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*Rapid communication*

# Multiple release of equimolar amounts of peptides from a polymeric carrier using orthogonal linkage-cleavage chemistry

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Peptides generated on polymeric beads that are attached to the support via cleavable linkers are released into solution in equimolar amounts in several independent steps and screening of the "peptide libraries" can be performed in the usual manner used for testing peptides in solution.

*Key words:* peptide libraries; multicleavable linkers; independently cleavable linkers; solid phase synthesis

Rapid methods for discovering novel peptide ligands as described recently (1–4) are based on building peptide libraries consisting of $10^6$ or more different peptides (1–5). Synthesis of completely random peptide libraries bound to polymeric solid supports (beads) and its screening in an ELISA type or immunofluorescence assays is the basis for the recently reported "Selectide" technology (3). In this approach, every bead of solid support contains a unique peptide, the structure of which is determined only after a particular bead is identified as positive in a color reaction. To apply the principle of a completely randomized library (versus a partially fixed library (4)) for screening in solution, it is necessary to release a defined portion of the peptide from each bead into solution for testing. To determine the structure of peptide responsible for biological activity by screening a library of millions of peptides, equal amounts of a peptide must be released repeatably from a single bead and still a portion of it must be retained bound to a polymeric carrier for sequencing. Using such approaches, reactions around beads immobilized in soft agar can be studied, or the libraries can be repeatably subdivided into multiple wells to identify released peptides with a desired biological activity.

In principle there are several ways to cleave part of the peptide from a solid carrier. Specifically, we were interested to apply independent methods which use cleavage that would not affect the remaining attached peptides. To this end we have tested the possibility of five levels of cleavability from the carrier in a model peptide (leucine-enkephalin). The structure of the peptide-resin construct is given in Fig. 1. We used combinations of two different protecting groups and two mechanisms of cleavage together with a photocleavable linker for the stepwise release of peptides for biological testing, and a so-called "safety catch" (SCAL) linkage (6) for attachment of the residual peptide used for sequencing. SCAL anchoring is completely stable to both piperidine and TFA (Fmoc and Boc chemistry) and can therefore be used as a stable attachment both in synthesis and side chain deprotection. However, after

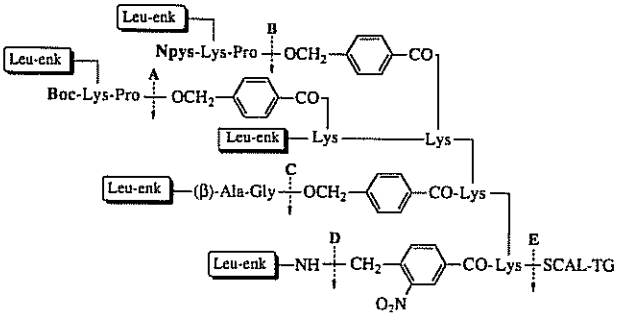


FIGURE 1

Structure of peptide-resin construct allowing four or five levels of cleavage of model peptide (Leucine-enkephalin, Tyr-Gly-Gly-Phe-Leu). Points of cleavage are marked A–E. Note that the released peptides in steps B–E have different *C*-termini, and identical peptides released in steps A and B contain at the *C*-terminus the diketopiperazine structure. TG stands for Tentagel.

reduction of the sulfoxide groups of this linker, it becomes extremely labile and can be cleaved by trifluoroacetic acid, producing a *C*-terminal amide. Two different protecting groups were also used in combination with the diketopiperazine (DKP) approach suggested and used for peptide release from Geysen's polyethylene pins (7). In combination with the hydroxymethylbenzoic acid type linker (8, 9) and the photocleavable linker (10) this gave us five levels of cleavability. We employed a combination of Boc and Npys (11) protecting groups. Under conditions of Boc cleavage (Fig. 1, see "A"), which has to be performed first since we are using this type of protection for functional groups on side chains, the Npys group was described to be stable and therefore the peptide fraction with this protection should not be influenced. However, we observed that the Npys group was partially cleaved under the conditions used for cleavage of Boc groups (50% TFA/DCM, 30 min), and therefore more than an equimolar amount of peptide was released in this step. In the second step the Npys group was cleaved selectively* (Fig. 1, "B") and the second part of the peptide was released (in less then equimolar amount). The third portion of the peptide was released by sodium hydroxide solution (Fig. 1, "C"). The fourth portion was released from the photocleavable linker (Fig. 1, "D") by the application of UV light. Although UV light release gave us a reasonable amount of the peptide, HPLC evaluation of released product showed that it contains an unacceptably high level of impurities**. The remaining peptide attached through the "safety catch" linkage (Fig. 1, "E") was either used for sequencing from the solid support or it was released after the reduction of the sulfoxide moieties by the application of conditions originally used for the removal of Boc groups and sequenced as a free peptide.

The above described quadruply cleavable construct showed that two mechanisms of release can be used with confidence, diketopiperazine formation and alkaline hydrolysis. An obvious disadvantage of the example given above is the fact that the released peptides have different *C*-terminal structures. Therefore, we have designed an alternative attachment of a peptide to a polymeric bead allowing the release of peptide having the same carboxy-terminus in both levels of the release. The principle of these "exclusive" linkers (in contrast to the linkers described above) is illustrated in Fig. 2.

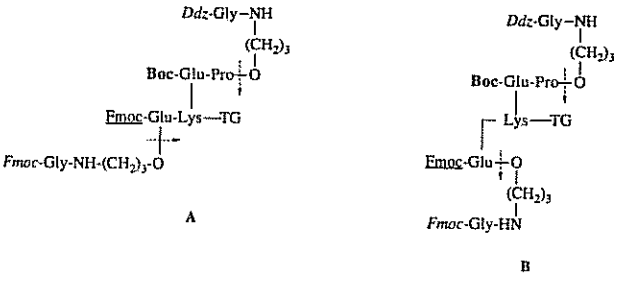


FIGURE 2

Structures of linker-resin assemblies allowing the release of identical peptides in two steps.

The dipeptide designed to form a diketopiperazine (DKP) is composed of Glu-Pro: the $\gamma$-carboxyl group of glutamic acid serves as the attachment site of the linker to the solid support (via branching Lys), Pro is known to accelerate DKP formation dramatically. The peptide is linked to Pro via an ester bond of the OH group of Ddz-Gly-NH-$(CH_2)_3$-OH. The $N^{\alpha}$ group of Glu is protected by a Boc group that is removed simultaneously with side-chain protecting groups on peptides. At this moment the internal nucleophile necessary for DKP formation loses its protecting group and becomes protonated, yet DKP formation is not possible until the pH is brought to ca. 7 or higher.

The second (hydrolytically cleavable) arm is composed of glutamic acid, its $\alpha$-carboxyl is engaged in linking to the support. The $\gamma$-carboxyl group bears Fmoc-Gly-NH-$(CH_2)_3$-OH attached via an ester bond as in the first arm (Fig. 2A). The reverse attachment of Glu (Fig. 2B) was found not to be completely satisfac-

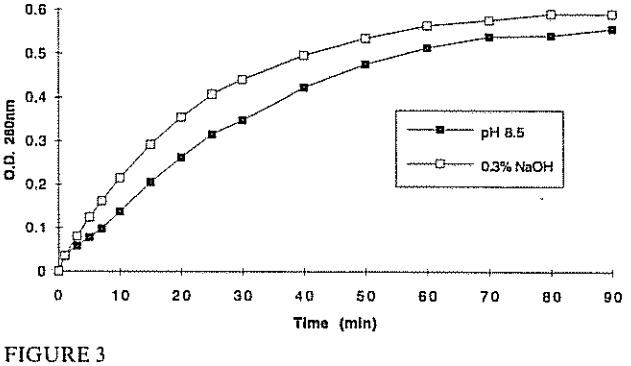


FIGURE 3

Time dependence of the release of Leu-enkephalin from a doubly cleavable linker-resin assembly. First step: 0.1 M buffer pH 8.5; second step: 0.3% NaOH (values corrected for different extinction coefficient of Tyr under these conditions).

* We examined three reagents to accomplish complete and mild deprotection of Npys group. With 0.1 M $PPh_3$/pyridine·HCl/DMF partial release of peptide from resin, probably due to the insufficient protonation of an $\alpha$-amino group, was observed. Unfortunately, with 3.5 M HCl/dioxane reduction of sulfoxide groups of SCAL linkage followed by cleavage from resin is a serious side-reaction. A system consisting of 0.1 M $PPh_3$/TsOH/DMF was found to be the best one since this reagent does not reduce sulfoxides and ensures complete protonation of an amino group.

** Photolytic cleavage experiments were performed at 30 °C for 5 h with 365 nm light on peptide-linker-resin suspended in: 1) trifluoroethanol-dichloromethane (3:7, v/v); 2) trifluoroethanol-dichloromethane-aqueous ammonia (5:14:1, v/v/v); 3) HEPES buffer pH 8.5, or in solid state. Composition of the crude material was almost independent of conditions used for cleavage.

tory since the second arm released by alkali was partially lost during peptide synthesis due to diketopiperazine formation after the first coupling and deprotection. The attachment of a hydrolytically cleavable arm via a α-carboxyl group eliminates this problem. The $N^{\alpha}$ amino group of Glu is protected by an Fmoc group and the third copy (noncleavable part) of the peptide is assembled on this amino group.

Synthesis of a doubly cleavable peptide then begins with elimination of Ddz (12) (2% TFA in $CH_2Cl_2$) and Fmoc groups. Both these cleavages are easily quantifiable and provided (together with the readings from Fmoc release in the next synthetic steps) a good basis for evaluating the quality of the assembled linker construction.

A model of a doubly cleavable library of pentapeptides – Leu-enkephalin bound to two different types of linkers and to a non-cleavable part – was synthesized and the two-step cleavage kinetics was followed (Fig. 3). In the first step diketopiperazine formation in buffer pH 8.5 was used for the release, in the second step hydrolysis in 0.3% NaOH was applied. One bead after the release was recovered and subjected to sequencing in an automatic sequencer, giving the expected sequence. The screening of a library showing applicability of the described technology in a specific molecular target recognition will be published elsewhere (12).

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