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Cryochemistry: Freezing Effect on Peptide Coupling in Different Organic Solutions

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Abstract: The freezing effect on peptide coupling in organic solutions of different polarity has been investigated and compared with the results obtained in liquid phase. The model reaction of DCC-activated coupling of Boc-Ala-Phe-OH with H-Ala-OBu^t has been carried out in dioxane, dimethylsulfoxide and formamide, as well as in mixtures (90%/10%, v/v) of dioxane with acetonitrile, dimethylformamide, dimethylsulfoxide and formamide.

The reactions have been traced and evaluated by RP-HPLC analysis. Freezing the reaction mixture resulted in all cases in a significant suppression of the *N*-dipeptidylurea side-product formation together with a slight decrease of tripeptide epimerization. The coupling yields and the side effects depended on the solvent, with the dioxane and dioxane/acetonitrile mixture produced the best results. The role of freezing and solvent in the improved results is discussed. © 1998 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: Cryochemistry; frozen organic solution; peptide coupling

INTRODUCTION

Rate and yield enhancements of quite different chemical and biochemical reactions have been described as running the procedures in frozen aqueous solution. This phenomenon is based mainly on the freeze-concentration of reactants in the diminished liquid phase of unfrozen micro-inclusions [1–3]. Although this type of reaction in ice has been demonstrated in several instances [1–6], there are only few examples of reactions proceeding in *frozen organic solution* [1,2,7]. Nevertheless, this possibility of running reactions in the smooth conditions of frozen organic solutions is a great challenge. Recently, the formation of peptide bonds in frozen dioxane solution at -18°C has been realized in our laboratory [8]. This new approach to peptide coupling included a 4-nitrophenyl active ester-mediated synthesis of dipeptides in dioxane. Because most of the protected peptides are soluble only in organic solvents, it seemed important to broaden the range of solvents.

In order to acquire further information on the applicability of our method, i.e. to elucidate the effect of freezing on the yield and on the conservation of chemical and chiral homogeneity of the peptide product, we present here a DCC-activated coupling of Boc-Ala-Phe-OH dipeptide with H-Ala-OBu^t at $+22$°C (liquid) and -18°C (frozen) temperature, respectively, in solvents of different polarity. The highly reactive carbodiimide coupling has been chosen for testing the tripeptide yield, epimerization and the extent of *N*-dipeptidylurea side-product formation. The results have been evaluated by RP-HPLC technique, because the peaks of LLL vs. LDL tripeptide epimers could be resolved without any difficulty by using a C_{18} column and mixture of ACN

Abbreviations: ACN, acetonitrile; DCC, dicylohexylcarbodiimide; DCU, dicyclohexylurea; DMF, dimethylformamide; DOX, 1,4-dioxane; DSO, dimethylsulfoxide; FMD, formamide.

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and TFA (0.1%, v/v) eluents [9,10]. In addition to our cryo-method of chemical peptide coupling in frozen organic solutions, several successful enzyme-catalyzed peptide syntheses in ice have been published recently [2]. In most cases a yield-increasing effect of freezing could be detected in these serine and cysteine protease-catalysed peptide couplings.

MATERIALS AND METHODS

Chemicals

All chemicals were of reagent grade. The solvents and the HC1.H-Ala-OBu^t derivative were purchased from Merck, Carlo-Erba, Italy, and Reanal, Hungary, respectively.

The reference peptides for RP-HPLC analysis were synthesized with solution chemistry in a range of 0.4–0.8 mmol. The coupling of both dipeptide diastereoisomers was performed by reaction of Boc-Ala-OSu with H-Phe-O$^-$. The synthesis of tripetide epimers was carried out by DCC-mediated coupling of Boc-Ala-Phe-OH with H-Ala-OBu^t in the presence of HOBt. All crude products were recrystallized and their purity was checked by TLC and RP-HPLC. The N-Boc-Ala-Phe-urea reference compound was obtained by the reaction of Boc-Ala-Phe-OH with DCC in the absence of the amine nucleophile. The crude product was then analysed by RP-HPLC without isolation and the chromatogram showed only some unreacted dipeptide (identified with the authentic sample) in addition to the dominating *N*-acylurea peak.

Procedure

All reactions were performed in 1.5 ml polypropylene centrifuge tubes in a total volume of 180–300 μl. Stock solutions of 150 mM Boc-Ala-Phe-OH, 150 mM H-Ala-OBu^t and 225 mM DCC were prepared immediately before the experiments in the given solvent or dioxane/solvent mixture (90%/10%, v/v). A suspension of HCl.H-Ala-OBu^t together with an equivalent of triethylamine was centrifuged before the use of its liquid part (the hydrochloride salt is soluble in DMSO and FMD). Identical volumes (60–100 μl) of the three stock solutions were mixed (i.e. the final concentration of the reactants became: 50, 50 and 75 mM, respectively) and, after a rigorous mechanical shaking, the reaction mixture was divided into two parts. One part was left at room temperature (22 ± 1°C), while the other was rapidly frozen in solid CO_2-methanol (30–40 s) and kept in the freezer (-18 ± 1°C). After 18–19 h of treatment the samples were diluted with acetonitrile, shaken rigorously and centrifuged from the DCU precipitate (DCU was soluble in DMSO and FMD). The diluted solutions were kept in the refrigerator (2–3 h) until the RP-HPLC analysis.

All experiments were repeated three times under rigorously identical conditions.

HPLC Analysis

Analytical RP-HPLC was performed on a programmable Knauer HPLC system using a Vertex column (250×4 mm) with C_{18}-silica as the stationary phase and a linear gradient elution with A = 0.1% TFA and B = 80% ACN in A as the mobile phase at a flow rate of 1 ml/min. The linear gradient was $20 \rightarrow 92$% B in 45 min at ambient temperature. A 20-μl volume of the diluted samples was injected and the peaks were monitored at 254 nm. Since the dipeptide and the products contain the same chromophore (-Phe-) the absorption coefficients were assumed to be equal. The peak identifications were carried out also by coinjection with the authentic substances.

RESULTS AND DISCUSSION

The RP-HPLC chromatograms of couplings carried out in liquid and frozen dioxane solutions demonstrate good resolution of all identified peaks (Figure 1), and similar separations were found in all experiments. Concerning the resolution of the tripeptide epimers, the mean retention times and capacity factor values together with the standard deviations (in brackets), as calculated from the different experiments, are: $t_R = 31.7(4)$ and 35.7(2); $k = 13.2(6)$ and 14.9(7). Although the *N*-dipeptidylurea peak may contain some Boc-Ala-Phe-urea (LD) diastereoisomer unseparated in the given eluent mixture, the extent of the side-product formation and not its racemization was the main point of these investigations. The unidentified peaks of the chromatograms indicate some impurities of the crude reaction mixtures.

The integrated areas under the chromatogram peaks compared with those of the authentic samples served for quantitative evaluation of the results (Table 1). In the special case of the *N*-acylurea reference compound, as also containing some unreacted dipeptide, the integrated area of the *N*-acyl-

urea has been extrapolated to 100%. Unreacted dipeptide was found in each experiment carried out in different solutions, except for couplings conducted at room temperature in dioxane and dioxane/acetonitrile, respectively. According to the chromatograms, the amount of the remained dipeptide has been proven to be smaller at 22°C (< 10%) and larger at − 18°C (> 20%).

The freezing of solutions caused a significant decrease of *N*-acylurea formation together with the appearance/increase of the unreacted dipeptide. It also resulted in a slight suppression of epimerization (Table 1). The highly reactive *O*-acylisourea intermediate of the DCC-activated synthesis produces the peptide coupling by the nucleophilic attack of the H-Ala-OBu^t amine component. A second, equally important bimolecular pathway of the coupling proceeds via symmetrical anhydride generated rapidly from the *O*-acyliosourea and the carboxylate anion of another dipeptide molecule. Beside the peptide coupling, however, the nucleophilic N-centre of the overactivated *O*-acylisourea intermediate competes with the amine nucleophile and this leads to the unreactive *N*-acylurea by-product [11]. This in turn means a competition between the bimolecular vs. monomolecular (intramolecular) reactions.

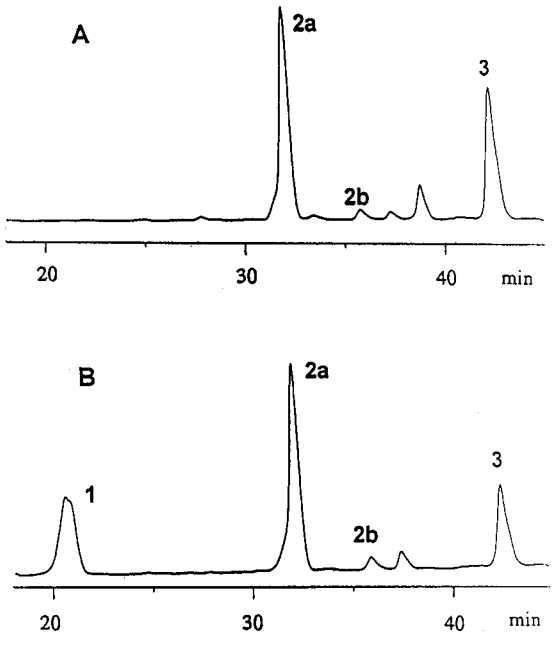

Figure 1 Analytical RP-HPLC chromatograms of the reaction mixtures of DCC-activated coupling of Boc-Ala-Phe-OH with H-Ala-OBu^t in (A) liquid and (B) frozen dioxane solutions. (1) Boc-Ala-Phe-OH; (2) Boc-Ala-Phe-Ala-OBu^t: (a) LLL and (b) LDL; (3) Boc-Ala-Phe-urea.

Therefore, the suppression of the side-product formation can be explained by the assumption that the freezing brings about an increase of the concentration of reactants in the liquid phase, i.e. the bimolecular reaction proceeds further at subzero temperature, hence the competing intramolecular side reaction depends only on the decreased energy of the lower temperature, and is independent of the concentration. This means, also, that for the bimolecular reaction the yield-decreasing effect of cooling is compensated by the freeze-concentration. On the other hand, the possibility of an intermolecular *N*-acylurea formation seems unlikely, comparing the rate of the intermolecular reaction with that of the *O*→*N*-acyl migration in the *O*-acylisourea molecule. The slight reduction of epimerization by freezing can also be attributed to an intramolecular reaction of the overactivated *O*-acylisourea and/or of the symmetrical anhydride, namely, the 5(4H)-oxazolone formation [11,12]. The amount of this chirally labile racemization prone intermediate may be reduced in the subzero frozen conditions.

The data of Table 1 show that reactions in dioxane and dioxane/acetonitrile mixture solutions led to the highest tripeptide yields. It can also be seen that the polarity change of the different solvents changes the relative importance of the various pathways as well. By arranging the solvents of Table 1 according to increasing polarity (Table 2), the data in neat solvents suggest a decreasing reactivity with the polarity increase, as there is practically no coupling in the most polar formamide. These findings may point to an inhibiting effect of the solvation shell around the reactant molecules in solvents of increased polarity [16]. Nevertheless, the experiments in solvent mixtures give quite scattered results as shown by the high coupling yield in the dioxane/acetonitrile solvent. The effect of polarity in liquid and frozen solvent mixtures is difficult to understand for two reasons: (i) the interaction of the solvent and solutes, as well as solvent/solvent and solute/solute, is very specific. This in turn means that because of the lack of understanding of these specific interactions, the success of the peptide coupling cannot be predicted from the macroscopic properties of the solvents [15,16]; (ii) the proportions of the solvent components of the mixtures change on freezing and therefore the real composition of the liquid phase in the cavities of frozen solutions is unknown. The phase diagrams of the applied mixtures are, unfortunately, not given in the literature.

Table 1 Peptide Yield, Epimerization and *N*-Acylurea Formation of Couplings in +22°C (liquid) and −18°C (frozen) Solutions[a]

Solvent	Peptide yield (%)[b]		LDL–Peptide (%)[c]		*N*-acylurea (%)[d]	
	+22°C	−18°C	+22°C	−18°C	+22°C	−18°C
Solvent						
DOX	67	58	5	5	38	19
DSO	17	16	11	7	85	25
FMD	3	2	66	42	2	1
Mixture[e]						
DMF	56	55	6	5	17	8
DSO	59	58	2	1.5	45	8
ACN	67	73	1.5	1	26	11
FMD	52	50	3	1.5	39	16

[a] Synthesis of Boc-Ala-Phe-Ala-OBu^t: coupling of 50 mM Boc-Ala-Phe-OH and 50 mM H-Ala-OBu^t by 75 mM DCC (the final concentrations in the reaction mixture are given) The diluted samples were evaluated by analytical RP-HPLC, monitored at 254 nm, data (%): integrated areas of identified peaks (standard deviations: 10%).
[b] LLL+LDL.
[c] (LDL)/(LLL+LDL).
[d] Boc-Ala-Phe-urea.
[e] Dioxane/solvent: 90%/10% (v/v).

CONCLUSIONS

It can be concluded that DCC-mediated coupling in frozen organic solutions may be a suitable method for peptide synthesis, as the *N*-acylurea formation is significantly suppressed by freezing without the addition of any auxiliary nucleophile such as HOBt. The application of different dioxane/solvent mixtures opens up new possibilities for the cryo-coupling of peptides.

Table 2 Physical Constants of the Applied Solvents Arranged with Increasing Solvent Polarity (E^N)[a]

Solvent	E^N	ε[b]	$t_{m.p.}$ (°C)[c]
DOX	0.164	2.2	11.8
DMF	0.386	38.2	−60.4
DSO	0.444	47.2	18.5
ACN	0.460	36.6	−43.8
FMD	0.775	111.0	2.5

[a] Empirical solvent polarity parameter, scale ranges from 0.000 for TMS to 1.000 for H_2O. Normalized $E_T(30)$ values, the excitation absorption energy of a pyridinium *N*-phenolate betaine solvatochromic dye [13].
[b] Relative permittivity for the pure liquid at 20°C [14].
[c] Melting point [15].

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REFERENCES

1. R.E. Pincock (1969). Reactions in frozen systems. *Acc. Chem. Res. 2*, 97–103.
2. M. Haensler and H.-D. Jakubke (1996). Nonconventional protease catalysis in frozen aqueous solutions. *J. Peptide Sci. 2*, 279–289.
3. H.M.R. Hatley, F. Franks and H. Day (1986). Subzero-temperature preservation of reactive fluids in the undercooled state. II. The effect on the oxidation of ascorbic acid of freeze concentration and undercooling. *Biophys. Chem. 24*, 187–192.
4. T. Vajda (1986). Role of Mn^{2+} ion in trypsin activity oscillations. *Cryo-Letters 7*, 23–30.
5. T. Vajda (1993). Cryochemistry. Large acceleration of the oxidation of hydroxylamine by iodate in frozen solution. *Int. J. Chem. Kinet. 25*, 1015–1018.

6. T. Vajda, F. Almási and T. Vántus (1993). Self-similarity of Mn(II)-induced trypsin activity oscillations. Experimental evidence. *J. Inorg. Biochem. 52*, 131–138.
7. N.H. Grant, D.E. Clark and H.E. Alburn (1966). Accelerated polymerization of *N*-carboxyamino acid anhydrides in frozen dioxane. *J. Am. Chem. Soc. 88*, 4071–4074.
8. T. Vajda (1996). Cryochemistry. Peptide coupling in frozen organic solution. *Cryo-Letters 17*, 295–302.
9. E. Lundanes and T. Greibrokk (1978). Reversed phase chromatography of peptides. *J. Chromatogr. 149*, 241–254.
10. Gy. Szókán, A. Török and B. Penke (1987). High-performance liquid chromatographic detection of side reactions in peptide synthesis. *J. Chromatogr. 387*, 267–280.
11. M. Bodánszky: *Peptide Chemistry. A Practical Textbook*, 2nd ed., p. 63–66, 115–128, Springer-Verlag, Berlin 1993.
12. Z. Wodecki, M. Slebioda and A. Kolodziejczyk (1995). Application of polarimetric detector for the HPLC determination of the optical purity of 5(4H)-oxazolones. *J. Chromatogr. 696*, 149–152.
13. C. Reichardt (1994). Solvatochromic dyes as solvent polarity indicators. *Chem. Rev. 94*, 2319–2358.
14. C. Wohlfarth in: *CRC Handbook of Chemistry and Physics*, 75th ed., D.R. Lide, Ed., p. 155–188, CRC Press, Boca Raton, FL 1994.
15. C. Reichardt: *Solvents and Solvent Effects in Organic Chemistry*, 2nd ed., VCH, Weinheim 1988.
16. F. Ruff and I.G. Csizmadia: *Organic Reactions. Equilibria, Kinetics and Mechanism*, p. 257–317, Elsevier, Amsterdam 1994.

