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Note

Alcalase-catalyzed Synthesis of Novel 2,6-Dimethoxyhydroquinone-3-mercaptoacetyl-peptide Conjugates

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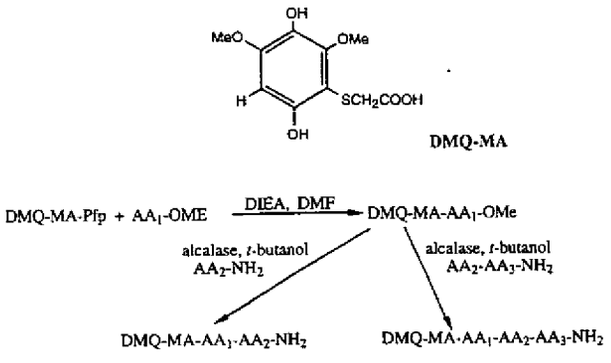
Seven dipeptide and one tripeptide conjugates of the cytotoxic drug 2,6-dimethoxyhydroquinone-3-mercaptoacetic acid (DMQ-MA) conjugates were prepared successfully by an alcalase-catalyzed reaction in alcohols, using DMQ-MA-X-OMe as the acyl donor and H_2N-Y-$CONH_2$ as the nucleophile (where X and Y are amino acids, and amino acid/dipeptides, respectively).

2,6-Dimethoxyhydroquinone-3-mercaptoacetic acid (DMQ-MA)[1-3] is a synthetic derivative of 2,6-dimethoxy-*p*-benzoquinone(DMQ)[4] which is a natural fermented product of wheat germ with significant antitumor activity under the synergistic activation of L-ascorbic acid as reported by Szent-Gyorgi.[5] Owing to the very low aqueous solubility of DMQ, which is an apparent disadvantage for the development as a vaccine, we prepared the thioglycolic acid derivative DMQ-MA.[1,2] A number of DMQ-MA congeners such as amino acid conjugates[2] and cyclic peptide conjugates[3] were synthesized by conventional chemical methods. Many of these conjugates displayed enhanced *in vitro* antitumor activities as compared to the parent compound DMQ-MA.[1-3]

A number of DMQ-MA derivatives of dipeptide conjugates were synthesized by reacting the dipeptides with the pentafluorophenyl ester of DMQ-MA in DMF. Owing to purification problems resulting from numerous side products that occurred during the coupling reaction, we investigated the possibility of employing an enzyme-catalyzed reaction to replace conventional chemical synthesis. Industrial alcalase is an inexpensive enzyme and the major active component is subtilisin Carlsberg which has a broad selectivity for the acyl donor and nucleophile.[6-8] Thus, we envisaged that a DMQ-MA-amino acid methyl ester can serve as an acyl donor whereas another amino acid or peptide with its *C*-terminal carboxyl group protected as an amide can act as a nucleophile. To test this hypothesis, we prepared a number of DMQ-MA-amino acid methyl esters and reacted these with different amino acid amides under the catalysis of alcalase. This study also served to probe the stereochemical requirements of the acyl donor and nucleophile in the synthesis of 2,6-dimethoxyhydroquinone-2-mercaptoacetyl peptides under the catalysis of alcalase.

The strategy for the synthesis of the DMQ-MA-peptide conjugates is shown in Scheme I. DMQ-MA-amino acid methyl esters were prepared by reacting the corresponding amino acid methyl ester with the pentafluorophenyl ester of DMQ-MA. The DMQ-MA-amino acid methyl esters were then employed as acyl donors and reacted with an amino acid amide or a dipeptide with its *C*-terminal protected as an amide under the catalysis of industrial alcalase in *t*-butanol.

Scheme I



In the synthesis of the DMQ-MA-peptide conjugates, the procedure is difficult because the 2,6-dimethoxyhydroquinone-3-mercaptoacetyl moiety is susceptible to minute amounts of water in the reaction medium, and gradually decomposed by the Michael retrograde reaction *in situ*. Thus, the kinetically controlled approach[8] was employed in the syntheses by monitoring the reaction with t.l.c. In general, the reaction time was limited to 1.5-2.0 h to minimize enzyme-catalyzed peptide bond cleavage and decomposition of the DMQ-MA moiety. Since water bound to alcalase can compete with the nucleophile in the alcalase-catalyzed peptide bond formation, water was removed from industrial al-

calase by washing with absolute ethanol promptly before use as previously described.[8] To minimize side reactions, industrial alcalase was purified by dialysis against phosphate buffer saline (pH 6.2) overnight and used immediately after preparation. For a typical reaction, 0.2 mmol of DMQ-MA-Ala-OMe and leucinamide (Leu-NH_2) were dissolved in *t*-butanol (3 mL) and dehydrated alcalase (prepared from 5 mL of dialyzed alcalase) was added and the mixture allowed to react at 30 °C for 2 hours. The mixture was filtered, lyophilized, and the product purified by silica gel column chromatography.

T.l.c. showed that the crude side reaction products of alcalase-catalyzed syntheses before column chromatography were much less than those by conventional chemical syntheses, and thus facilitated subsequent purification. This benefit is attributed to the proximity and orientation effects typical of enzymatic reactions. In addition, the time required by alcalase-catalyzed reactions ranged from 1.5-2.0 hours, which is significantly less than that by chemical coupling (2.0-4.0 hours). Capital investment can also be kept down for alcalase-catalyzed reactions since industrial alcalase is much less expensive than the pentafluorophenol (Pfp) and carbodiimides commonly used in chemical coupling. However, the yields from alcalase-catalyzed syntheses were in general less than those by chemical coupling.

The data in Table 1 indicate that the steric effect of the nucleophile is important in determining whether the alcalase-catalyzed coupling reaction will be successful. For example, the acyl donor DMQ-MA-Ala-OMe coupled successfully with nucleophiles such as Gly-NH_2, Ala-NH_2, Leu-NH_2, and Gly-Leu-NH_2, but failed to couple with Ala-Phe-NH_2, in which Ala has a more bulky side chain than the glycyl residue. Besides, the van der Waals radius of Ala-Phe-NH_2 is greater than those of the other nucleophiles. This may be attributed to the suggestion that the S_1' site of

Table 2. Physicochemical Properties of Various DMQ-MA-Peptide Conjugates Synthesized *via* Alcalase-catalysis

Compound	$[\alpha]_D^{29}$	M.p.°C	HRMS[MH^+]
DMQ-MA-Gly-Gly-NH_2		153-154	374.1028
DMQ-MA-Gly-Ala-NH_2	-27.3	134-135	388.1113
DMQ-MA-Ala-Gly-NH_2	-31.8	134-135	388.00(LRMS)
DMQ-MA-Phe-Gly-NH_2	25.32	142-143	464.1458
DMQ-MA-Ala-Ala-NH_2	-20.4	125-126	402.1332
DMQ-MA-Ala-Leu-NH_2	-16.4	150-152	444.1809
DMQ-MA-Phe-Ala-NH_2	-18.7	137-138	478.1662
DMQ-MA-Ala-Gly-Leu-NH_2	-22.6	148-150	501.2025

Abbreviations: HRMS: molecular ion from high resolution mass spectra (FAB). LRMS: molecular ion from low resolution mass spectra (FAB).

subtilisin Carlsberg has a rather tight steric environment.[9] In addition, the affinity of the acyl donor to the S_1 site of the enzyme is also important since the nucleophile Gly-Leu-NH_2 failed to react with the acyl donors DMQ-MA-Gly-OMe and DMQ-MA-Phe-OMe, despite the fact that the glycyl residue is less bulky than the alanyl residue. It is noteworthy that the acyl donor DMQ-MA-Tyr-OMe failed to react with all of the nucleophiles listed in Table 1, suggesting that steric effect or ionization of the tyrosyl phenolic group was involved (compare to the fact that the acyl donor DMQ-MA-Phe-OMe could react satisfactorily with two nucleophiles listed in Table 1).

The present results suggest that the synthetic strategy shown in Scheme I can be used to prepare novel drug-peptide conjugates with few side products and in moderate yields under the catalysis of alcalase. This methodology should be of value in the preparation of other peptide-drug conjugates, particularly if the drug moiety is sensitive during conventional chemical coupling resulting in purification problems. The lower capital investment in industrial appli-

Table 1. Synthetic Yields (%) of Various DMQ-MA-Peptide Conjugates *via* Alcalase Catalysis and Chemical Methods

	Nucleophile									
	Gly-NH_2		Ala-NH_2		Leu-NH_2		Gly-Leu-NH_2		Gly-Ala-NH_2	
Acyl donor	E	C	E	C	E	C	E	C	E	C
DMQ-MA-Gly-OMe	30	54	31	62	-	-	0[a]	-	0	-
DMQ-MA-Ala-OMe	43	40	38	54	48	64	43	66	0	-
DMQ-MA-Phe-OMe	36	-	54	-	-	-	0	-	0	-
DMQ-MA-Tyr-OMe	0	-	0	-	-	-	0	-	0	-

E: synthesized by alcalase-catalyzed reactions.

C: synthesized by conventional chemical methods (coupling *via* active esters).

- Reaction not performed.

[a] Trace amount of products were detected by T.L.C. for all these reactions but could not be isolated by silica gel chromatography.

cations is an additional advantage.

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Key Words

Alcalase-catalyzed syntheses; Peptide conjugates.

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