acid.

Comparison of Different Strategies for Assembly of Peptides on the HYCRAM™-resin

To examine the stability of the HYCRAM™-linker under different conditions used for removing the N^{α}-protecting groups we synthesized bradykinin and some of its analogues by three different strategies. Scheme 2 shows the strategies used: Fmoc-, Ddz- and Boc-strategy. By the Fmoc-strategy the guanidino group of the arginine was blocked by the Mtr-residue and the hydroxyl group of serine by *tert*-butyl residue. The repeated deprotection with piperidine leads to some extent to a cleavage of the peptide from the resin, therefore the increase in weight during the assembly of the peptide is low. The resin bound peptides showed the correct amino acid analysis, but, their removal from the resin gave only impure products.

To avoid this loss of peptide we attempted to use the Boc-strategy in combination with Aloc-protection for the guanidino group of arginine residues and the hydroxyl group of serine (Scheme 2). In contrast to the Fmoc-removal the HYCRAM™-linker is absolutely stable under conditions used for Boc-deprotection. In this combination Ser(Aloc) seems to be a problematic residue. Reduced increase in weight, reduced content of Ser in the amino acid analysis and troubles with Ser by sequenzing the nonapeptide indicate side reactions. In the case of Thr(Aloc) in another sequence we observed under basic conditions as used for Fmoc-removal a migration of the Aloc-group from the hydroxyl to the amino group. Thus, we would assume a similar side

Table 1 Comparison of different methods used for simultaneous removal from HYCRAM™-linker and deprotection from allyl groups studied on Arg(Aloc)$_2$-Pro-Pro-Gly-Phe-Ser(Aloc)-Pro-Phe-Arg(Aloc)$_2$-OCr-βAla-AM-P

method number	catalyst	used conditions	cleavage from resin and Aloc-deprotection remaining peptide; perc. cleavage (mmol/g)	
1	$Pd[P(C_6H_5)_3]_4$	morpholine; DMF/THF/0.5N HCl 2 : 2 : 1	0.08	76
2	$Pd[P(C_6H_5)_3]_4$	morpholine; $CHCl_3/H_2O$ 5 : 0.25	0.08	76
3	$Pd[P(C_6H_5)_3]_4$	$CHCl_3/CH_3COOH$/N-methyl-morpholine 5 : 0.25 : 0.12	0.18	48
4	$PdCl_2[P(C_6H_5)_2$-CH_2-CH_2-$(C_6H_5)_2P]$	morpholine; DMF/THF/0.5N HCl 2 : 2 : 1	0.28	18
5	$PdCl_2[P(C_6H_5)_2$-CH_2-CH_2-$(C_6H_5)_2P]$	$CHCl_3/CH_3COOH$/N-methyl-morpholine 5 : 0.25 : 0.12	0.31	9
6	$Pd(CH_3COO)_2$/TPPS	diethylamine; CH_3CN/H_2O	0.11	68

Table 2 Analytical Characterization of analogues of BK and $BPP_{9\alpha}$

No	structure	strategy	yield %	TLC [a] R_f values S5	S6	electrophoresis [b] E(Arg)	HPLC [c] t_R(min)	MALDI-MS [MH$^+$] calcd.	obsd.
1	Arg-Pro-Pro-Gly-Phe-Ser-*m*Aba-Phe-Arg	HYCRAM™ [d])	70	0.70	0.25	0.85	34.6	1082.5	1084.0
2	Arg-*m*Aba-Pro-Gly-Phe-Ser-Pro-Phe-Arg	HYCRAM™ [e])	65	0.75	0.25	0.85	37.3	1082.5	1083.3
3	Arg-*cis*Hyp-Pro-Gly-Phe-Ser-Pro-Phe-Arg	HYCRAM™ [f])	50	0.70	0.25	0.85	29.5	1076.2	1077.8
4a	Pro-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro	HYCRAM™ [g])	80	0.80	0.25	0.7	31.5	1087.3	1088.2
4b	Pro-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro	Wang-resin [h])	break						
4c	Pro-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro	Cl-Trityl [h])	75						
5	Pro-Trp-Ala-Arg-Pro-Gln-Ile-Pro-Pro	HYCRAM™ [g])	75	0.80	0.3	0.7	30.4	1061.3	1062.7
6	Pro-Trp-Pro-Leu-Pro-Lys-Ile-Pro-Pro	HYCRAM™ [g])	80	0.85	0.35	0.7	35.8	1044.3	1045.4
7	Pro-Trp-Pro-Lys-Pro-Lys-Tyr-Pro-Pro	Cl-Trityl [h])	80	0.80	0.3	0.85	33.6	1109.2	1109.9

[a]) Silica gel 60 F_{254} 250µm (Merck 5715) glass plates were used, together with the following solvent systems: S5, pyridine/ethyl acetate/acetic acid/H_2O, 5/5/1/3 (v/v/v/v); S6, *n*-butanol/acetic acid/water, 48/18/24 (v/v/v). [b]) Paper electrophoresis with 6% aqueous acetic acid. R_f values are calculated relatively to Arg. [c]) HPLC conditions: Vydac C18 reversed phase column (250 × 4.6mm), Solvents: A, 0.1% aqueous TFA; B, 0.1% TFA in acetonitrile; gradient 0% B to 60% B in 60 min; flow rate 0.8 mL, monitored at 220nm.
[d]) Boc-. [e]) Boc-/Ddz-. [f]) Boc-/Aloc-. [g]) Boc-/Ddz-/Aloc-. h) Fmoc- .

reaction during the coupling for serine, too.The finding agrees with those from other authors [29]. The observed side reaction limits the use of Fmoc-Ser(Aloc)-OH in the peptide synthesis.

Good results were obtained with the Ddz-strategy, using Mtr for side chain protection of Arg and *tert*-butyl protection of serine hydroxyl group (Scheme 2). The use of protecting group combinations with Ddz- and Aloc-groups enhances the number of possible combinations. This additional possibility is important for syntheses with trifunctional amino acids, for syntheses of cyclic peptides, as well as peptides with side chain or backbone cyclization. The Ddz-group is removed with 5% TFA in dichlormethane (DCM) during 20 min. We found that the Mtr-group and the *tert*-butyl ether of serine are stable under these conditions. After assembly of the peptide the Mtr- and *tert*-butyl residues were removed with 80-proc. TFA in the presence of anisole from the resin bound peptide. But, after cleavage from the resin the crude product showed some by-products in the HPLC elution profile. Thus, we cleaved at first the protected peptide from the resin and removed the protecting groups in a second step. The crude products obtained by this way are more homogenous in the HPLC diagram.

To avoid strong acidic conditions which lead to lacton formation in peptides with *cis*-Hyp, we protected the amino group with Ddz and assembled the nonapeptide on a HYCRAM™-linker together with Aloc-protection for the side chains of Ser and Arg. We reduced the lacton formation to a low amount by coupling of Ddz-*cis*Hyp to the heptapeptide with *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluroniumhexafluorophosphate (HATU)/7-*aza*-benzotriazole (HOAt).

Avoiding of Trp Substitution by the Use of Aloc-protection for the Guanidino Group of Arginine and either N^{α}-Ddz- or Fmoc- protection for the N^{α}-Group

Complete protection of the arginine guanidino group and smooth removal of the protecting group are still a difficult task for peptide chemists [22, 29, 30]. Arginine in combination with tryptophane in the sequence creates an additional complication by the intramolecular migration of the protecting group from the guanidino group to the indole ring of tryptophane under deprotecting conditions. Stierandová *et al*. [22] published the migration of the Pmc- group from arginine to tryptophane in peptides containing the sequence Trp-X-Arg. This migration occurs intramolecularly under the conditions of deprotection with TFA. With the aim to prevent this substitution at the Trp residue we used arginine derivatives with Aloc-protection of the guanidino group. As shown in [22] the intramolecular migration is facilitated in the sequence Trp-Ala-Arg. To study this kind of migration we synthesized an analogue of $BPP_{9\alpha}$ with that sequence.

The sequenation of the polymer bound as well as of the free peptide showed that the removal of the N^G-Aloc-group did not lead to a substitution at the indole ring (Figure 1). This finding clearly demonstrates the advantage of the Aloc-HYCRAM™-strategy compared with other linkers and guanidino protecting groups. Our

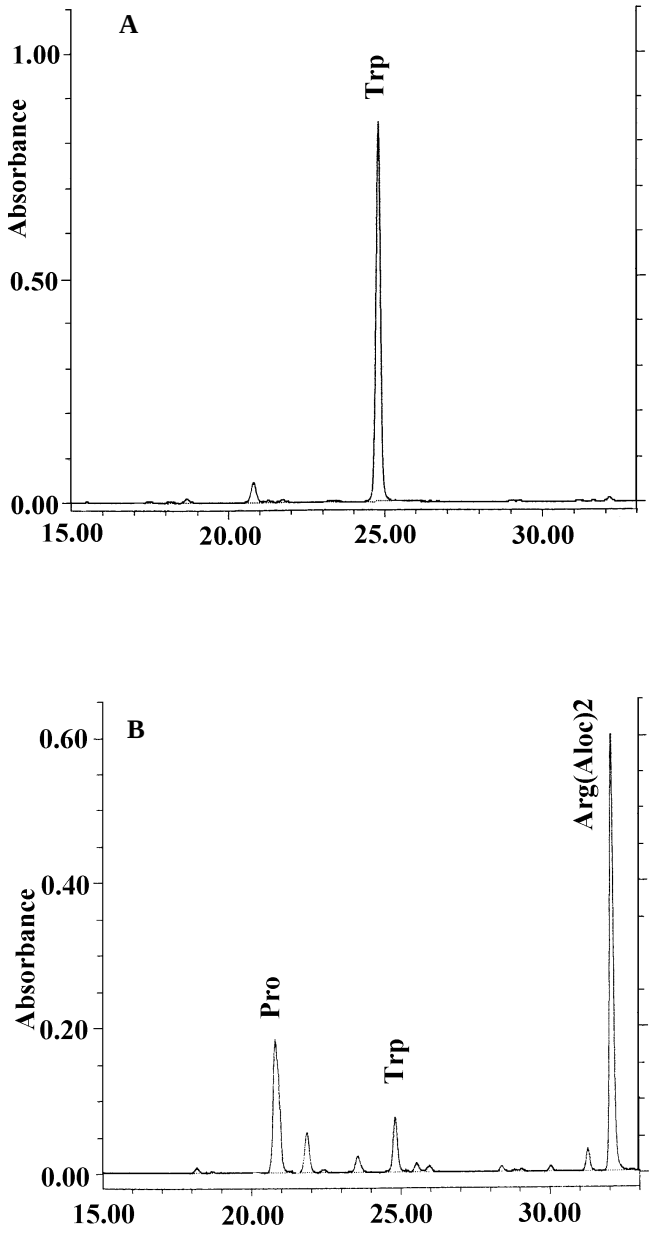


Fig. 1 Sequence data showing the intactness of Trp and $Arg(Aloc)_2$ residues by the synthesis of the $BPP_{9\alpha}$-analogue Pro-Trp-Ala-Arg-Gln-Ile-Pro-Pro using N^{α}-Ddz-protection. Removal of Ddz-residues was carried out with 5% TFA in DCM. The amino acids and their derivatives were estimated by Edman degradation of the resin bound peptide with the sequenator Beckman LF 3000. A: Trp; B: $Arg(Aloc)_2$

methodology requires neither strong acids for cleavage from the resin nor for protecting group removal. The removal of the N^{G}-Aloc-groups showed no attack on the indole ring. Under basic conditions, used for Fmoc-removal, one Aloc group was removed from the $Arg(Aloc)_2$-residue (Figure 2). This finding is in agreement with results obtained by Loffet and Zhang [31]. The Edman degradation of the polymer bound octapeptide after Fmoc-removal with 20% piperidine in DMF clearly shows a peak for N^{G}-(mono-Aloc)arginine. Deprotection of the octapeptide Ddz-Trp-Pro-$Arg(Aloc)_2$-Pro-Gln-Ile-Pro-Pro from the Ddz-group by 5% TFA in DCM gives no side reaction on Trp (Figure 1).

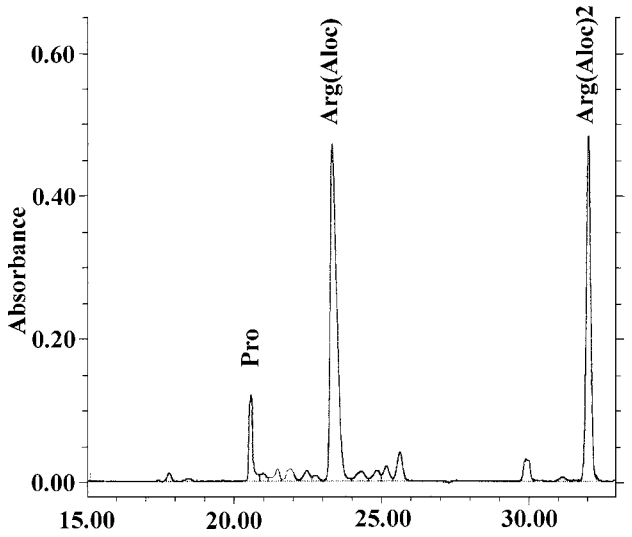


Fig. 2 Partial deprotection of the $Arg(Aloc)_2$ residue by the synthesis of the $BPP_{9\alpha}$-analogue Pro-Trp-Ala-Arg-Gln-Ile-Pro-Pro using the Fmoc-strategy. $Arg(Aloc)_2$ and Arg(Aloc) were estimated by Edman degradation of the resin bound peptide using the sequenator Beckman LF 3000.

Search for Optimized Conditions to Remove Allyl Protection and Hydroxycrotonoyl-linker Simultaneously

The combination of the HYCRAM™-linker technology with allyl protection of amino acid side chains should allow the simultaneous removal of the allyl protecting group and cleavage of the peptide from the resin by the same procedure. Various conditions were investigated for the removal of allyl side chain protection and cleavage of the HYCRAM™-linker. In the literature a number of procedures are described using various catalysts, different solvents, nucleophiles and pH values. Thus, the following catalysts are described: $Pd[P(C_6H_5)_3]_4$ [1, 4, 32, 33], $Pd(CH_3COO)_2$ [34], $PdCl_2[P(C_6H_5)_3]_2$ [35], $RhCl[P(C_6H_5)_3]_3$ [1], $(CH_3)_2CuLi$ [36] and $PdCl_2$ $[P(C_6H_5)_2\text{-}CH_2\text{–}CH_2\text{-}(C_6H_5)_2P]$. Nucleophiles like morpholine, *N*-methyl-morpholine, *N*-methyl aniline, sodium triphenylphosphine *m*-sulfonate (TPPTS) [37, 38], dimedone, sodium azide, sulphur nucleophiles, silylated nucleophiles and hydride donors are used (for review see [6]).

Starting with a procedure using $Pd[P(C_6H_5)_3]_4$, dimethysulfoxide (DMSO), dimethylformamide (DMF), morpholine and $P(C_6H_5)_3$, described for cleavage from the HYCRAM™-linker [6], we observed an incomplete deprotection of the arginine derivative $Arg(Aloc)_2$. By

sequenation of the peptide we found Arg(Aloc). By other procedures the use of Bu_3SnH as hydride donor in the Pd° catalysis [31, 39] leads to formation of a voluminous brown sludge, difficult to separate. Thus, we searched for optimized conditions without Bu_3SnH. Table 1 lists the results with some of the used procedures. The catalysts 4 and 5 are recommended as more active than $Pd[P(C_6H_5)_3]_4$. Comparing the completeness of cleavage from the resin both catalysts are under the used conditions not only less active than the normally used one, but also nearly unable to cleave the allyl linker bond. Since the synthesis started with a loading of the Boc-βAla-aminomethyl-(βAla-AM) resin from 0.63 mmol/g the calculated loading with the fully protected nonapeptide yields 0.34 mmol/g. Thus, values for the remaining polymer bound peptide from about 0.30 mmol/g clearly indicate the low ability of the above discussed catalysts to cleave the peptide from the HYCRAM™-linker. The listed three procedures 1, 2 and 3 with $Pd[P(C_6H_5)_3]_4$ give good results in the peptide cleavage from the resin. The obtained peptides were characterized by HPLC, TLC, Edman degradation and paper electrophoresis. The procedure 2 using morpholine–$CHCl_3$–water gives the most homogeneous crude product but, despite the low content of remaining peptide a lower yield of final product. Thus, considering the yields of obtained free peptides and their purities we recommend procedure number 1 for simultaneous removal. We applied therefore this procedure for the formation of free peptides in the case of bradykinin and $BPP_{9\alpha}$ analogues.

Conclusions

Our studies about the usefulness of HYCRAM™-technology for syntheses of peptides with sensitive nonproteinogenic amino acids clearly demonstrate the advantage of the very mild conditions, required for the linker cleavage, in comparison with other linkers. The catalytic cleavage of this allylic linker shows no side reactions on labile amino acids like Trp and *cis*-4-hydroxyproline, and on labile peptide bonds such as anilide like amides with *m*-amino benzoic acid. As shown by syntheses of analogues of the very proline rich nonapeptide $BPP_{9\alpha}$ the HYCRAM™-linker as well as the chlorotrityl resin prevents the dioxopiperazin formation in the *C*-terminal dipeptide Pro-Pro. Using Wang-resin the *C*-terminal dipeptide is nearly completly cleaved from the resin. In combination with N^{α}-protection by the Boc- and Ddz-group good results were obtained with regard to the increase of weight and to the purity of the end product. Thus, Boc-Arg$(Aloc)_2$-OCr–OH and Ddz-Arg(Mtr)-OCr–OH are useful linkers for syntheses of peptides with *C*-terminal Arg. In contrast to this result Fmoc-Arg(Mtr)-OCr–OH used for assembly of peptides by Fmoc-methodology leads to a distinct loss of peptide. To avoid this loss by repeated treatment with piperidine the use of the more stable HYCRON-linker was recommended [9, 40]. The HYCRAM™-linker is stable under conditions used for many coupling procedures like DIC, TBTU/HOBt, HBTU/HOBt, HATU/HOAt and PyBrop. Ddz-protection as well as N^{G}-Mtr protection can be removed from the resin bound peptide or from the free peptide. From various methods with $Pd[P(C_6H_5)_3]_4$ for removal of allyl type protecting groups described in the literature the treatment of the HYCRAM™-resin with a mixture of dimethylformamide/tetrahydrofurane/0.5N HCl/morpholine provides both, good cleavage from resin and nearly complete removal of all side chain protecting groups from allyl type. Furthermore, the use of the HYCRAM™-linker in combination with Aloc-side chain protection and Ddz-protection for N^{α}-groups prevents the modification of the Trp residue in analogues of $BPP_{9\alpha}$. Compared with the bradykinin analogues the bradykinin potentiating peptides are obtained in slightly higher yields (Table 2). This result indicates a facilitated removal of the *C*-terminal proline from the HYCRAM™-linker compared with *C*-terminal arginine derivatives in bradykinin analogues.

In summary the HYCRAM™-technology in combination with Boc-, Ddz- or Aloc/All-protecting groups facilitates the synthesis of peptide fragments or free peptides with labile amino acids or modified peptide bonds. Using these advantages we synthesized dipeptide building units from arginine esters with hydroxycrotonic acid and assembled successfully backbone cyclic bradykinin analogues on a HYCRAM™-linker. This application will be published separately [41].

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Experimental

Synthesis of the Peptides

Boc-, Fmoc- and Ddz-amino acids were purchased from BACHEM (Switzerland) and ORPEGEN-Pharma (Germany). Boc-Ser(Aloc)-OH and Boc-Arg$(Aloc)_2$-OH were obtained from Propeptide (Frankreich). Boc-Arg$(Aloc)_2$-OCr–OH, Fmoc-Arg(Mtr)-OCr–OH, Ddz-Arg(Mtr)-OCr–OH and Boc-Pro-OCr–OH were synthesize according to the general procedure published in [6]. These hydroxycrotonic esters are now available from ORPEGEN-Pharma (Germany). The used resins were purchased from the following companies: amino methyl polystyrene, Tentagel-S NH_2 and Wang-resin from RAPP Polymere (Germany), chlorotrityl resin from NOVA-Biochem (Germany). The peptides were synthesized on a PSS-80 automatic synthesizer (Applied Protein Technologies, USA) or on the semiautomatic synthesizer SP-650 (BACHEM, Switzerland). Each step was monitored by the Kaiser test. In some cases we used additionally to the Kaiser test the internal monitoring of the synthesizer PSS-80 with dimethoxytritylchlorid.

Coupling Procedures

Diisopropylcarbodiimide (DIC)

Couplings were carried out after neutralization by repeated washings (5 to10 times) of the resin with 5% DIEA in DCM in a 2-fold excess of N^{α}-protected amino acid and DIC in DCM for 4 h.

2-(1H-Benzotriazol-1-yl) 1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU)/1-hydroxybenzotriazole (HOBt)

Boc- or Fmoc- protected amino acids were used in a 4-fold excess, solved in DMF or DCM/ DMF 1:1. TBTU and HOBt were applied in the same excess, diisopropylethylamine (DIEA) in a 6-fold excess. The reaction time was between 4 and 12 h.

O-(7-Azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU)/1-(7-aza)benzotriazole (HOAt)

HATU and HOAt were used for couplings in the same manner as TBTU/HOB*t*.

Deprotecting Procedures

Boc-deprotection was performed with TFA/DCM 1:1 without any scavenger in two steps, 5 min treatment followed by washing with DCM and second treatment for 20 min.

Ddz-deprotection was carried out with 5% TFA in DCM in two steps, 5 min and 20 min.

Fmoc-deprotection was achieved with 20% piperidine in DMF in two steps, 5 and 20 min.

Bradykinin Analogues by the Boc-strategy

Amino methyl polymer (RAPP-Polymers) with a capacity of 0.8 mmol/g was loaded with Boc-βAla (capacity 0.63 mmol βAla/g Boc-βAla-resin). After capping with acetic anhydride the Boc-group was removed with TFA/DCM 1:1. The resin was thoroughly washed, neutralized with DIEA and coupled with Boc-Arg$(Aloc)_2$-OCr–OH (2 equ.) by TBTU (3 equ.), HOBT (3 equ.) and DIEA (4 equ.) for 2h. The resin was again capped. Then the sequence of the bradykinin analogue was assembled by double couplings, using a twofold excess of each amino acid. The couplings were performed in the case of Boc-Phe, Boc-Gly and Boc-Pro with DIC, and in the case of Boc-Ser(Aloc) with TBTU, HOBt. Couplings to sterically hindered or less active nonproteinogenic amino acids like *m*-Aba were carried out with HATU/HOAt (3 fold excess of amino acid and 6 fold exess of HATU/HOAt). The assembly of the peptide occurred nearly quantitatively (calculated loading: 0.34 mmol/g, by amino acid analysis estimated loading for protected BK: 0.33 mmol/g). After finishing the assembly the peptide was cleaved from the resin and deprotected from all Aloc-groups with $Pd[P(C_6H_5)_3]_4$ by a procedure described below.

Bradykinin Analogues by the Ddz-strategy

Ddz-Arg(Mtr)-OCr–OH (1.89g, 2.79 mmol) was coupled to 1 g amino methyl polystyrene resin (capacity: 1.21 mmol/g, RAPP-Polymere) with DIC in DCM in 4 h. The resin was capped with acetic anhydride. After removal of the Ddz-group with 5% TFA in DCM the resin was 5 times washed with 5% DIEA in DCM, and the following amino acids were coupled as Ddz-derivatives (3 fold excess) with DIC (3 equ.) and DIEA (3 equ.). The reaction time for all coupling steps was calculated to 4 h. The following protected amino acids were coupled with DIC: Ddz-Phe, Ddz-Pro and Ddz-Gly. Ddz-Pro at position 3 requires a recoupling step. Ddz-Ser(Bu^t)-OH as well as bulky or less active nonproteinogenic amino acids were coupled with HATU, HOAt (4 fold excess) and DIEA (6 fold excess). Ddz-Ser(Bu^t)-OH required a recoupling with HATU/HOAt.

Analogues of the Bradykinin Potentiating Peptide $BPP_{9\alpha}$ by HYCRAM™-Technology

Boc-Pro-OCr–OH was coupled to amino methyl Tentagel using TBTU. The capacity of the loaded resin comes to 0.16 mmol/g. The following amino acids were coupled as Boc-derivatives with HBTU/HOBt. In the case of Arg the derivative Boc-Arg $(Aloc)_2$-OH was used. Trp was coupled either as Fmoc-Trp or as Ddz-Trp. The *N*-terminal Pro was coupled as Aloc-Pro. Cleavage from the resin and removal of all protecting groups were performed under conditions described below.

Cleavage from the Resin and Removal of the Protecting Groups

a) Simultaneous cleavage from the resin and deprotection from Alloc-groups

200 mg peptide resin were suspended in 10 mL of a mixture of DMF/THF/0.5N HCl (2:2:1) and 50 equivalents of morpholine. The mixture was degassed under vacuum five times and purged away from oxygen with argon. A point of a spatula of $Pd[P(C_6H_5)_3]_4$ from yellowish color was added under argon, and the reaction mixture was again purged with argon. Then the mixture was shaken for 12 h at room temperature in the darkness. After removing the resin by filtration and washing with DMF (3×10 mL) and ethanol (3×10 mL) the filtrate was evaporated to dryness *in vacuo*. The dry residue was solved in 3 ml ethanol and the product precipitated with ether. The precipitate was thoroughly washed with ether, solved in 80 perc. *tert*-butanole and lyophilized.

b) Simultaneous Cleavage of N^{α}*-Ddz-, -Bu^t and* N^{G}*-Mtr-protecting Groups from the Resin Bound Peptide*

250 mg of dry resin bound protected peptide *e.g.* Ddz-Arg(Mtr)-*m*Aba-Pro-Gly-Phe-Ser(Bu^t)-Pro-Phe-Arg(Mtr)-OCr-βAla-AM-resin were suspended in a solution of 2 ml DCM, 8 ml TFA and 0.5 ml anisole, stirred for 4 h at room temperature, washed with DCM (5×), ethanol (2×) and dried *in vacuo*. The free peptide was removed from the HYCRAM™-linker with $Pd(P(C_6H_5)_3)_4$ by a procedure decribed in detail above.

c) Simultaneous cleavage of the N_{α}*-Ddz-, -Bu^t and* N^{G}*-Mtr protected peptide from the HYCRAM™-resin and subsequent removal of protecting groups*

The protected peptide was separated from polymeric support by catalytic cleavage of the crotonoyl linker with $Pd[P(C_6H_5)_3]_4$ using the procedure described above. The peptide was treated with TFA, DCM, anisole (4:1:0.2 v/v) for 4 h. The solvent was evaporated *in vacuo*, the residue dissolved in ethanol and precipitated with ether.

Purification and Chemical Characterization of the Peptides

The crude peptide was dissolved in 20 ml *tert*-butanol and lyophilized. The lyophilized product was dissolved in 2% aqueous acetic acid (1 mg/5 mL), passed through anion exchange resin (Amberlite IRA 410, 12 mL resin/mequ. peptide) and relyophilized. The analogues were purified by gel filtration on Biogel P_2 using 2% aqueous acetic acid and by RP-HPLC (Shimadzu LC-8A, SPD-6A) using Vydac C8, 5 µm reversed phase columm (250 × 22 mm) and a gradient from 30% CH_3CN in 0.1% aqueous trifluoracetic acid to 80% CH_3CN at a flow rate of 4 ml/min with UV detection at 233 nm. The purity of the free peptides was detected by TLC in two solvent systems, by paper electrophoresis at pH 2.3 and by analytical HPLC. Additionally Table 2 contains the strategy used and final yields of obtained peptides. Mass spectra were recorded by MALDI-technique (Lasertec, Perseptive Biosystems, USA). Amino acid analyses were carried out on LC 300 (Eppendorf-Biotronik, Germany), the peptide sequenation was performed on a Beckman sequenator LF 3400 (Beckman Instruments, USA). Amino acid analysis gave acceptable results for all peptides. The $(M+1)^+$ molecular ions were in good agreement with the calculated molecular weights for each peptide (Table 2).

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Address for correspondence:
Prof. Dr. S. Reissmann
Friedrich-Schiller-University of Jena
Institute of Biochemistry and Biophysics
Philosophenweg 12
D-07743 Jena
Fax: Internat. code (0)3641 949352
e-Mail: reissmann@merlin.biologie.uni-jena.de