Novabiochem®

Guide to selection of building blocks

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Building blocks for solid phase synthesis

1 Choosing amino acid derivatives

Choosing the "best" amino acid derivative is one of the most important and sometimes difficult aspects of peptide synthesis. Often compromises must be made in yield, purity, or both when choosing the most appropriate derivatives for a particular synthesis. We can not overemphasize the importance of taking the time to plan a synthesis carefully. Inappropriate choice of amino acid derivatives can lead to insurmountable problems later in the synthesis or during purification.

Not all amino acids require side-chain protection. Those which do can undergo irreversible side reactions, either during the synthesis or during cleavage, if the protecting group is not compatible, or if an inappropriate cleavage method is used. The choice of side-chain protection is not only dependent on the chemistry used, but also on the coupling and cleavage methods employed, solubility of the derivative in question, and the sequence of the peptide being synthesized. Because of the sheer number of different amino acid derivatives available, it would be impossible to list all their properties and applications here. For that reason, this section will concentrate only on the "problem species". All the information provided is a starting point and is not meant to be an extensive review of the literature. We encourage you to read the literature cited and the reviews by Stewart and Young [1], Barany, *et al.* [2], Fields & Noble [3], Atherton & Sheppard [4] and Chan & White [5].

Table 1 shows Novabiochem®'s recommendation for Boc-amino acids, whilst Table 2 shows our recommended Fmoc-amino acids. In both cases, only the tri-functional amino acids are listed.

1.1 Arginine

The tri-functional guanidino side-chain group of arginine is strongly nucleophilic and as such is easily acylated during SPPS if not protected. Ideally all three side-chain nitrogen atoms should be blocked; however, in practice, the majority of protecting groups only block the ν-nitrogen.

These groups can be divided into several classes [6, 7]: nitro, urethane (acyl), arenesulfonyl and trityl. The NO_2 group protects the ν-nitrogen of arginine and is normally removed using H_2/Pd [8]. Unfortunately this group is prone to several side reactions during acylation and cleavage [9]. Arg(NO_2) is available in both N-α-Boc and N-α-Fmoc protected forms. For urethane protection of the Fmoc-Arg side chain, blocking of a single ν-nitrogen with Boc [10] or δ- and ν-nitrogens with Adoc groups [11] has been investigated. However, the protection offered by these derivatives does not appear to be sufficient to mask the nucleophilicity of the guanidino function, leading to substantial ornithine formation through acylation of the unprotected ν-nitrogen during coupling and subsequent intramolecular decomposition during deprotection [12, 13]. This problem has been overcome with the introduction by Verdini [14] of a derivative in which both ν-nitrogens have been protected by Boc, but this compound is hindered and often requires extended coupling times to ensure complete reaction.

In contrast to urethane, a single arene sulfonyl-based arginine protecting group appears to offer complete blocking of the guanidino side chain and this class includes the most commonly used groups: Tos, Mts, Mtr, Pmc and Pbf. Arg(Tos) prepared from tosyl chloride is widely used in Boc SPPS. This group is extremely stable, and may only be removed by high HF/anisole [15] or Na/NH_3 [16]. Peptides containing this group normally require extended cleavage times which can have a deleterious impact on sensitive residues.

In order to design more acid-labile protecting groups, the effects of adding electron-donating substituents to the phenyl ring of the benzenesulfonyl group have been studied [17, 18], leading to the development of the methoxy-2,3,6-trimethylbenzenesulfonyl (Mtr) group. Mtr protection has been extensively used in Fmoc synthesis, despite the fact that Mtr removal often requires several hours of treatment with TFA/thioanisole [19] and can lead to modification of Trp residues. Several different cleavage mixtures have been developed to overcome these problems, and the best of these, especially for the cleavage of peptides containing multiple Arg and Trp residues, is 1 M TMSBr in TFA [20]. This reagent has been shown to remove up to four Mtr groups in 15 minutes without modification of Trp residues [21].

To overcome the obvious limitations of the Mtr group, Ramage [22, 23] undertook a thorough examination of the mechanistic factors controlling lability of arene sulfonyl protecting groups. These studies led to the development of the Pmc group. It has comparable acid sensitivity to *t*-butyl, making it ideally suited to the preparation of peptides containing multiple arginine residues, but like Mtr, it also causes sulfonation of Trp residues during TFA mediated cleavage [24]. This side reaction does appear to be less serious with Pmc; presumably the rapid cleavage of this protecting group limits the opportunity for intramolecular transfer of the Pmc group from Arg to Trp. This problem can be eliminated by the use of Fmoc-Trp(Boc)-OH for the introduction of Trp residues [25].

Table 1: Recommended protected derivatives for Boc synthesis.

Boc-Arg(Tos)-OH[1]	Boc-Asp(OBzl)-OH
Boc-Asp(OcHx)-OH[2]	Boc-Cys(4-MeBzl)-OH[3]
Boc-Cys(Acm)-OH[4]	Boc-Glu(OBzl)-OH
Boc-His(Dnp)-OH[5]	Boc-Lys(2-Cl-Z)-OH
Boc-Ser(Bzl)-OH	Boc-Thr(Bzl)-OH
Boc-Trp(For)-OH[6]	Boc-Tyr(2-Br-Z)-OH

1 Recommended for long peptides.
2 Recommended for protection of Asp in sequences prone to aspartimide formation, Asp-Gly, Asp-Ser, Asp-Asn. Requires high HF for removal.
3 Generates a cysteinyl peptide on HF treatment.
4 Stable to HF. Enables peptide to be purified prior to liberation of air sensitive thiol groups.
5 Dnp group should be removed prior to liberation of N-terminal or side-chain amino functions. Cleaved by treatment with thiophenol.
6 Removed during low-high HF cleavage if thiophenol is used. Can be removed prior to cleavage by treatment of the peptidyl resin with 10% piperidine in DMF, or after high HF cleavage by treatment with 30 mM hydroxylamine.

During their work on the development of the Pmc group, Ramage and co-workers [26] had originally discounted dihydrobenzofuran derivatives in favor of those based on chromans, as X-ray crystallographic data indicated the latter to have a more favorable oxygen lone pair-aromatic p-system orbital overlap. This assumption has been challenged by Carpino, *et al.* [27], who found Pbf, the dihydrofuran analog of Pmc, to possess superior deprotection kinetics. The rate of removal of Pbf during TFA cleavage appears to be 1.2 - 1.4 times faster than that of Pmc. More importantly, Pbf has been shown to give rise to lower levels of Trp sulfonation than Pmc, particularly when used in combination with Trp(Boc) and silane scavengers [28]. Pbf can also be used for side-chain protection of monomethyl and asymmetric dimethyl-arginine (ADMA) in Fmoc SPPS. Using Fmoc-Arg(Me,Pbf)-OH and Fmoc-ADMA(Pbf)-OH, peptides containing monomethyl and asymmetric dimethyl-arginine can be prepared without modification of standard protocols. For symmetric dimethyl-arginine (SDMA) di-Boc protection is used.

O-Sulfonation of Ser and Thr residues has been noted during the deprotection of sulfonyl-based protecting groups, such as Mtr and Pmc [29]. This side reaction was suppressed by the addition of thiocresol to the cleavage cocktail.

The Trt group is well known in peptide chemistry for its acid-labile properties and was investigated for Fmoc-Arg protection [30]. However, Fmoc-Arg(Trt) is not readily soluble in DCM/DMF [3] and is therefore not normally used.

Bernatowicz and Matsueda have introduced a novel approach to the introduction of arginine involving the guanylation of ornithine side chains using 1-guanylpyrazole [31].

Related products

853036	Boc-Arg(di-Z)-OH
853013	Boc-Arg(Tos)-OH
853037	Boc-D-Arg(Tos)-OH
852107	Fmoc-ADMA(Pbf)-OH
852310	Fmoc-SDMA(Boc)$_2$-ONa
852101	Fmoc-Arg(Boc)$_2$-OH
852105	Fmoc-Arg(Me,Pbf)-OH
852067	Fmoc-Arg(Pbf)-OH
852165	Fmoc-D-Arg(Pbf)-OH
852034	Fmoc-Arg(Pmc)-OH

1.2 Asparagine and glutamine

Asparagine and Glutamine derivatives can be incorporated without side-chain protection. However, they are known to undergo several side reactions, of which the most important is nitrile formation *via* dehydration of the carboxamide side chain during acylation [32 - 34]. This side reaction has been reported to occur with a variety of activating reagents, including carbodiimides [32, 34], BOP [33, 34], PyBOP® [35] and HBTU [35], but fortunately this can be minimized by the addition of HOBt to the coupling reaction. Acylation with activated esters results in minimal dehydration of both Asn and Gln [33, 36, 37, 38].

Blocking the free amide side chain also eliminates dehydration, and a variety of groups have been exploited for this purpose, including Dmcp, Mbh, Xan, Tmob and Trt. In Fmoc synthesis, the trityl protected derivatives Fmoc-Asn(Trt)-OH and Fmoc-Gln(Trt)-OH are the most widely used [39, 40]. These compounds are more soluble in DMF and DMA than their Mbh or Tmob counterparts [39] and the trityl group, once liberated by TFA (usually 90 - 95% TFA with water and scavengers) does not alkylate Trp or other sensitive side chains. Coupling protocols using DIC/HOBt, PyBOP®, TBTU or HBTU have given excellent results, even in peptides containing multiple Asn or Gln derivatives. Trt protection does, however, have certain limitations. Firstly, cleavage of the N-Trt group can be sluggish, particularly in the case of N-terminal Asn residues which can require over 4 hours for complete Trt removal with 95% TFA [41]. Secondly, these derivatives have low solubility in DMF compared to other Fmoc-protected amino acids. Thirdly, protected peptides containing Asn(Trt) and Gln(Trt) often have poor solubility. Finally, Asn(Trt) in particular is notorious for inducing aggregation during solid phase synthesis. Carpino's N-dimethylcyclo-propylmethyl (Dmcp) derivatives of Fmoc-protected asparagine and glutamine [42] solve these issues. Cleavage of the Dmcp group from the side-chains of Asn and Gln is rapid, even when the residue is located at the N-terminus of a peptide. Coupling of Dmcp-protected derivatives appears to be faster than that of the corresponding hindered Trt derivatives: for example, coupling of Fmoc-Asn(Trt)-OH with TFFH in the synthesis of ACP gave significant quantities of des-Asn peptide, whereas with Fmoc-Asn(Dmcp)-OH none of this by-product was generated [42]. Furthermore, protected peptides containing Asn(Dmcp) and Gln(Dmcp) residues appear to have enhanced solubility. This observation would indicate that peptides containing these residues would be less prone to aggregation during SPPS. Finally, Fmoc-Asn(Dmcp)-OH and Fmoc-Gln(Dmcp)-OH are also more soluble in DMF than Fmoc-Asn(Trt)-OH and Fmoc-Gln(Trt)-OH, thereby facilitating coupling reactions at higher concentration.

Intramolecular cyclization of Gln to pyroglutamate is an important cause of chain termination. This side reaction is particularly problematic in the Boc strategy as it is catalyzed by weak acids such as Boc-amino acids during coupling and TFA after deprotection [1]. Fortunately, this problem can be minimized by using high concentrations of TFA for Boc removal

Table 2: Recommended protected derivatives for Fmoc synthesis.

Fmoc-Arg(Pbf)-OH	Fmoc-Asn(Dmcp)-OH[1]
Fmoc-Asn(Trt)-OH	Fmoc-Asp(OtBu)-OH
Fmoc-Asp(OMpe)-OH[2]	Fmoc-Cys(Trt)-OH[3]
Fmoc-Cys(Acm)-OH[4]	Fmoc-Cys(STmp)-OH[5]
Fmoc-Gln(Trt)-OH	Fmoc-Glu(OtBu)-OH
Fmoc-His(Clt)-OH[6]	Fmoc-His(Trt)-OH
Fmoc-Lys(Boc)-OH	Fmoc-Ser(tBu)-OH
Fmoc-Thr(tBu)-OH	Fmoc-Tyr(tBu)-OH
Fmoc-Trp(Boc)-OH	

1 Recommended for N-terminal Asn residues.
2 Recommended for peptides containing Asp-Xaa, where Xaa=Asp, Asn, Cys, Gly, Thr.
3 Recommended for the routine preparation of cysteinyl peptides.
4 Stable to TFA. Can be removed with I_2 to form cyclic disulfide peptides in a single step. Has been used in combination with Trt to prepare peptides containing multiple disulfide bonds.
5 Removed with thiols or tributylphosphine. Can be removed whilst the peptide is still attached to the resin to allow on resin disulfide bond formation. Not compatible with cleavage mixtures containing thiol scavengers.
6 Reduced enantiomerization compared to His(Trt). Ideal if coupling is slow or hindered.

and by separately activating the incoming amino acid [43]. Side-chain protection of Gln residues also prevents pyroglutamate formation, and Boc-Gln(Xan)-OH can be used to this effect. Base catalyzed pyroglutamate formation in Fmoc chemistry is extremely low [3].

Related products

853039	Boc-Asn-OH
853088	Boc-D-Asn-OH
853007	Boc-Asn(Xan)-OH
852119	Fmoc-Asn(Dmcp)-OH
852044	Fmoc-Asn(Trt)-OH
852159	Fmoc-D-Asn(Trt)-OH
853040	Boc-Gln-OH
853100	Boc-D-Gln-OH
853016	Boc-Gln(Xan)-OH
852120	Fmoc-Gln(Dmcp)-OH
852045	Fmoc-Gln(Trt)-OH
852160	Fmoc-D-Gln(Trt)-OH

1.3 Aspartic and glutamic acids

The most common side reaction of Asp residues is cyclization to form aspartimide, with concomitant epimerization, and the subsequent reopening of the ring to yield undesirable **b**-aspartyl peptides [446]. In Fmoc chemistry, it was originally believed this could be overcome by the use of Asp(OtBu) [47] and Asp(O-1-Ada) [48] derivatives. However, a number of authors have reported observing aspartimide and piperidide formation [49 - 54] and the problem is clearly more widespread than originally thought. Dölling, *et al.* [49] detected piperidides and aspartimides in peptides containing the Asp(OX)-Asn(Trt) sequence [X=tBu, Ada]. In a more systematic study Lauer, *et al.* [50] found sequences of the type Asp(OtBu)-X, where X is Gly, Thr(tBu), Cys(Acm), Asn(Trt) and Asp(OtBu) to be most problematic. The addition of either HOBt or 2,dinitrophenol to the piperidine reagent was shown to suppress these side reactions. More recently, have shown the addtion of 1 M Oxyma Pure to be particularly effective in this regard [51]. However, ultimately total elimination of the problem in difficult cases can only be achieved by employing amide protected derivatives for the introduction of the residue preceding the Asp [53] or Fmoc-Asp(OtBu)-(Dmb)Gly-OH (see section 3.6, page 3.23).

Karlström and Undén [54] have introduced the extremely hindered Mpe (**b**-3-methylpent-3-yl) protecting group in an attempt to overcome this problem. This group was shown to drastically reduce aspartimide formation compared to tBu in model systems, and the routine use of Fmoc-Asp(OMpe)-OH is strongly recommended. For further information, please see section 3.6, page 3.23, Novabiochem Catalog.

In Boc chemistry, Asp(OcHx) provides protection against succinimide formation in sensitive sequences, such as Asp-Gly and Asp-Ser [55, 56]. Temperature and time play an important role in suppressing aspartimide formation when using strong acids. Please refer to the section on cleavage and deprotection for more details.

Like Asp, Glu can also undergo cyclization, leading to the subsequent formation of **g**-Glu peptides. Although not as serious a problem as with Asp, Glu is normally protected using the same side-chain protecting groups as Asp. Glu dehydration, followed by anisylation of the resulting acylium ion can occur during cleavage with HF. Both succinimide formation (Asp) and acylation of scavenger molecules by the glutamyl side chains can be prevented by using the low-high cleavage procedure of Tam, *et al.* [57].

Related products

853045	Boc-Asp(OBzl)-OH
853105	Boc-D-Asp(OBzl)-OH
853030	Boc-Asp(OcHx)-OH
852005	Fmoc-Asp(OtBu)-OH
852154	Fmoc-D-Asp(OtBu)-OH
852104	Fmoc-Asp(OMpe)-OH
853010	Boc-Glu(OBzl)-OH
853089	Boc-D-Glu(OBzl)-OH
852009	Fmoc-Glu(OtBu)-OH
852155	Fmoc-D-Glu(OtBu)-OH

1.4 Cysteine

The synthesis of peptides containing Cys residues is often complicated because some peptide products require the Cys residue to be in the free sulfhydryl form while others require inter- or intramolecular disulfide bonds. For a discussion on the management of cysteine containing peptides, the reader is referred to the excellent article by Albericio, *et al.* [58] which contains many detailed protocols.

There are currently a number of different groups which can effectively protect cysteine. In Fmoc chemistry, these have included Trt, Mmt, Acm, tBu, tButhio, and STmp, of which the most commonly used is Trt. This group generates the free thiol upon deprotection with TFA and is particularly useful for the preparation of peptide antigens which require conjugation of the peptide to carrier proteins. Table 3 summarizes the deprotection methods for the most commonly used groups.

Unlike Trt and Mmt, the Acm, tBu, tButhio and STmp protecting groups require additional procedures for deprotection after assembly of the peptide. When used in combination, these derivatives enable differential deprotection of Cys residues in polycysteinyl peptides, thus permitting selective formation of disulfide bridges [59]. The *t*-butyl protecting group of cysteine is labile to TFMSA [60, 61], TMSBr/thioanisole [62] and HF at 20°C [58], and like the Acm group, it can be deprotected using mercury (II) acetate [60, 61, 63, 64] (Method 3-39, page 3.34, Novabiochem Catalog). Cys(Acm), Cys(Mmt) and Cys(Trt) residues can be oxidized directly to cystine (disulfide) using iodine or thallium (III) trifluoroacetate [58]; Trp and Met should be protected to prevent side-reactions. The rates of iodine oxidation of Cys(Acm) and Cys(Trt) are strongly solvent dependent, and this fact can be utilized synthetically for the selective conversion of Cys(Trt) residues to disulfide bridges in the presence of Cys(Acm) groups [65]. Iodine oxidation can cause the formation of Trp-2-thioesters and Met sulfoxides [66] and iodination of Tyr residues [67]. However, these side reactions can be minimized through the appropriate choice of reaction conditions [66, 68]. In addition, iodine and thallium oxidation of Cys(Acm) has recently been found to cause migration of the Acm group to the side chains of Ser, Thr or Gln residues [67-70].

Cys(tBu), Cys(MeBzl) and Cys(MeOBzl) residues may be directly converted to cystine by treatment with $MeSiCl_3/Ph_2SO$/TFA [58, 71]. This method provides an extra level of selectivity, and has been used to prepare regioselectively peptides containing up to three disulfide bridges [71]. Similarly, Cys(tBu), Cys(MeBzl) residues may be converted to cystine by TFA/DMSO/anisole [72]. At room temperature only Cys(tBu) is affected; Cys(MeBzl) requires heating to 70°C to react. This selectively has been exploited to achieve in one-pot the regioselective formation of two disulfide bridges [73].

Table 3: Deprotection methods for cysteine. Key: + cleaved; - not cleaved; n not tested. [1]Reaction should be conducted in TFA. [2]Product of the reaction is cystine. [3]1-3%TFA.

Protecting group	Reagent						
	TFA	TFMSA	Hg^{2+}	Ag^{+}	I_2	Tl^{3+}	RSH
tBu	-	+	+[1]	-	-	n	-
Trt	+	+	+	+	+[2]	+[2]	-
Mmt	+[3]	+	+	+	+[2]	+[2]	-
StBu	-	-	n	n	n	n	+
Acm	-	-	+	+	+[2]	+[2]	-
STmp	-/+	+	n	n	n	n	+

Yoshida, ***et al.*** have reported the use of silver tetrafluoroborate ($AgBF_4$) in TFA for the deprotection of Tacm and Acm protecting groups of cysteine [74]. In an improved approach, Tamamura, ***et al.*** instead used silver trifluoromethanesulfonate; treatment of the product generated in this reaction with DMSO-aq. HCl results in removal of the silver ions as AgCl and disulfide bond formation (Method 3-36, page 3.32, Novabiochem Catalog) [75].

Disulfide bond formation from free sulfhydryls can be performed using a number of different methods. The two most popular are air oxidation [1, 58, 61] or oxidation using potassium ferricyanide ($K_3Fe(CN)_6$) [58, 61, 76]. Both procedures use a dilute solution of peptide in water to prevent the formation of aggregates and polymers. The potassium ferricyanide method is faster than air oxidation which may require several days. In both cases disulfide bond formation can be monitored by HPLC. Another mild method has been described which makes use of DMSO in TFA [58, 72, 77] or aqueous buffered solutions [78] for disulfide bond formation. No side reactions were observed with amino acids such as Met, Tyr, Trp with aqueous DMSO [78], but DMSO in TFA should be avoided with Trp and Met.

In Boc chemistry, the pMeOBzl, pMeBzl, and Acm groups are often employed. Cys(Acm) residues are resistant to HF cleavage and require additional methods for their deprotection.

Another approach is to perform the cyclization while the protected peptide is still anchored to polymeric supports used for solid phase synthesis [58]. Varying results were obtained for Boc- and Fmoc-strategies using either thallium (III) trifluoroacetate in TFA or iodine for the cyclization step [79] with Acm protection. Cys(tButhio) and Cys(STmp) can be deprotected on the solid phase by reduction with thiols (e.g. b-mercaptoethanol [76])(Method 3-41, page 3.34, Novabiochem Catalog) or tributylphosphine [59], whereas Mmt can be selectively removed on the solid phase with 1-3% TFA in DCM (Method 3-42 page 3.34). The combination of STmp and Mmt have been used to form two disulfide bridges on the solid phase [80]. The method involved first removing the STmp group with mercaptoethanol, and then conversion of the liberated thiols to a disulfide with N-chlorosuccinimide (NCS). Treatment with 2% TFA in DCM cleaved the Mmt groups and the second disulfide formed with oxidation again with NCS.

Hondal and colleagues [81] have shown that DTNB in TFA can cleave MeOBzl, tBu, StBu, and Acm from side chain of cysteine to form the corresponding Cys(Npys) derivative. In the case of StBu and Acm, the reaction requires the addition of thioanisole. A similar approach was adopted by Wade and co-workers [82] in the synthesis of relaxin. Here, the A chain containing one disulfide bridge, a Cys(tBu) and Cys(Acm) residues, was converted by treatment with TFA/TFMSA/DPDS to the activated disulfide bridged Cys(Pys), Cys(Acm) peptide, which could then be selectively linked *via* another disulfide bridge to the B-chain.

Considerable enantiomerization has been noted during the introduction of Fmoc-Cys(Trt)-OH using base mediated *in situ* activation with reagents such as TBTU [83 - 85]. Coupling under neutral conditions using preformed symmetrical anhydrides [83], OPfp esters [84], DIPCDI/HOBt [85]; or the use of HBTU or PyBOP®/HOBt in DMF/DCM without preactivation [84, 85] appears to minimize this problem. In automated synthesis where preactivation is often unavoidable, substitution of DIPEA for TMP has been shown to reduce enantiomerization to within acceptable levels (1.1%) [85]. Enantiomerization can also occur during coupling of the first residue and during chain extension. When Cys is the C-terminal residue of peptide acids, the use of 2-ClTrt resin is recommended, since the loading of this support does not require carboxy activation and is consequently free from enantiomerization. The trityl resin also protects the Cys residue from enantiomerization during piperidine-mediated Fmoc removal [86]. The use of Wang-type resins for the anchoring of Cys is not recommended.

Related products

853049	Boc-Cys(Acm)-OH
853109	Boc-D-Cys(Acm)-OH
853050	Boc-Cys(4-MeOBzl)-OH
853033	Boc-Cys(4-MeBzl)-OH
853005	Boc-Cys(Trt)-OH
853115	Boc-D-Cys(Trt)-OH
852006	Fmoc-Cys(Acm)-OH
852158	Fmoc-D-Cys(Acm)-OH
852007	Fmoc-Cys(tBu)-OH
852022	Fmoc-Cys(tButhio)-OH
852031	Fmoc-Cys(Mmt)-OH
852373	Fmoc-Cys(STmp)-OH
852008	Fmoc-Cys(Trt)-OH
852143	Fmoc-D-Cys(Trt)-OH
852126	Fmoc-Cys(Trt)-OPfp

1.5 Histidine

Histidine is one of the most problematic amino acids in peptide synthesis, by both Boc and Fmoc strategies. There are two main problems associated with the use of histidine: acylation and enantiomerization.

Acylation

Acylation of unprotected histidine during subsequent coupling cycles occurs when temporary protection of histidine is used (e.g. Boc-His(Boc)), or when the protecting group is not completely stable to the conditions of subsequent coupling or deprotection (e.g. Boc-His(Tos)). It is a relatively minor problem as the products are normally removed during either the deprotection step of each cycle or the final deprotection of the peptide, although there can be wastage of reagents as a result of this side reaction. However, the imidazole must remain protected for use in segment condensation. The unprotected imidazole group can also catalyze peptide formation at free hydroxyl groups on the resin. This emphasizes the importance of capping the derivatized resin.

Enantiomerization

This is the most serious problem during coupling of histidine due to the reactivity of the imidazole nucleus. It has been shown that enantiomerization can be suppressed by blocking the **p**-nitrogen [87] (Figure 1). However, most protecting groups for histidine actually block the **t**-nitrogen. The fact that these suppress enantiomerization, to a greater or lesser extent, is due to electronic effects reducing the basicity of the **p**-nitrogen (steric effects may also play a part).

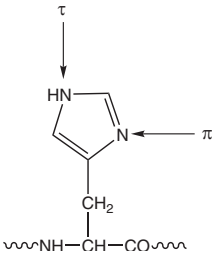


Fig. 1: Histidine: imidazole nitrogens.

The various histidine derivatives in current use (mostly offered by Novabiochem®) are described overleaf.

Boc-His(Tos)-OH

Boc-His(Tos)-OH is probably the most popular of the Boc derivatives due to its solubility and the ease with which the tosyl group can be removed by the normal deprotection conditions (liquid HF or TFMSA) [88]. It does, however, have a number of disadvantages. The N-im-tosyl group on histidine is susceptible to cleavage by HOBt. This can cause problems in subsequent coupling cycles if DCC/HOBt or the PyBOP® reagent are used, leading to chain termination by tosylation of the amino terminus. This only has to happen to the extent of a few percent each cycle to cause appreciable reduction in yield in a long peptide. For this reason, many people prefer to remove the tosyl group with 1 M HOBt in DCM after the coupling reaction. Another serious side reaction with Boc-His(Tos)-OH has been reported, when it is used in conjunction with HOBt and acetic anhydride capping [89].

Boc-His(Tos)-OH is not stable for long periods at room temperature. For this reason, the material must be stored in the freezer as soon as it is received. Boc-His(Tos)-OH does not couple satisfactorily in some sequences. In these cases, Boc-His(Dnp)-OH has been found to work.

Method 1: Conversion of a CHA/DCHA salt to the free acid

1. Suspend 5 mmole of CHA or DCHA salt in 20 ml ethyl acetate in a separating funnel.
2. Add approx. 1.2 equivalents of ice-cold 10% H_3PO_4 until pH 3 is reached. Shake until all solid material has dissolved.
3. Remove the top (ethyl acetate) layer and set aside. Add a further 10 ml of cold water to the aqueous layer and extract with 2 x 20 ml of ethyl acetate.
4. Combine all the ethyl acetate layers and wash with 2 x 20 ml of 1% H_3PO_4 and 4 x 20 ml of water.
5. Dry with Na_2SO_4 and remove the ethyl acetate in a rotary evaporator at not more than 40 °C.

Boc-His(Dnp)-OH

The advantage of Dnp for protection of histidine is that it is stable to almost all coupling conditions and, indeed, may succeed in difficult couplings where Boc-His(Tos)-OH has failed. However, its main drawback is the fact that it is also stable to liquid HF and TFMSA, so a separate deprotection step is necessary at the end of the synthesis (Method 3-18, page 3.26, Novabiochem Catalog). Another disadvantage of Dnp is that it leaves the peptide bright yellow after deprotection. The color (due to Dnp-thiophenol) can be removed by gel filtration on Sephadex G-25 in 10-30% acetic acid. This simple purification step is well worth incorporating routinely in the work-up of all peptides, as it removes scavengers and nonvolatile products of the deprotection reaction before proceeding to further purification by ion exchange or HPLC.

Boc-His(Boc)-OH•DCHA

This derivative provides protection for only the critical coupling step as both Boc groups are removed simultaneously during deprotection [90]. This leads to acylation of the imidazole at every coupling and, although the adduct is removed by TFA at each cycle, this is wasteful of reagents.

Fmoc-His(Trt)-OH

Although the Trt group blocks the τ-nitrogen rather than the π-nitrogen, it has been shown to be effective at suppressing enantiomerization [91], except in those instances where coupling is sluggish due to steric hindrance or aggregation [92]. In the coupling of His to Pro with TBTU/DIPEA 5% enantiomerization was observed [93], which could be reduced to >0.8% with DEPBT and 2.8% with DCC/HOBt activation respectively [94]. The Trt group is stable to the conditions of coupling and deprotection, but is readily removed by 95% TFA for 1 hour at 20°C. This derivative couples well as the symmetrical anhydride, OPfp ester or using the PyBOP® reagent or TBTU/HBTU. Enantiomerization has been observed when using Fmoc-His(Trt)-OH for attachment to Wang resin. Therefore, it is better to use the 2-chlorotrityl resin as attachment of Fmoc-His(Trt)-OH to this resin is without risk of enantiomerization.

Fmoc-His(Clt)-OH

The use of Fmoc-His(Trt)-OH for the production of protected peptide fragments can result in the formation of a mixture of fully and partially protected peptides because loss of the τ-trityl group can occur during the HFIP/DCM or 1% TFA in DCM treatments used to release products from 2-chlorotrityl or HMPB-AM resins. In constrast, the τ-Clt group in Fmoc-His(Clt)-OH is completely stable to these mild acids, enabling the preparation of protected peptides free from partially deprotected by-products.

Furthermore, in model studies we have found Fmoc-His(Clt)-OH to give about a third less enantiomerization than Fmoc-His(Trt)-OH.

Fmoc-His(Boc)-OH•CHA

Like Fmoc-His(Trt)-OH, the Boc-group protects the τ-nitrogen. However, the smaller bulk of the Boc group compared to Trt, coupled with the greater stability of Trt towards nucleophiles, suggests that Boc will be less effective than Trt in suppressing enantiomerization [19]. Several side reactions leading to a C-terminal truncated sequence and the formation of succinimide from Asp-His were encountered when using the above-mentioned derivative on a continuous flow instrument during the synthesis of an N-acetylated undecapeptide [95].

Related products

853008	Boc-His(Dnp)-OH · isopropanol
853041	Boc-His(Tos)-OH
852052	Fmoc-His(Boc)-OH · CHA
852371	Fmoc-His(Clt)-OH
852211	Fmoc-His(Mtt)-OH
852032	Fmoc-His(Trt)-OH
852161	Fmoc-D-His(Trt)-OH

1.6 Methionine

The principal side reaction with methionine is the acid catalyzed oxidation of Met to Met(O) during cleavage and deprotection [96, 97]. In Fmoc chemistry Met is introduced without side-chain protection. The formation of Met(O) is prevented by using ethylmethylsulfide [98] or thioanisole [99] during cleavage and deprotection.

In Boc chemistry, methionine can be introduced in its unprotected form. However, spontaneous oxidation can occur during cleavage with strong acids. Alternatively methionine can be introduced as Met(O) during the synthesis and then reduced to Met using the low-high HF (or TFMSA) cleavage procedure outlined in Method 3-17, page 3.25, Novabiochem Catalog.

Alkylation of methionine during HF cleavage can be prevented by using DMS in combination with *p*-thiocresol or anisole [100]. Met(O) can also be reduced to Met after cleavage using N-methylmercaptoacetamide (MMA)[101-103], ammonium iodide/DMS [104], ammonium fluoride/2-mercaptoethanol [105], $TiCl_4$ [106] or TMSBr/EDT [58, 107]. Reduction with ammonium iodide/DMS has been shown not to affect disulfide bonds [108].

Related products

853054	Boc-Met-OH
853093	Boc-D-Met-OH
853035	Boc-Met(O)-OH
852002	Fmoc-Met-OH
852140	Fmoc-D-Met-OH
852225	Fmoc-Met-OPfp
852054	Fmoc-Met(O)-OH
852212	Fmoc-Met$(O)_2$-OH

1.7 Tryptophan

There are two main side reactions which occur with tryptophan residues: oxidation of Trp during synthesis and alkylation of the indole ring by carbonium ions generated during cleavage [1]. In Boc chemistry, the Trp(For) derivative has been used to overcome both of these problems [1]. The formyl group is stable to acid cleavage reagents and can be deprotected either before normal HF cleavage or by thiolytic deprotection using the low-high HF or TFMSA procedure [109]. These procedures are provided in Method 3-19, page 3.25, and Method 3-22, page 3.27, Novabiochem Catalog. Avoid the use of thioanisole during HF cleavage if your peptide contains tryptophan. In Fmoc chemistry, the Trp has been incorporated without side-chain protection. Problems due to *t*-butylation were normally controlled using scavenging mixtures containing EDT [110-112]. However, sulfonation by the by-products from the deprotection of Mtr [113], Pmc [24] and Pbf [28] protected Arg residues, and reattachment of the peptide to the resin support *via* the side chain of a C-terminal Trp residue [114] can only be reduced, but not eliminated, by varying the scavenger mixtures. These problems were resolved with the introduction by Novabiochem®'s own research group of Fmoc-Trp(Boc)-OH [25]. This derivative has proven to be extremely effective in preventing these side reactions, and is now firmly established in the repertoire of standard reagents for Fmoc SPPS.

Mechanism of Boc removal

During normal deprotection with TFA the *t*-butyl moiety is removed, leaving the indole protected with an N-carboxy group [115], thereby preventing alkylation, peptide re-attachment or sulfonation. Tryptophan is subsequently regenerated during the course of the normal lyophilization procedure, since this carbamic acid intermediate readily decarboxylates in acidic aqueous media (Figure 2).

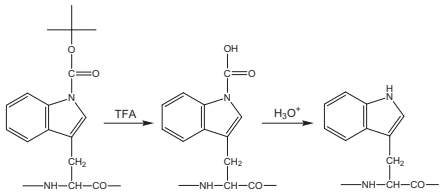


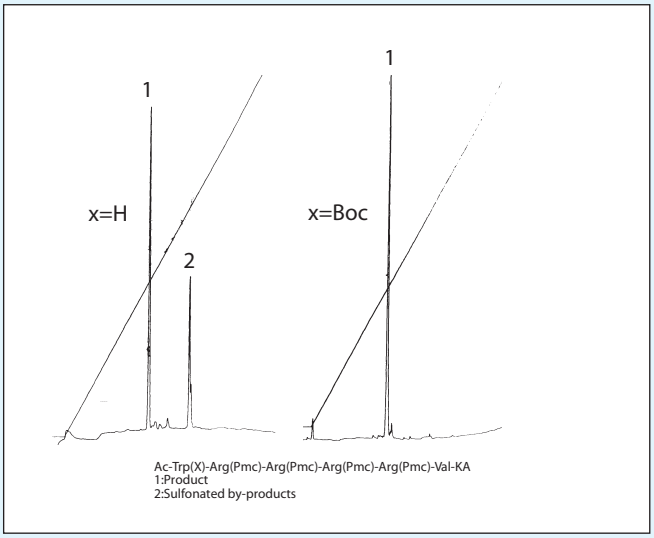
Fig. 2: Mechanism of Boc removal.

Cleavage of Trp and Arg-containing peptides

N^{in}-Boc protection prevents sulfonation of Trp, even with sequences containing multiple Arg residues [116] (Application 1). It is especially effective when used in conjunction with thiol-containing scavenger mixtures such as Reagent K [117], but also works well with silanes [28,116]. The efficacy of using Trp(Boc) in Fmoc synthesis has now been confirmed by a number of independent studies [28, 118, 119]. The combination of Trp(Boc) and Arg(Pbf) seems to be optimal [28].

Application 1: Synthesis of peptides containing multiple Arg(Pmc) residues with Trp(Boc)

The peptides shown in Figure 3 were prepared automatically on a NovaSyn® Crystal using either N^{in}-Boc protected or unprotected tryptophan. Coupling reactions were carried out using a mixture of Fmoc amino acid/HOBt/PyBOP® (1:1:1) activated with DIPEA (2 eq.) or Fmoc amino acid pentafluorophenyl esters. Peptidyl resins were cleaved by treatment with TFA/thioanisole/phenol/EDT/water (87.5:5:2.5:2.5:2.5).



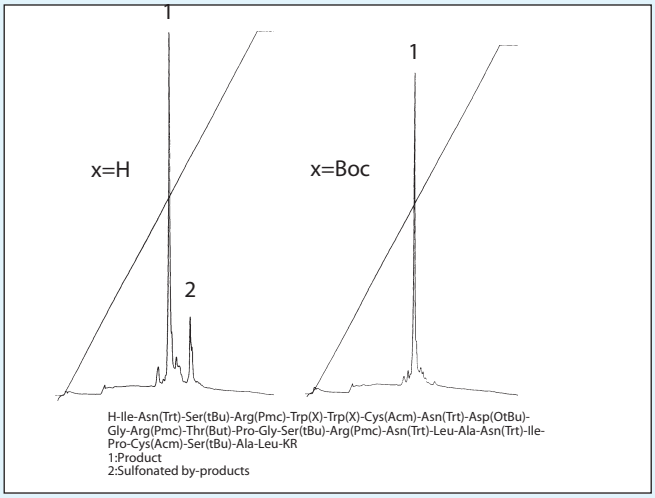


Fig. 3: HPLC profiles of total crude products obtained from syntheses of model peptides containing Trp and Arg residues.

Reduction of Trp by silanes

Trialkylsilanes are very effective scavengers, especially for those highly stabilized cations which are not irreversibly scavenged by thiols, such as trityl [120], Tmob [121] and the Rink linker. However, these reagents can cause reduction of tryptophan residues [122], converting the side-chain indole to an indoline. This effect is greatly reduced with Trp(Boc), as demonstrated in Application 2.

Peptide re-attachment

This can be a particular problem for peptides containing C-terminal Trp, giving severely reduced yields in some cases. Trp(Boc) is effective in limiting this side reaction.

Application 2: Cleavage with silanes

The peptide was prepared automatically on a NovaSyn® Crystal using either N^{in}-Boc protected or unprotected tryptophan. Coupling reactions were carried out using a mixture of Fmoc amino acid/HOBt/PyBOP® (1:1:1) activated with DIPEA (2 eq.). Peptidyl resins were cleaved by treatment with TFA/TES/water (90:5:5). The HPLC elution profile of the crude product is shown in Figure 4 .

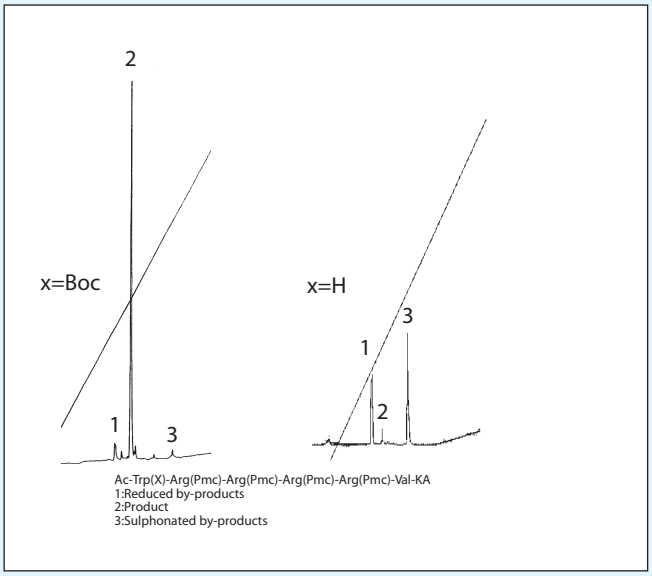


Fig. 4: Cleavage of a model peptide with TFA mixtures containing silanes.

Related products

853038	Boc-Trp-OH
853086	Boc-D-Trp-OH
853078	Boc-Trp(Boc)-OH
853022	Boc-Trp(For)-OH
853108	Boc-D-Trp(For)-OH
852207	Fmoc-Trp-OH
852150	Fmoc-D-Trp-OH
852050	Fmoc-Trp(Boc)-OH
852164	Fmoc-D-Trp(Boc)-OH
852131	Fmoc-Trp(Boc)-OPfp

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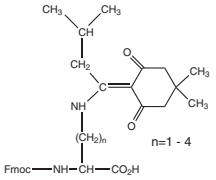
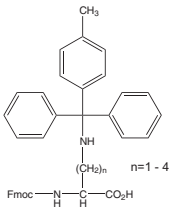
2 Selectively protected amino acid building blocks

Novabiochem® has one of the largest collections of orthogonally and quasi-orthogonally protected tri-functional amino acids. These derivatives have many interesting applications in peptide and combinatorial chemistry, such as synthesis of cyclic [1] and branched peptides [2] and the preparation of peptides carrying side-chain modifications [3]; the construction of orthogonal multi-release resins for solution screening of one bead-one compound libraries [4, 5]; the preparation of dendritic or cyclic scaffolds for ligand [6, 7] or protein motif presentation [8, 9]; libraries of cyclic glycopeptides [10]; and synthesis of b-turn mimetics [11]. The properties of Novabiochem®'s selectively protected amino acids are given in Table 4, page 11.

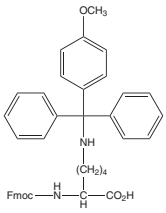
2.1 Diaminopropionic/butanoic acid, ornithine and lysine derivatives



Fmoc-Dpr(ivDde)-OH, Fmoc-Dab(ivDde)-OH, Fmoc-Orn(ivDde)-OH, Fmoc-Lys(ivDde)-OH, ivDde-Lys(Fmoc)-OH



Fmoc-Dpr(Mtt)-OH, Fmoc-Dab(Mtt)-OH, Fmoc-Orn(Mtt)-OH, Fmoc-Lys(Mtt)-OH



Fmoc-Lys(Mmt)-OH

The side chains of lysine, ornithine, diaminopropionic acid (Dpr) and diaminobutanoic acid (Dab) are very useful for the introduction of a range of modifications, e.g. biotin, fluorescent labels, etc., as well as for the synthesis of branched and cyclic peptides.

Symmetrically branched peptides or MAP core molecules can be produced using the symmetrically protected Fmoc-Lys(Fmoc)-OH, in which both the α- and ε-amino groups are simultaneously deprotected with 20% piperidine [12].

However, for specific modification of side-chain amino groups, the use of selective, or ideally orthogonal, protecting group strategies are required. For such applications Novabiochem is able to offer the following derivatives.

Dde/ivDde

Since the introduction of the Dde [2] and ivDde [13] amino-protecting groups in 1993 and 1998, respectively, the Fmoc/xDde strategy has become the standard approach for the synthesis of branched, cyclic and side-chain modified peptides by Fmoc SPPS, with over 200 publications citing the use of these protecting groups [14].

Their utility stems from the fact that Dde and ivDde-protected primary amines are stable to 20% piperidine and TFA but are cleaved with 2% hydrazine in DMF. Thus, amino groups protected by these groups can be selectively unmasked on the solid phase without affecting the side-chain protecting groups of other residues, facilitating subsequent site-specific modification. Furthermore, the reaction can be monitored by spectrophotometry since the indazole cleavage product absorbs strongly at 290 nm (Figure 1).

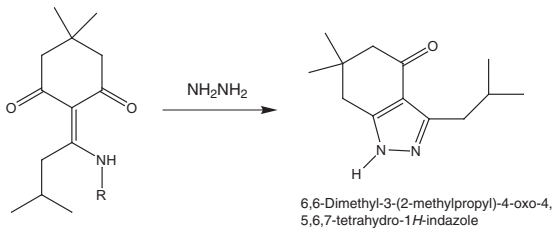


Fig. 5: Removal of ivDde.

Examples of the use of the Fmoc/Dde strategy include: branched [2] and di-epitopic peptides [15]; MAP core molecules and lipo-MAPs [16 -18; cyclic peptides [1, 19]; TASP molecules [8]; templates for combinatorial chemistry [6, 7]; synthetic proteins [9]; fluorescently-labeled peptides [3]; modified Lys and Dpr derivatives [20, 21]. the synthesis of multi-functional probes [22]; and ubiquinated peptides [23].

The choice between Dde or ivDde depends on the application. Dde is easier to remove than ivDde but is much less robust. Dde has been observed to undergo migration during piperidine mediated deprotection of N-e-Fmoc-Lys and N-terminal Dpr residues, leading to scrambling of its position within the peptide chain; and partial loss has been noted during the synthesis of long sequences [24]. The more hindered ivDde on the otherhand does not undergo leaching or side-chain migration to any significant extent, except in the special case of Dpr [25] (see Method 3), but can occasionally prove extremely hard to remove, particularly if located at the *C*-terminus of a peptide or in an aggregated region of the sequence.

Fmoc derivatives of lysine are available in which the α- and ε-amino groups are protected with Dde and ivDde: Fmoc-Lys(Dde)-OH, Dde-Lys(Fmoc)-OH, Fmoc-Lys(ivDde)-OH; and ivDde-Lys(Fmoc)-OH. For Dpr and Dab, only side-chain ivDde-protected derivatives are available: Fmoc-Dpr(ivDde)-OH and Fmoc-Dab(ivDde)-OH.

As hydrazine can also remove Fmoc groups as well as Dde and ivDde groups, when preparing cyclic or side-chain modified peptides by the Fmoc/Dde strategy, assembly of the peptide backbone is generally completed prior to deprotection of the Dde/ivDde groups. The *N*-terminus of the peptide should be protected with Boc, which can be achieved either by direct incorporation of the *N*-terminal residue as a Boc protected amino acid or acylation of the free *N*-terminal amino group with Boc_2O.

Removal of Dde or ivDde is achieved by treating the resin with 2% hydrazine in DMF, although in cases where ivDde removal has proved difficult, solutions of as much as 10% hydrazine have been employed. The process can be followed spectrophotometrically at the same wavelength used for monitoring removal of Fmoc, since the reaction-product of the Dde or ivDde group with hydrazine is a chromophoric indazole derivative (Figure 5). Typical traces obtained for Dde removal on the NovaSyn® Crystal and Millipore 9050 peptide synthesizers are shown in Figure 6; ivDde gives similar results. Dde and ivDde also stable to the normal reagents employed for Boc cleavage (TFA or 50% TFA in DCM) and to DBU at the normal concentrations (approx. 2%) used for Fmoc removal.

As previously mentioned, if the ivDde group is close to the *C*-terminus of the peptide or the peptide has aggregated, removal ivDde can be very sluggish and often incomplete. The problem can be avoided for peptides containing side-chain modified lysine by using ivDde-Lys(Fmoc)-OH instead of Fmoc-Lys(ivDde)-OH for incorporation of those residues. The use of the former allows side-chain modification of lysine to be accomplished during chain extension. Following incorporation of ivDde-Lys(Fmoc)-OH into the peptide chain, the side-chain Fmoc group can be removed with piperidine, the side-chain amino group reacted with the desired carboxylic acid-functionalized moiety, before removal of the ivDde with hydrazine, and chain extension in the usual manner.

Recently, complete orthogonality of Dde with Fmoc has been demonstrated if hydroxylamine hydrochloride/imidazole (1.3:1) in NMP instead of hydrazine in DMF is used for Dde removal [26].

Method 2: Selective removal of Dde/ivDde with 2% hydrazine in DMF

Batch

1. Place the peptidyl-resin in a flask and treat with 2% hydrazine monohydrate in DMF (25 ml/g). Stopper the flask and leave to stand at rt for 3 min.
2. Filter the resin and repeat the hydrazine treatment two more times. Wash the partially protected resin with DMF.

Continuous flow

1. Flow 2% hydrazine monohydrate in DMF at 3 ml/min through the peptidyl resin packed in a 1 cm diameter reaction column. Deprotection can be followed by monitoring spectrophotometrically at 290 nm the absorbance of the column eluant using a 0.1 mm path length cell.
2. When the reaction is complete, as indicated by return of the absorbance to its original value, flush the column with DMF.

Method 3: Special procedures for chain extension following introduction of Fmoc-Dpr(ivDde)-OH

Fmoc removal

1. Treat the Fmoc-Dpr(ivDde)-peptidyl resin with morpholine-DMF (1:1) (2 x 15 min).
2. Quickly wash resin with DMF.

Addition of next amino acid

1. Treat resin with Fmoc-A.A.-OH (1.5 eq.) and DIPEA (3 eq.) in DMF at -20°C.
2. Add PyBOP (1.6 eq.) to the slurry and allow to warm to rt.

In the worst case (Fmoc-Val), migration observed when using this procedure is <10%.

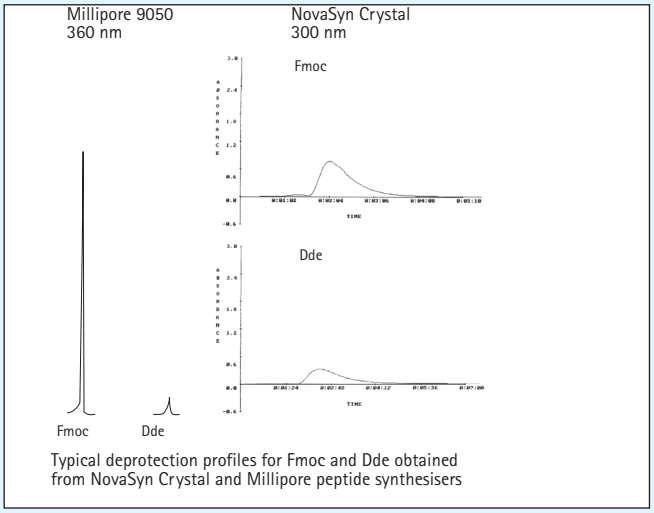


Fig. 6: Spectroscopic monitoring of the removal of Dde based protecting groups.

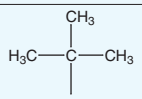
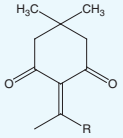
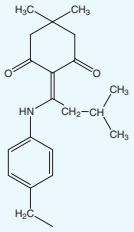
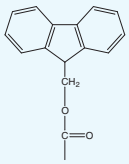
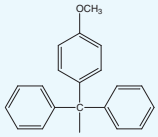
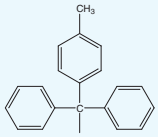
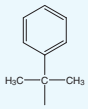
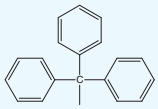
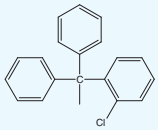
Mtt

The Mtt group can be removed from the side chain of lysine or ornithine using 1% TFA in DCM (Method 4) [27, 28] or with DCM/HFIP/TFE/TES (6.5:2:1:0.5) (Method 5) [29], allowing selective removal in the presence of other side-chain protecting groups which require up to 95% TFA for removal. When using TFA, addition of 1-5% TIS or MeOH is recommended to quench the trityl cations released. Preliminary results indicate MeOH scavenging prevents loss of t-butyl groups and premature cleavage from Rink Amide resins [30] (Method 4b).

The application of Fmoc-Lys(Mtt)-OH is illustrated by the synthesis of a branched peptide in which the sequence Ile→Leu was assembled on an amide resin (Application 3). The *N*-terminal Ile residue was introduced as the Boc-amino acid. The Mtt group was removed from the ε-amino group of the lysine residue with 1% TFA (Method 4a). The Gly→Glu branch was then assembled on the lysine side chain using normal Fmoc chemistry. The peptide was cleaved with 95% TFA, removing all other side-chain protecting groups, as well as the N-α-Boc group from the Ile.

Applications of Mtt derivatives include the use of Lys(Mtt) in conjunction with Lys(Dde) to prepare fluorescence-quenched substrates [3], and of Lys(Mtt) and Orn(Mtt) in the preparation of peptidomimetic macrocycles [11, 31].

Table 4: Protecting groups.

Name	Structure	Removed by	Stable to
O-All/Alloc		3 eq. $Pd(Ph_3P)_4$ in $CHCl_3$/AcOH/NMM (37:2:1)	TFA, piperidine, hydrazine
Azido		3 eq. Me_3P in dioxane/water	TFA, piperidine
O-tBu		95% TFA	1% TFA, piperidine,Pd(O), hydrazine
STmp		b-Mercaptoethanol, 0.1 NMM in DMF	TFA(partial), piperidine
N-xDde		2% hydrazine in DMF or 20% hydroxylamine/ 15% imidazole in NMP/DCM (5:1)	TFA, piperidine, Pd(0), DBU
O-Dmab		2% hydrazine in DMF	TFA, piperidine, Pd(0), DBU
N-Fmoc		20% piperidine in DMF	TFA, Pd(0)
S-Mmt N-Mmt		1% TFA in DCM containing 1-5% TIS AcOH/TFE/DCM (1:2:7)	Pd(0), hydrazine, piperidine
N-Mtt		1% TFA in DCM containing 1-5% TIS	Pd(0), hydrazine, piperidine
O-2-(PhiPr)		2% TFA in DCM containing 1-5% TIS	Pd(0), piperidine
O-Trt		1% TFA in DCM containing 1-5% TIS	Pd(0), hydrazine, piperidine
O-2-ClTrt		1% TFA in DCM containing 1-5% TIS	Pd(0), hydrazine, piperidine

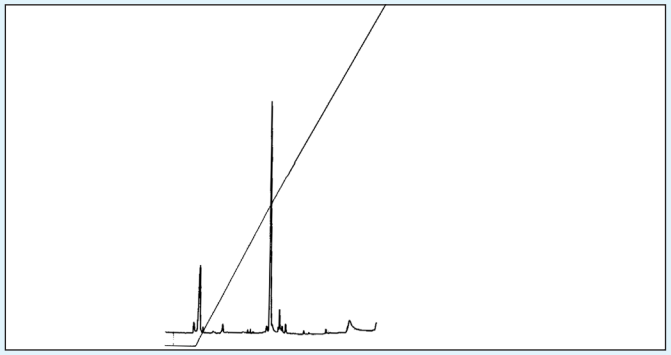


Fig. 7: HPLC of crude branched peptide.

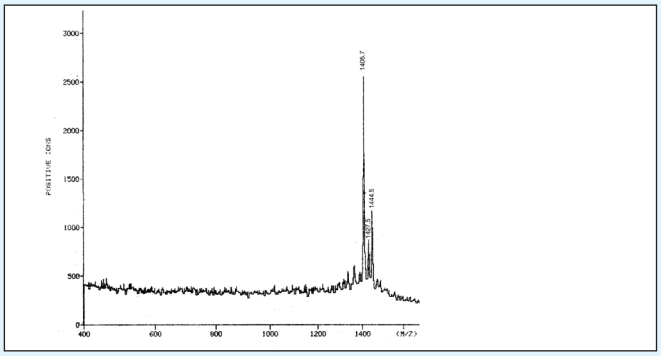


Fig. 8: PD-MS of crude product; peaks at 1427.5 and 1444.5 are due to $M+Na^+$ and $M+K^+$, respectively.

Application 3: Synthesis of H-Ile-Pro-Glu-Lys(H-Gly-Asp-Phe-Glu-Glu)-Glu-Thr-Leu-NH_2

Boc-Ile-Pro-Glu(OtBu)-Lys(Mtt)-Glu(OtBu)-Thr(tBu)-Leu-NovaSyn® KR was prepared using PyBOP®/HOBt chemistry on a NovaSyn® Crystal fully automatic, continuous flow synthesizer. Lys(Mtt) was deprotected by flowing 1% TFA in DCM through the reaction column and monitoring the removal of Mtt spectrophotometrically at 470 nm.

After neutralization of the TFA salt with piperidine, the synthesis continued in the usual way on the side chain of lysine.

The peptide was cleaved using TFA/water/TIS (95:2.5:2.5) for 3 h This treatment also removed all side-chain protecting groups as well as the N-a-Boc group on Ile.

The crude product was analyzed by HPLC (Figure 4-7) and PD-MS, expected 1406.5, found 1405.7 (Figure 8).

Method 4-4: Removal of trityl groups on solid phase

Batch-wise method

1. Pre-swell the dry resin (1 g) with DCM in a sintered glass funnel (of a type with a tap and stopper). Remove excess DCM.
2. Add 94:1:5 DCM/TFA/TIS (10 ml), seal funnel and shake for 2 min. Remove solvent by applying N_2 pressure.
3. Repeat step 2 three times.
4. Wash resin with DCM and dry under vacuum.

Flow method

1. Pre-swell resin (1 g) with DCM and pack into reaction column.
2. Pump 1% TFA in DCM (2 ml/min) through resin. The reaction can be followed by measuring the absorbance of the column eluant using a 0.1 mm flow cell at 460 nm[a].
3. Once reaction is finished, as indicated by the absorbance returning to baseline, flush column with DCM.

[a]If the peptide contains other trityl-based protecting groups, the level will not return to baseline owing to slow leaching of Trt groups [27].

Method 5: Removal of Mtt from Lys on solid phase

a) DCM/HFIP/TFE/TES [29]

1. Add DCM/HFIP/TFE/TES (6.5:2:1:0.5) (20 ml/g of resin) to peptidyl resin.
2. Leave mixture to stand with gentle agitation for 1 h.
3. Remove a small sample of resin and wash with DCM. Add 1% TFA in DCM to resin sample. If an orange color is formed instantly then leave the reaction for a further hour.
4. Once the trityl test is negative, wash the resin with DMF, 10% DIPEA in DMF, DMF and use in next step of synthesis.

b) TFA/DCM/MeOH [28]

1. Add TFA/DCM/MeOH (1:98:1) (15 mg/ml) to peptidyl resin. Drain after 1 min.
2. Add fresh TFA/DCM/MeOH (1:98:1) (15 mg/ml) to peptidyl resin and leave for 16 h.
3. Wash the resin with DMF, 10% DIPEA in DMF, DMF and use in next step of synthesis.

Mmt

The Mmt group is considerably easier to remove than Mtt. It is cleaved rapidly from the side-chain of lysine using the same methods that were described for Mtt but can be even removed using AcOH/TFE/DCM (1:2:7) [32] or HOBt in DCM/TFE (Method 5-4). The use of Fmoc-Lys(Mmt)-OH is ideal for those cases where removal of Mtt is problematic. Owing to the extreme acid sensitivity of the Mmt group, coupling of Fmoc-Lys(Mmt)-OH is best carried out using PyBOP®/DIPEA or other base-mediated coupling methods.

Alloc

The Alloc group is stable to treatment with piperidine and TFA, but can be easily removed under mild conditions by Pd(0) catalyzed allyl transfer [33]. A variety of methods have been used for this step, but perhaps the most useful is that of Kates, ***et al.*** utilizing $Pd(Ph_3P)_4/CHCl_3$/HOAc/NMM [34] (Method 7). The Alloc group is not compatible with the conditions employed for removal of ivDde [35]. It is thought that the presence of a small amount of diazine in hydrazine causes reduction of the double bond in the allyl group. Fortunately, this side reaction can be easily overcome by the addition of allyl alcohol to the hydrazine reagent.

Azido

Azides can be reduced under mild conditions with thiols or phosphines to the corresponding amines. Therefore, Fmoc-protected amino acids bearing side-chain azido functionalities are useful tools for preparing branched and side-chain modified peptides. The azido group is stable to coupling conditions and to treatment with piperidine. It is also stable to TFA cleavage conditions provided thiols are omitted from the cocktail [36]. Reduction of the azide on the solid phase selectively unmasks the side-chain amino group without affecting any other amino-acid residues [37, 38].

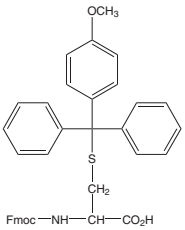
Method 6: Reduction of azido group on solid phase

1. Wash resin with three times with dioxane and dioxane/water (4:1).
2. Drain resin and add 1 M Me_3P in toluene (6 eq.) in dioxane/water (4:1). Gently agitate for 30 mins.
3. Remove a small sample of resin and wash with dioxane then DCM. Add 95% TFA to leave to cleave for 1.5 h. Analyze by HPLC to check completeness of reduction of azido group.

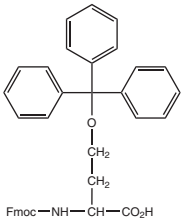
Related products

852321	Fmoc-γ-azidohomoalanine
852326	Fmoc-ε-azidonorleucine
852322	Fmoc-δ-azidonorvaline
852352	(2S,3S)-Fmoc-Abu(3-N_3)-OH
852084	Fmoc-Dab(ivDde)-OH
852167	Fmoc-D-Dab(ivDde)-OH
852092	Fmoc-Dab(Mtt)-OH
852083	Fmoc-Dpr(ivDde)-OH
852243	Fmoc-D-Dpr(ivDde)-OH
852089	Fmoc-Dpr(Mtt)-OH
852124	Fmoc-Lys(Alloc)-OH
852057	Fmoc-Lys(Dde)-OH
852147	Fmoc-D-Lys(dde)-OH
854000	Dde-Lys(Fmoc)-OH
852082	Fmoc-Lys(ivDde)-OH
852369	Fmoc-D-Lys(ivDde)-OH
852370	ivDde-Lys(Fmoc)-OH
852065	Fmoc-Lys(Mtt)-OH
852094	Fmoc-Lys(Mmt)-OH
852088	Fmoc-Orn(ivDde)-OH
852075	Fmoc-Orn(Mtt)-OH
852351	cis-Fmoc-Pro(4-N_3)-OH

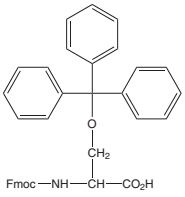
2.2 Hse, Ser, Thr, Tyr and Cys derivatives



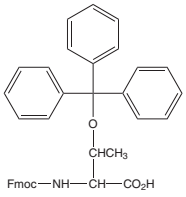
Fmoc-Cys(Mmt)-OH



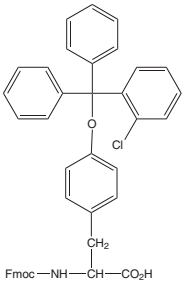
Fmoc-Hse(Trt)-OH



Fmoc-Ser(Trt)-OH



Fmoc-Thr(Trt)-OH



Fmoc-Tyr(2-ClTrt)-OH

2-ClTrt/Trt/Mmt

2-ClTrt, Trt and Mmt groups can be removed of the side chains of Tyr, Hse/Ser/Thr [39] and Cys [40] using only 1% TFA in DCM, leaving all other protecting groups intact (Method 4). This allows the selective deprotection of a single residue for subsequent side-chain modification. In products with multiple Ser, Thr or Tyr residues, selectivity is possible by incorporating all other such residues, with the exception of the residue to be modified, as the *t*-butyl ether. Trityl group removal is an equilibrium process, so must be performed using silanes to scavenge the trityl cations, or in a continuous flow mode to drive the equilibrium. For Ser(Trt), Coba, ***et al.*** [41] have described the use of 20% dichloroacetic acid in DCM for 10 min for Trt removal. This procedure may also have wider application for the deprotection of other trityl-protected amino acids.

The most common application of the Ser/Thr/Tyr derivatives is in the preparation of phosphopeptides by the post-synthetic method using a phosphoramidite reagent (section 3.11.3, page 3.41, Novabiochem Catalog).

In a comparative study, purer products were obtained using side-chain trityl protected amino acids than with standard *t*-butyl protected amino acids [42].

tButhio/STmp

The tButhio/STmp can be removed from the side chain by reduction with thiols [43, 44] (Method 3-41, page 3.34, Novabiochem Catalog).

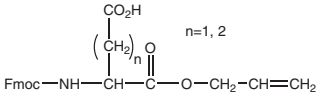
Related products

852022	Fmoc-Cys(tButhio)-OH
852031	Fmoc-Cys(Mmt)-OH
852373	Fmoc-Cys(STmp)-OH
852059	Fmoc-Hse(Trt)-OH
852046	Fmoc-Ser(Trt)-OH
852166	Fmoc-D-Ser(Trt)-OH
852066	Fmoc-Thr(Trt)-OH
852241	Fmoc-D-Thr(Trt)-OH
852080	Fmoc-Tyr(2-ClTrt)-OH

2.3 Asp and Glu derivatives

Orthogonally and quasi-orthogonally protected Asp and Glu derivatives have numerous applications in peptide synthesis and combinatorial chemistry. a-Esters are particularly useful for the preparation of head-to-tail cyclic peptides by on-resin cyclization, whereas selectively protected b- and g- esters of Asp and Glu can be utilized in the synthesis of glycopeptides and side chain-to-side chain or head-to-side chain lactam bridged peptides [33, 34, 45-48]. Similar strategies can also be applied to the construction of cyclic library templates [6, 7].

Allyl



Fmoc-Asp/Glu-OAll/Fmoc-Asp/Glu(OAll)-OH

Allyl esters are stable to treatment with piperidine and TFA but can be easily removed under mild conditions by Pd(0) catalyzed allyl transfer [33], as previously described for the Alloc group (Method 7).

The allyl group is not compatible with the conditions employed for removal of ivDde [35]. It is thought that the presence of a small amount of diazine in hydrazine causes reduction of the double bond in the

allyl group. Fortunately, this side reaction can be easily overcome by the addition of allyl alcohol to the hydrazine reagent.

Method 7: Removal of allyl protecting groups

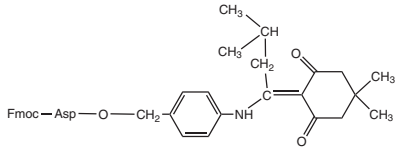
IMPORTANT: This reaction is air-sensitive and all manipulations should be carried out under Ar.

1. Weigh the peptidyl resin into a test tube and dry at 40°C under high vacuum. Seal the tube with a rubber septum. Flush the vessel with a stream of Ar delivered via a needle inserted through the septum.
2. Weigh $Pd(PPh_3)_4$ (3 eq.) into a dry test tube, add $CHCl_3$-AcOH-N-methylmorpholine (37:2:1) (15 ml/g of resin), dissolve catalyst by bubbling a stream of Ar through the solution, and seal the tube with a rubber septum.
3. Transfer this mixture using an Ar flushed gas-tight syringe to the tube containing the resin. Leave to stand for 2 h with occasional gentle agitation.
4. Transfer the resin to a sintered glass funnel and wash consecutively with 0.5% DIPEA in DMF and sodium diethyldithiocarbamate (0.5% w/w) in DMF to remove the catalyst.

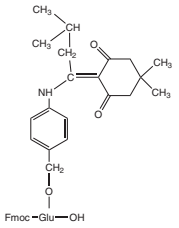
The above procedure can also be carried out on any automated peptide synthesizer which uses N_2 agitation for dissolution and transfer of reagents. The $Pd(PPh_3)_4$ catalyst should be weighed out into an amino acid cartridge and dissolved in $CHCl_3$-AcOH-N-methylmorpholine (37:2:1) with Ar agitation. The cartridge should be sealed and placed in the instrument autoloader as normal. The instrument should then be programmed to transfer the contents of the vial to reaction vessel or column without the addition of further reagents. If the instrument has any spare solvent reservoirs, these can be filled with 0.5% DIPEA in DMF and sodium diethyldithiocarbamate (0.5% w/w) in DMF to allow automated washing of the resin following allyl deprotection.

NOTE: If the N-terminal Fmoc group is removed after cleavage of the allyl ester or if a carbodiimide is to be used to effect cyclization, then the resin should also be washed with HOBt/DMF.

Dmab



Fmoc-Asp-ODmab, Fmoc-Glu-ODmab



Fmoc-Asp(ODmab)-OH, Fmoc-Glu(ODmab)-OH

Dmab esters were developed in collaboration between Prof. B. W. Bycroft and Dr. W. Chan, of the University of Nottingham, and Novabiochem®, to provide a complement to Dde amino protection. The design of the Dmab blocking group is based on the safety-catch principle and the known propensity of *p*-aminobenzyl esters to undergo 1,6-elimination [49, 50]. The safety catch is provided by the ivDde group, which protects the amino function of the unstable *p*-aminobenzyl ester during synthesis.

Deprotection

Dmab protection is quasi-orthogonal to the Fmoc/tBu strategy, since Dmab esters are stable to 20% piperidine in DMF and to TFA, but are quantitatively cleaved with 2% hydrazine in DMF within minutes. Removal of Dmab involves a two step process: treatment with hydrazine initially removes the N-ivDde group; this is then followed by collapse of the resultant *p*-amino benzyl ester, with concurrent release of the carboxylic acid (Figure 9).

The deprotection reaction can either be carried out in a batch-wise or continuous flow manner. In the latter case, the reaction can be monitored spectrophotometrically at 290 nm by following release of the indazole by-product. Sluggish cleavage of the aminobenzyl moiety has been occasionally observed [51-54], and appears to be very sequence dependent. In these instances washing the support with 20% DIPEA in DMF/water (90:10) [51] or 5 mM sodium hydroxide in methanol [54] has been found to be efficacious. As hydrazine will remove Fmoc, assembly of the peptide backbone must be completed prior to deprotection of the Dmab side chain. The N-terminus of the peptide should be protected with Boc. This can be achieved either by direct incorporation of the N-terminal residue as a Boc protected amino acid or acylation of the free N-terminal amino group with Boc_2O.

Fmoc-Asp-ODmab has been employed to prepare a cyclic analog of pyrrhocoricin [55], a 29-mer head-to-tail cyclic peptide [56], and chlorofusin peptide [57].

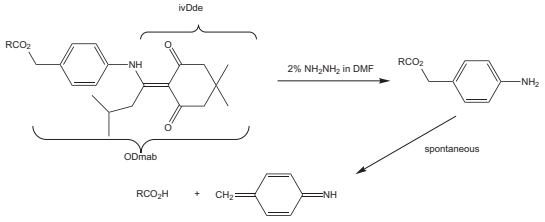


Fig. 9: Removal of Dmab.

2-PhiPr

Fmoc-Glu(O-2-PhiPr)-OH Fmoc-Asp(O-2-PhiPr)-OH

The 2-phenylisopropyl group (2-PhiPr) [58] can be removed from the side chain of Asp and Glu using 1% TFA as in the protocol given in Method The use of this protecting group in combination with Mtt on Lys or Orn provides an excellent strategy for the synthesis of side chain-to-side chain lactam bridged peptides. In contrast to Dmab and All, the 2-PhiPr group offers significant protection against aspartimide formation, which makes Fmoc-Asp(O-2-PhiPr) the derivative of choice for the synthesis of cyclic peptides that are prone to this side reaction.

Cyclic peptide synthesis

In recent years the synthesis and biological properties of cyclic peptides has attracted considerable interest. Introduction of conformational restraint through head-to-tail cyclization has become a standard strategy in medicinal chemistry for increasing the receptor affinity and selectivity of peptide ligands. Furthermore, cyclization has often been employed as a means of prolonging the duration of action of peptide hormones, since

in general cyclic peptides are more stable to proteolysis than their linear counterparts [59]. Cyclic peptides are also used as synthetic immunogens [60], as by restricting conformational flexibility, the peptide is thought to adopt a conformation which more closely mimics that of the epitope as presented on the surface of the native protein.

There are two approaches currently available for the preparation of head-to-tail cyclic peptides. The first involves cyclization of a protected fragment in solution [61], and the second, on-resin cyclization of a peptide attached *via* the side chain of an Asp or Glu residue [45-48, 49, 62]. The solid phase technique is the more straightforward, as excess reagents and by-products can be easily removed by washing of the resin. In addition, this method often gives superior results due to resin induced pseudo-dilution effects. The synthesis of cyclic peptides has been recently reviewed [63].

Peptide assembly

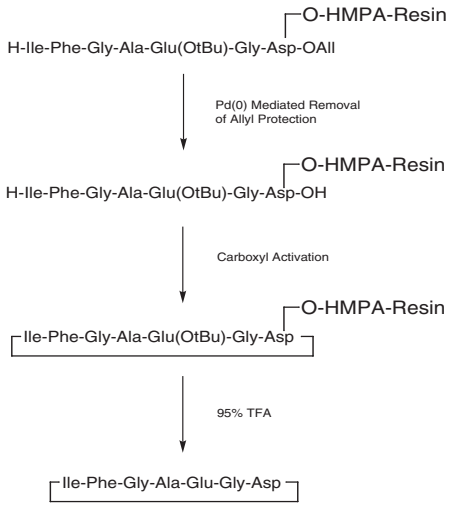


Fig. 10: Head-to-tail cyclization using a-allyl esters.

Figure 10 shows the synthesis of cyclic peptide using Fmoc amino acid allyl esters.

For the production of head-to-tail cyclic peptides, the C-terminal amino acid, either Fmoc-Asp-OX or Fmoc-Glu-OX, is attached to the resin via its side chain. If an Asn or Gln residue is required then an amide resin, such as NovaSyn® TGR, should be employed. Chain elongation can be carried out using standard Fmoc SPPS procedures.

Problems of aspartimide or piperidide formation can occur with both Dmab and allyl [666] esters of aspartic acid, involving the nitrogen of the preceding residue or linker. In the former case, this side reaction can be overcome by employing a N-Hmb protected derivative for introduction of the preceding residue [64, 66].

The yield and purity of peptides obtained by this approach are heavily influenced by the choice of solid support, with higher loading resins giving the poorest results [33]. PEG-PS composite resins, such as NovaSyn® TGA and TGR, are particularly recommended as these supports swell well in the chlorinated solvents used for allyl deprotection and the tentacle nature of the resin matrix helps minimize inter-chain oligomerization.

Cyclization

Cyclization occurs upon activation of the free resin-bound carboxy group with coupling reagents such as TBTU, PyBOP® or DIPCDI/Oxyma Pure. The reaction normally takes between 2-5 hours, and can be monitored using TNBS or ninhydrin.

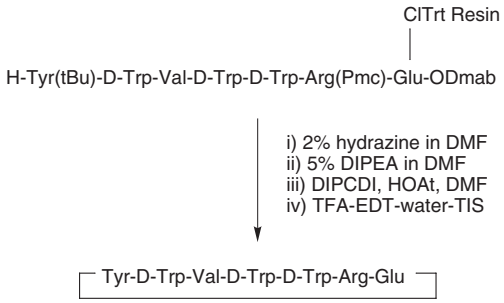


Fig. 11: Synthesis of cyclo(Tyr-D-Trp-Val-D-Trp-D-Trp-Arg-Glu) using a Dmab ester.

NOTE: An excess of uronium coupling reagents (i.e. TBTU, HBTU) should be avoided as this can result in capping of resin amino groups. Furthermore, in common with other fragment condensation reactions, epimerization may accompany activation of the carboxy-terminal residue. The extent of this side reaction will vary depending on the solvent, coupling reagent used and peptide sequence. Some experimentation may be required to optimize reaction conditions. The use of Oxyma Pure/DIPCDI activation, for this step is particularly recommended.

The strategy for using Dmab is exemplified through synthesis of a cyclic peptide (Application 4).

Related products

852072	Fmoc-Asp-OAll
852122	Fmoc-Asp(OAll)-OH
856023	Fmoc-Asp(Wang resin)-OAll
856121	Fmoc-Asp(Wang resin LL)-OAll
852079	Fmoc-Asp-ODmab
852078	Fmoc-Asp(ODmab)-OH
856123	Fmoc-Asp(Wang resin LL)-ODmab
852086	Fmoc-Asp(O-2-PhiPr)-OH
852335	Fmoc-Asp-O-2-PhiPr
852073	Fmoc-Glu-OAll
852123	Fmoc-Glu(OAll)-OH
856024	Fmoc-Glu(Wang resin)-OAll
856022	Fmoc-Glu(Wang resin)-ODmab
852077	Fmoc-Glu-ODmab
852076	Fmoc-Glu(ODmab)-OH
852117	Fmoc-Glu-O-2-PhiPr
852085	Fmoc-Glu(O-2-PhiPr)-OH

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Application 4: Synthesis of cyclo(Tyr-D-Trp-Val-D-Trp-D-Trp-Arg-Glu)

H-Tyr(tBu)-D-Trp-Val-D-Trp-D-Trp-Arg(Pmc)-Glu(O-2-ClTrt resin)-ODmab was prepared automatically using a Perseptive Millipore 9050 peptide synthesizer on 2-ClTrt resin (Figure 4-11). All acylation reactions were carried out using Fmoc-amino acids activated with HBTU (1 eq.) in the presence of DIPEA (2 eq.) and HOBt (1 eq.). Removal of Dmab was effected by flowing 2% hydrazine monohydrate in DMF through the resin bed, until no further release of indazole by-product could be detected by spectrophotometric monitoring at 290 nm. To assess the purity of the intermediate peptide prior to cyclization, a small sample of peptidyl resin was treated with 1% TFA in DCM and the partially protected peptide so released was analyzed by HPLC (Figure 4-12). The resin bound hydrazine salt of glutamic acid was then converted to a DIPEA salt by washing the remainder of the resin with 5% DIPEA in DMF. On-resin cyclization was achieved by treatment of the resin with DIPCDI (1.1 eq.) and HOAt (1.1 eq.) for 18 h. Cleavage was carried out by treatment of the peptidyl resin with TFA/TIS/water/EDT (90:1:5:4) for 2h. The crude peptide was analyzed by HPLC (Figure 13) and PD-MS (Figure 14) [expected $M+H^+$ 1107.2, found 1107.7].

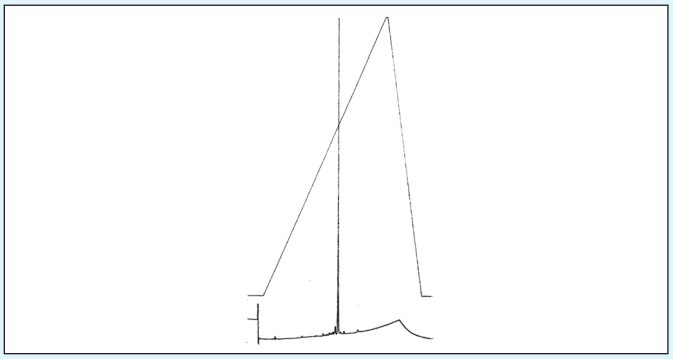

Fig. 13: HPLC elution profile of crude H-Tyr(tBu)-D-Trp-Val-D-Trp-D-Trp-Arg(Pmc)-Glu-OH.

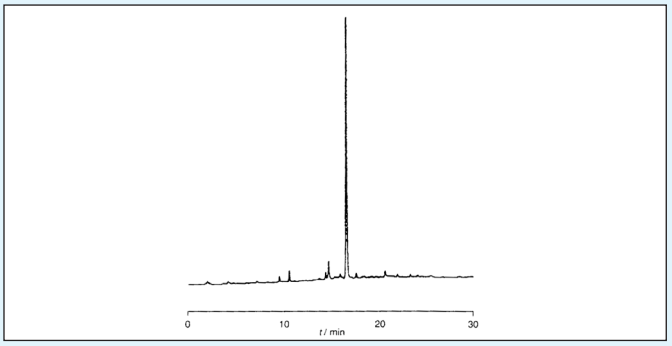

Fig. 14: HPLC elution profile of crude cyclo(Tyr-D-Trp-Val-D-Trp-D-Trp-Arg-Glu).

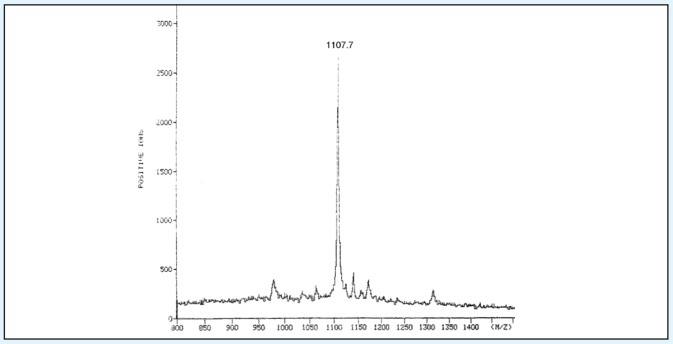

Fig. 15: PD-MS of total crude peptide.

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