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Activation of D-Asparagine and D-Glutamine Derivatives Using the Mitsunobu Reaction

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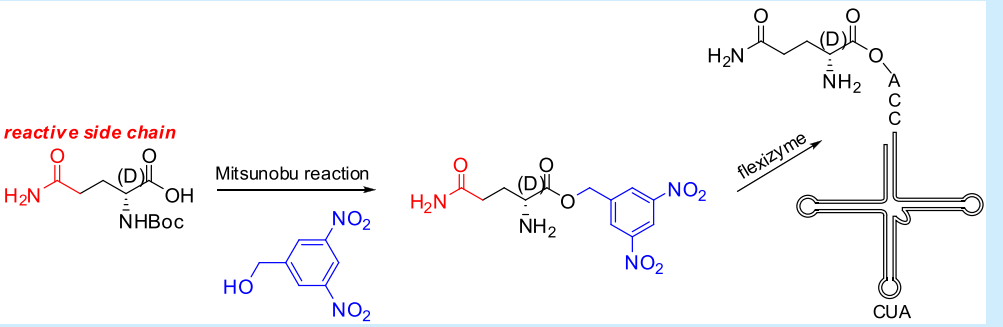

ABSTRACT: Seven D-amino acid derivatives having reactive side chains have been activated to afford their respective 3,5-dinitrobenzyl esters using the Mitsunobu reaction. This esterification was found to be difficult using traditional methods involving 3,5-dinitrobenzyl chloride under alkaline conditions. The conversion of a tRNA to the respective D-glutaminyl-tRNA using D-glutamine 3,5-dinitrobenzyl ester was catalyzed by a flexizyme, followed by purification to remove all the unacylated tRNAs and other byproducts. Both D- and L-glutamine were incorporated from their aminoacyl-tRNAs into a model peptide structurally related to IFN-β.

The incorporation of D-amino acids into peptides and proteins holds the promise of creating more diverse polypeptide structures and has attracted significant research attention for decades.[1] One strategy has involved the use of chemically misacylated tRNAs prepared by the condensation of *E. coli* $tRNA^{Phe}$ lacking cytidine-75 and adenosine-76 with chemically acylated derivatives of p(d)CpA via the agency of T4 RNA ligase.[2] Such misacylated tRNAs have been used to incorporate many noncanonical amino acids into proteins, but initial efforts documented quite low incorporation of D-amino acids.[1g−i]

Our laboratory demonstrated that D-Met and D-Phe could be incorporated into DHFR from the activated suppressor tRNAs using an *E. coli* S-30 system containing modified ribosomes in yields of 23% and 12%, respectively, compared to 5% and 3%, respectively, in a native *E. coli* cell free system.[3] More recently, the development of ribozymes (flexizymes) capable of acylating tRNAs with a variety of amino acids has enabled the preparation of noncanonical aminoacyl-tRNAs using chemically activated amino acid esters.[4] Using tRNAs activated with D-amino acids by flexizymes in a system reconstituted from purified components provided further improved yields of modified peptides, including the introduction of multiple D-amino acids into a single polypeptide.[4d,e]

Both strategies employed for tRNA aminoacylation are dependent on the use of chemically activated amino acids in the form of cyanomethyl esters or 3,5-dinitrobenzyl esters. Accordingly, chemical methods for amino acid esterification are of considerable interest in this regard.

Traditional synthetic methods for preparing these types of activated amino acid esters involve the use of chloroacetonitrile and 3,5-dinitrobenzyl chloride under alkaline conditions (e.g., as provided by Et_3N, Scheme 1), and these generally work well for amino acids with unreactive side chains.[5] However, only a low yield of activated amino acid was isolated when D-asparagine was employed under alkaline reaction conditions (Scheme 1), presumably due to competing intramolecular participation of the reactive side chain.[6] In fact, a γ-amino acid dinitrobenzyl ester was found to function poorly in the flexizyme-mediated acylation of a minihelix RNA due to the rapid intramolecular cyclization between the amino group and the activated carboxyl group of the esterified amino acid.[7] Thus, the efficient activation of noncanonical amino acids under mild and neutral reaction conditions has become of

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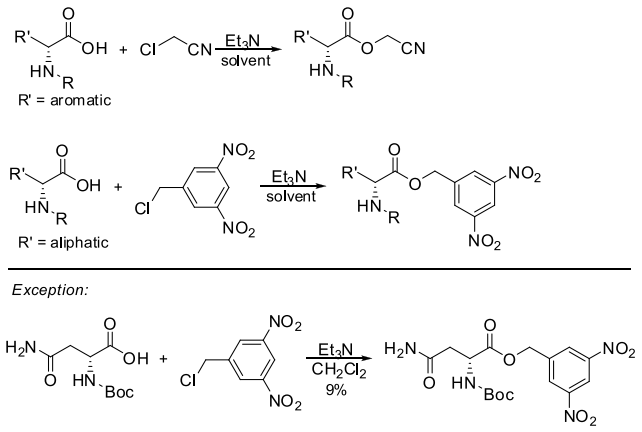
Scheme 1. Traditional Esterification Methods for Activating D-Amino Acids



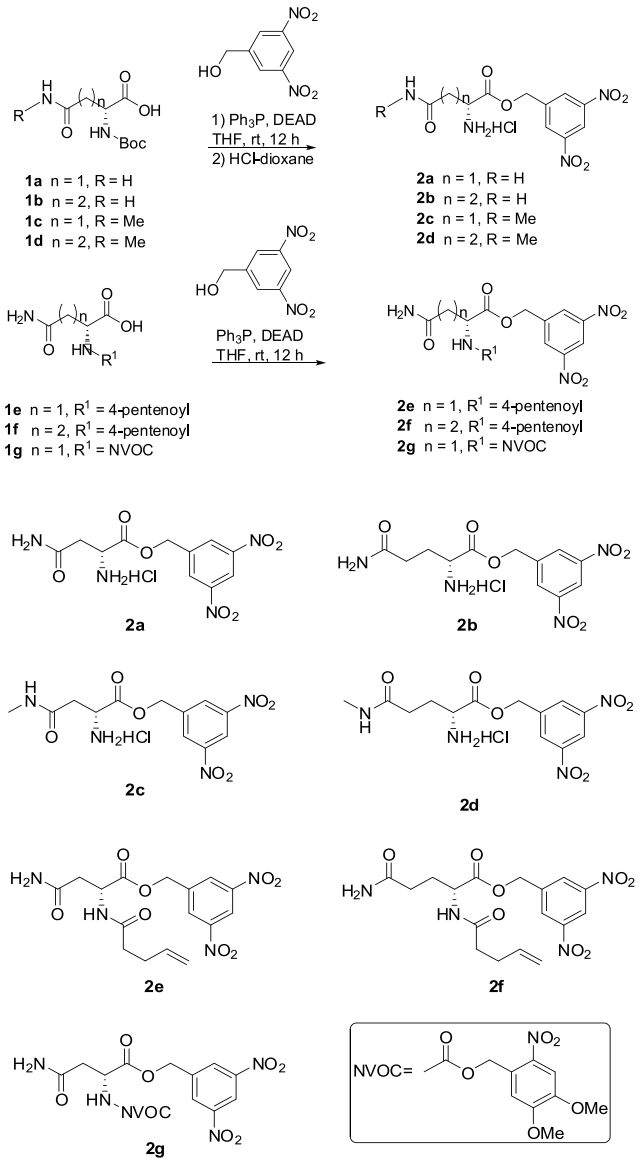
great practical importance to the successful *in vitro* biochemical synthesis of structurally diverse peptides and proteins.

The well-known Mitsunobu reaction enables the conversion of primary and secondary alcohols to amines, esters, ethers, and thioethers, which proceed with inversion of configuration at the potentially chiral carbon atom.[8] The phosphine-mediated, S_N2-type substitution mechanism has been described in detail by Hughes.[9] Applications in peptide and protein chemistry include transformations of hydroxy amino acids, the synthesis of *N*-alkylated amino acid derivatives, and the synthesis of free or protected amino acids.[10] α-Carboxylic acids were converted to *N*-acyloxyphthalimides or *N*-acyloxysuccinimides using the Mitsunobu reaction to activate −COOH during peptide synthesis.[11] In comparison, only a single example has been described for the *p*-nitrobenzyl esterification of D-asparagine using a Mitsunobu reaction.[12] This provides a potential strategy for the general synthesis of amino acid 3,5-dinitrobenzyl esters, which could be used directly as the chemical precursor in flexizyme-catalyzed aminoacyl-tRNA formation. Herein, we report the efficient 3,5-dinitrobenzyl esterification of D-Asn and D-Gln and representative derivatives by the Mitsunobu reaction, enabling the preparation of D-aminoacyl-tRNAs by flexizyme dFx.[4]

The esterification of selected *N*-Boc-D-amino acids was carried out by treatment with triphenyl phosphine (Ph_3P) and diethyl azodicarboxylate (DEAD) in the presence of 3,5-dinitrobenzyl alcohol in anhydrous THF under a nitrogen atmosphere at room temperature. Several substituted D-amino acids were selected for activation as their 3,5-dinitrobenzyl esters (Scheme 2). Starting with the simplest structures, *N*-Boc-D-asparagine (**1a**) and *N*-Boc-D-glutamine (**1b**) were converted to their respective 3,5-dinitrobenzyl esters, and the crude products were isolated by extractive workup. The final activated products (**2a**, 85% yield, and **2b**, 74% yield; Table 1) were obtained as their hydrochloride salts following Boc group deprotection in 4 M HCl in dioxane at room temperature for 30 min. D-Asn and D-Gln derivatives having *N*-methylated side chains (**1c** and **1d**, respectively) were also converted to their 3,5-dinitrobenzyl esters using the same Mitsunobu conditions; following Boc group removal with 4 M HCl in dioxane, the hydrochloride salts were isolated as colorless solids (**2c**, 78% yield, and **2d**, 84% yield; Table 1).

Misacylated tRNAs are also prepared with protecting groups on N^{α} of the aminoacyl moiety attached to the 3′-end of

Scheme 2. 3,5-Dinitrobenzyl Esterification of Selected D-Amino Acids Utilizing the Mitsunobu Reaction[a]



[a]Mitsunobu conditions: **1** (0.1 M), 1.1 equiv of 3,5-dinitrobenzyl alcohol, 1.5 equiv of Ph_3P, and 1.1 equiv of DEAD in anhydrous THF, at room temperature, stirred for 24 h under N_2.

tRNA.[13,14] These can facilitate tRNA purification and stabilize the activated tRNA, thus permitting storage until the material is employed experimentally. The 4-pentenoyl[13] and NVOC[14] groups have been used extensively as aminoacyl-tRNA protecting groups following activated tRNA preparation using T4 RNA ligase and can be removed easily in aqueous iodine solution and by UV irradiation, respectively. Since these groups may well prove to be compatible with flexizyme-mediated tRNA activation, we also explored the activation of representative *N*-protected derivatives. Accordingly, esterification of *N*-pentenoylated D-asparagine (**1e**) and D-glutamine (**1f**), as well as NVOC-protected D-asparagine (**1g**), was investigated in an effort to prepare their N^{α}-protected 3,5-dinitrobenzyl esters. As shown in Scheme 2 and Table 1, these

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Table 1. Comparison of Traditional Methods and the Mitsunobu Reaction in 3,5-Dinitrobenzyl Esterification of D-Amino Acids

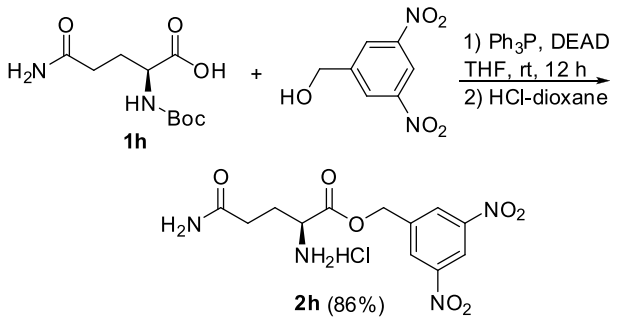
entry	D-amino acid	esterified product	yield of traditional method (%)[a]	yield of Mitsunobu reaction (%)[b]
1	**la**	**2a**	9	85
2	**lb**	**2b**	7	74
3	**lc**	**2c**	<5	78
4	**Id**	**2d**	<5	84
i	**le**	**2e**	<5	56
6	**If**	**2f**	<5	53
7	**lg**	**2g**	<5	48

[a]Traditional method conditions: **1** (0.10 mmol), 1.2 equiv of 3,5-dinitrobenzyl chloride, 2.0 equiv of Et_3N in 200 μL of anhydrous CH_2Cl_2, room temperature, stirred for 72 h under N_2. [b]Isolated yield.

three derivatives could all be converted to their respective 3,5-dinitrobenzyl esters in yields ranging from 48 to 56%.

Also prepared was the 3,5-dinitrobenzyl ester of L-glutamine. As shown in Scheme 3, this was accomplished in analogy with

Scheme 3. 3,5-Dinitrobenzyl Esterification of L-Glutamine Utilizing the Mitsunobu Reaction



the synthesis of D-glutamine 3,5-dinitrobenzyl ester. The yield of L-glutamine 3,5-dinitrobenzyl ester (**2h**) was 86%, not dissimilar to the 74% yield obtained for D-isomer **2b** (Table 1).

In order to assess the effectiveness of the Mitsunobu reaction for activation of D-amino acids with reactive side chains, all of the D-amino acids employed in the Mitsunobu reaction were also tested by a more traditional method as well. The yields of products **2a** and **2b** obtained by the traditional method were 9% and 7%, respectively, while those prepared by the Mitsunobu reaction were 85% and 74%, respectively (Table 1, entries 1 and 2). For the synthesis of side chain methylated products **2c** and **2d**, the Mitsunobu reaction provided the esters of D-amino acids in 78% and 84% yields, while only poor yields (<5%) resulted from use of the traditional method (Table 1, entries 3 and 4). Products **2e**, **2f**, and **2g** were synthesized in moderate and comparable yields using the Mitsunobu reaction, while only trace amounts of **2e**, **2f**, and **2g** were obtained using the traditional method (Table 1, entries 5−7).

Finally, to demonstrate the utility of the activated esters reported here in preparing activated tRNAs, D- and L-glutamine 3,5-dintrobenzyl esters (**2b** and **2h**) were used in the presence of flexizyme dFx to prepare D- and L-glutaminyl-$tRNA_{CUA}$s (Figure 1). The reaction mixture contained flexizyme dFx and $tRNA^{Phe}$ at 25 μM final concentrations and glutamine 3,5-dinitrobenzyl ester at a 5 mM final concentration. After incubation on ice for 3 h, the crude product was purified by the

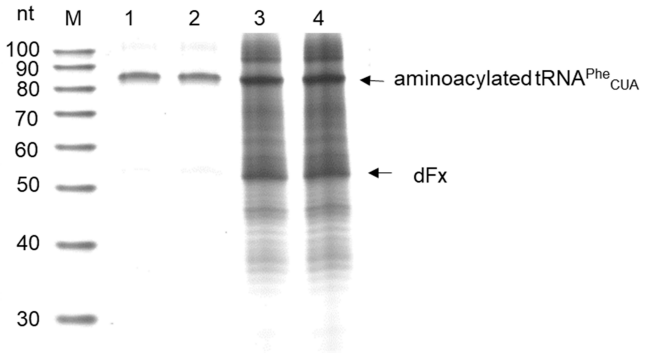


Figure 1. Scanned image of a 15% acid polyacrylamide electrophoretic gel (125 V, 2 h) demonstrating D- and L-glutaminyl-tRNAs formed using a flexizyme (dFx) reaction and purified using *m*-aminophenylboronic acid agarose resin. Lane 1: purified D-glutaminyl-$tRNA^{Phe}$. Lane 2: purified L-glutaminyl-$tRNA^{Phe}$. Lane 3: untreated flexizyme reaction mixture containing D-glutaminyl-$tRNA^{Phe}$. Lane 4: untreated flexizyme reaction mixture containing L-glutaminyl-$tRNA^{Phe}$. Lane M: oligonucleotide ladder.

use of *m*-aminophenylboronic acid agarose beads, which sequestered unreacted tRNA as well as the flexizyme. As is clear from the figure, the aminoacyl-tRNAs purified with the agarose beads were largely homogeneous.

The purified D- and L-glutaminyl-$tRNA_{CUA}$s were used in a protein synthesis experiment in the presence of a commercial reconstituted protein-synthesizing system (PURExpress system, New England Biolabs) to incorporate both amino acids into position 3 of an 18-amino-acid peptide structurally related to part of murine interferon β. As shown in Figure 2, both

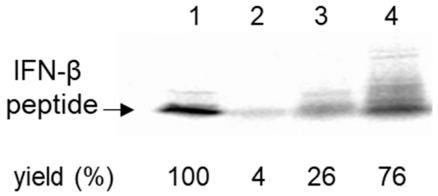


Figure 2. Scanned image of a 20% SDS polyacrylamide electrophoretic gel analysis of the *in vitro* translation of an interferon-β peptide containing D- or L-glutamine at position 3, formed by suppression of a UAG codon in the mRNA using the PURExpress system. Lane 1: unmodified IFN-β peptide. Lane 2: negative control prepared without misacylated suppressor tRNA. Lane 3: IFN-β peptide with D-glutamine in position 3. Lane 4: IFN-β peptide with L-glutamine in position 3.

enantiomers of glutamine were incorporated, although the natural L-isomer was incorporated more efficiently. The observation was consistent with the reports that D-amino acids can be incorporated into peptides using a reconstituted system.[4c−e]

In conclusion, D-amino acids with reactive amide side chains could be converted to their respective 3,5-dinitrobenzyl esters in good yields using the Mitsunobu reaction. The resulting D-amino acid 3,5-dinitrobenzyl esters should be of utility as the activated amino acid source for the flexizyme-catalyzed activation of suppressor tRNA transcripts, as illustrated for a D-glutaminyl-tRNA. This tRNA activation was performed at pH 8.8, achieving good activation efficiency. Moreover, by the use of *m*-aminophenylboronic acid agarose beads, it was

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possible to remove unactivated tRNA and flexizyme dFx from the sample containing the activated D-glutaminyl-tRNA.

■ ASSOCIATED CONTENT

Data Availability Statement

The data underlying this study are available in the published article and its online Supporting Information.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.orglett.3c00232.

Detailed experimental procedures, characterization data for novel compounds, and copies of NMR spectra (PDF)

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Notes

The authors declare the following competing financial interest(s): As indicated in the manuscript, Sidney Hecht is a consultant for Ionis Pharmaceuticals.

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