Development of Economical and Efficient Mixed Reactive Carbonates for Peptide Synthesis through Scientific Planning

TITUS SLAVICI[1,2]*, DOINA DARVASI[1], ALIN MNERIE[1], PERSIDA CECHIN-CRISTA[1]

[1] „Ioan Slavici" University of Timisoara, 144 Dr. A. Paunescu-Podeanu Str., 300587, Timisoara, Romania

[2] Politehnica University of Timisoara, 1 Mihai Viteazu Str., 300222, Timisoara, Romania

The determination of efficient and improved procedures for the synthesis of N-protective groups in peptide synthesis using two types of reactive carbonates with different leaving groups (N,N'-disuccinimidyl carbonate and N,N'-diphthalimidyl carbonate) and different alcohols is reported. Good yields were obtained for succinimidyl derivatives because of the higher reactivity of N,N'-disuccinimidylcarbonate compared with N,N'-diphthalimidyl-carbonate. By using principles of the scientific programming of the laboratory experiments and by identifying the discrete and continuous variables that influence the processes, it has been achieved the improvement and optimization of the synthesis reactions yield and costs reduction.

Keywords: N-protective groups; peptide synthesis; scientific research planning; influencing factors

Peptide Synthesis

In chemistry, the underlying principles of sustainable chemical development (green chemistry) imposed the necessity for the identification of new methods concerning the reduction of reagents and energy consumption in the chemical processes, the reduction of emissions of toxic chemical products into the environment, the extension of the use of renewable resources.

Peptides and proteins have many important therapeutic applications, such as in the treatment of cancer, infectious and viral diseases, AIDS-related diseases, heart disease, respiratory diseases, autoimmune disorders, transplantations, skin disorders, diabetes, genetic disorders, digestive disorders, blood disorders, infertility, growth disorders, and eye conditions. These peptides are present in numerous physiological and biochemical processes of life where they play major roles. Peptides allow for communication between cells through interactions with receptors and are involved in different biochemical and metabolic processes, including pain, reproduction and immune response.

There are many known types of intermediary groups that are used for the temporary protection of amino and carboxyl functional groups involved in peptide bond formation. These groups must be selectively cleaved under conditions that do not interfere with the stability of the peptide bonds or semi-permanent protecting groups on the amino acid side chains. Although a wide range of amino protecting groups are known, the development of mild and efficient procedures using environmentally friendly reagents represents a continuous challenge in peptide synthesis [1, 2].

During the synthesis of peptides, the incorporation of amino acids that retain optical activity is a key factor in obtaining compounds that are identical to natural peptides. For this reason, alkoxycarbonyl-type (carbamate) groups are preferred [3].

Among the carbamates, the succinimidyl and phthalimidyl derivatives are particularly important because of the high reactivity of their leaving groups (*N*-hydroxy imidic cycles) and weakly acidic character, which inhibits amino acid racemisation. The use of mixed carbonates of succinimidyl and phthalimidyl derivatives represents an alternative to traditional amino acid protection strategies [2, 4–9].

The reactive organic carbonates are an ecological alternative to halogenated compounds, reagents like phosgene and its reactive chlorinated derivatives (chloroformates, diphosgene, triphosgene) in alkoxycarbonylation and carbonylation reactions [22].

The reactive organic carbonates have numerous applications in fine organic chemistry: intermediates in the synthesis of other derivatives of carbonic acid (asymmetric carbonates, polycarbonates, carbamates, ureas) and in peptide synthesis. There are many fundamental and applicative scientific studies on the physical and chemical properties (reactivity, stability of molecule) of reactive organic carbonates [17].

Optimisation of the Scientific Research

In many situations, the development of the scientific research uses empirical algorithms that are a waste of human resources, time, and money. To follow the economic impact of how material resources are used for conducting experiments and calculating the specific cost per experiment, the costs for each experiment must be analysed separately. Direct costs for each test performed can be approximated from the sum of the material costs, utility costs and human resource costs. This approximation omits administration costs, building use and equipment depreciation. Therefore, considering that scientific planning can lower the number of experimental tests necessary to obtain the best objective function values and empirical planning requires a large number of experimental tests, a direct cost reduction of approximately 88% can be realised.

The goal of this paper was to convert empirical planning of scientific resources into scientific planning. For this purpose, only experiments that were relevant and contained a wealth of information were used. For example, it is possible to obtain more information from 16 scientifically planned experiments than from 50 empirically planned experiments. This study sought to identify the influencing factors (xi) that assured maximal objective functionality. In many situations, empirical planning can even be considered preliminary research and serve as a starting point for future scientific planning. The second step

* *email: titusslavici@yahoo.com; Tel.: +40 0256 213108*

consisted of scientific research planning, and the optional final step involved improving research planning to reach the optimum cost/benefit ratio.

Experimental part

Melting points were determined in a Boetius apparatus (Carl Zeiss, Jena). The IR spectra were recorded as KBr pellets for solid compounds, and the reaction was monitored in thermostated cells that were 0.137 mm thick on a Vertex 70 (Bruker) FT/IR instrument. TLC analyses were performed on plates pre-coated with silica gel 60 F254 (Merck) using an acetonitrile:ethyl acetate (7:3) solvent system. Spot visualisation was achieved by UV fluorescence quenching under a UV 254 lamp. Mass spectrometry was conducted on a High Capacity Ion Trap Ultra (HCT Ultra, PTM discovery) mass spectrometer from Bruker Daltonics (Bremen, Germany). HCT-MS was interfaced to a PC running the Compass TM 1.2 integrated software package, which included the HystarTM 3.2.37 and Esquire 6.1.512 modules for instrument control and chromatogram/spectrum acquisition. The Data Analysis 3.4.179 portal was used for storing the ion chromatograms and processing the MS data. The ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker DPX instrument at 200 MHz in DMSO-d6 using TMS as the reference. The coupling constants were normal for vicinal couplings (CH-CH and CH-NH) and in the range of 6.5 to 7 Hz. Detailed IR, ^{1}H NMR and ^{13}C NMR spectra are available by request from the authors. All reagents were purchased from chemical suppliers and used without further purification.

The reactive carbonate (1 mmol) was dissolved in acetonitrile (10 mL), and alcohol (1 equiv.) and amine (1 equiv.) were added. The reaction mixture was stirred at room temperature for 5-48 hours. Next, the solvent was removed under vacuum, and the residue was purified. The residue was dissolved in ethyl acetate (15 mL) and washed three times with 20% citric acid (5 mL), 5% $NaHCO_3$ (5 ml) and saturated NaCl (5 mL). The organic phase was dried ($MgSO_4$) and concentrated to dryness. The residue was crystallised from ethyl acetate-hexane.

Results and discussions

The procedure describes the preparation of reactive *N*-succinimidyl and *N*-phthalimidyl carbonates [9, 22-25], and the results of the scientific research planning were used to develop an improved scheme for the synthesis of *N*-protective groups used in peptide synthesis.

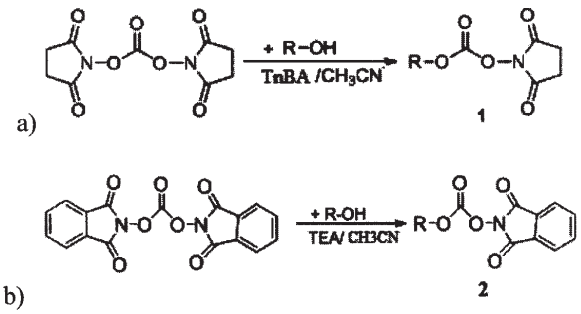

Fig. 1. General reaction of a) succinimidyl derivatives; b) phthalimidyl derivatives

This scheme employed two types of reactive carbonates with leaving groups (*N,N'*-disuccinimidyl carbonate and *N,N'*-diphthalimidyl carbonate) and a series of alcohols (table 1) to generate the protected functionality in one step under basic catalysis (i.e., triethylamine [TEA]). The molar ratio of carbonate:alcohol:amine used in this scheme was 1:1:1. The reaction was performed in acetonitrile at room temperature for 5 to 48 h depending on the substrate and alcohol type (table 1). The reaction co-products (N-succinimimide and *N*-phthalimide) can be recoverd and reused.

Synthesis of Succinimidyl Mixed Carbonates

The one-pot syntheses using commercial *N,N'*-disuccinimidyl-carbonate (DSC) and alcohols with different chemical structures have been demonstrated to yield a wide range of succinimidyl derivatives in good yield at room temperature. These intermediates are used in peptide synthesis reactions as alkoxycarbonyl amino acid *N*-protection groups [10-23]. The results from the syntheses are shown in table 2.

Synthesis of Phthalimidyl Derivatives

The synthesis of phthalimidyl derivatives was performed under similar reaction conditions as the succinimidyl derivatives (fig. 3).

The *N*-phthalimidyl derivatives were obtained from the corresponding symmetrical carbonates, an alcohol (six different types of alcohols were used) and an aliphatic amine (TEA) mixed at a 1:1:1 molar ratio at room temperature and resulted in good yields (table 3).

No.	Alcohol (R-OH)	Chemical formula
1	Benzyl alcohol	C_6H_6-CH_2-OH
2	α,α-dimethyl-3,5-dimetoxy phenylcarbinol	$(CH_3)_2$-C_6H_5-C-$(CH_3)_2$-OH
3	β-phenyl-ethyl alcohol	C_6H_5-CH_2-CH_2-OH
4	Benzhydrol	$(C_6H_5)_2$-CH-OH
5	2-ethoxyethanol	CH_3-CH_2-O-CH_2-CH_2-OH
6	2,2,2-trifluoroethanol	CF_3-CH_2-OH
7	4-nitro-phenyl-methanol	O_2N-C_6H_4-CH_2-OH
8	2-phenyl ethanol	C_6H_5-CH(CH_3)-OH
9	3-phenyl propen 2-ol	C_6H_5-CH=CH-CH_2-OH
10	Cyclohexylmethanol	C_6H_{12}-CH_2-OH
11	2-methyl-2-propanol	$(CH_3)_3$-CH-OH
12	Tert-amyl alcohol	$(CH_3)_3$-CH-CH_2-OH

Table 1
ALCOHOL SERIES

Compound	1a	1b	1c	1d	1e	1f	1g	1h	1i	1j	1k	1l
Yield (%)	70.5	64.1	66.8	75	79.6	62.1	73.8	81.2	75.8	74.6	82.8	80.6

Table 2
REACTION YIELDS FOR SYNTHESIZED *N-SUCCINIMIDYL DERIVATIVES*

Compound	2a	2b	2c	2d	2e	2f
Yield (%)	63.7	58.3	56.7	53.4	55.7	28.7

Table 3
REACTION YIELDS FOR THE SYNTHESIZED N-PHTHALIMIDYL DERIVATIVES

No. exp.	Substrate	Alcohol	Catalyst	Catalyst quantity [ml]	Reaction time [h]
	X1	X2	X3	X4	X5
1	DSC	1	TEA	0.2049	26
2	DSC	2	TnBA	0.524	5
3	DSC	3	TnBA	0.93	5
4	DSC	4	TnBA	0.93	5
5	DSC	5	TnBA	0.93	5
6	DSC	6	TnBA	0.93	5
7	DPC	1	TEA	0.38	24
8	DPC	2	TEA	0.395	24
9	DPC	3	TnBA	0.212	24
10	DPC	4	TnBA	0.212	24
11	DPC	5	TnBA	0.212	24
12	DPC	6	TnBA	0.212	48

Table 4
PRELIMINARY RESULTS

Legend: DSC = N,N'-disuccinimidyl carbonate; DPC = N,N'-diphthalimidyl carbonate; TEA = triethylamine; TnBA = tri-n-butylamine

Substrate	DSC	DPC
Cycle type	Aliphatic	Aromatic

Table 5
SUBSTRATE CHARACTERISATION

Alcohol (R-OH)	Alcohol Type	Aromatic core	Carbon chain Length
1	Aromatic alcohol	yes	7
2	Tertiary alcohol	yes	11
3	Secondary alcohol	yes	8
4	Secondary alcohol	yes	13
5	Primary aliphatic alcohol	no	4
6	Primary aliphatic alcohol	no	2
7	Primary alcohol	yes	7
8	Secondary alcohol	yes	8
9	Primary alcohol	yes	9
10	Primary alcohol	no	7
11	Secondary alcohol	no	4
12	Primary alcohol	no	5

Table 6
ALCOHOL CHARACTERISATION

The decreased reactivity of the symmetrical *N,N'*-diphthalimidyl carbonate (DPC) compared with *N,N'*-disuccinimidyl-carbonate (DSC) resulted in reduced yields for the final products. In addition, the quantitative removal of the *N*-hydroxyphthalamide and unreacted *N,N'*-diphthalimidyl-carbonate (DPC) from the final products was also an impediment.

The reaction yields were strongly influenced by the alcohol type, and aromatic alcohols and aliphatic alcohols with different electron withdrawing groups were used as well as primary, secondary, and tertiary alcohols.

Lower yields were also observed for fluoro-derivatives (fluoro-succinimidyl and fluoro-phthalimidyl) because of the electronegativity of the fluoro-alkyl group, which had decreased reactivity as a result of a sterically hindered secondary or tertiary alcohol.

Scientific Planning of the Experiment

The major objective of experimental modelling is to obtain more accurate experimental models for technological purposes. The achievement of this objective is supported by a rational program of experiments. The first step is to perform preliminary experiments to determine the most significant factors that influence the technological process of interest. Based on the developed experimental models, influencing factors (xi) capable of improving the objective function in terms of optimisation criterion are selected.

The multifactorial space proposed for investigation included five influencing factors: X_1, the nature of the substrate with two levels: 1 (DSC), 2 (DPC); X_2, the alcohol with twelve levels: R_1-R_{12} (table 1); X_3, the catalyst type: C_1 (TEA) and C_2 (tri-n-butylamine [TnBA]); X_4, catalyst quantity; and X_5, reaction time. Among the influencing

factors are discrete variables (substrate (X_1), alcohol (X_2), and catalyst (X_3)) and continuous variables (reaction time (X_5) and catalyst quantity (X_4)). Assuming the assignment of two levels of variation (minimum and maximum) for the two continuous factors, the number of theoretically possible combinations for a complete experiment would be 192 (2x12x2x2). However, only part of the experimental points that correspond to a systematic exploration of the multifactorial space is feasible.

The preliminary experiment consisted of the selection of six reactants from a total of twelve reactants, and the generation of the most convenient experimental method from this set was performed. This assumption resulted in twelve preliminary experimental methods, which are shown in table 4.

Each of the substrates was described by the type of ring (table 5), and the reactants were described by the alcohol type, aromatic ring and number of carbon atoms (table 6).

From the results of the preliminary analysis, the most convenient combination was *N,N'*-disuccinimidylcarbonate (DSC) as the substrate, 2-phenyl-ethyl alcohol as the reactant, and tri-n-butylamine as the catalyst. A series of conclusions can be drawn from the preliminary analysis. The best performance was obtained using DSC as the substrate in combination with 2-phenyl-ethyl alcohol and tri-n-butylamine (*TnBA*) as the catalysts (fig. 2 and table 2).

These results represent a starting point for the analysis of the performance of the other six alcohols (alcohol 6–12, table 1) not used in the preliminary experiment. This second set of experiments led to the results presented in table 2. The results presented in table 2 show that the yields were similar to or higher than the best result obtained in the preliminary test. The results from tables 1 and 2 also allowed for the classification of the reactants. Specifically, the yields could be increased by approximately 78% by carefully selecting the type of reactant used. The trend observed in reactivity could possibly be explained by the stability of the corresponding radicals: allylic>benzylic> tertiary>secondary> primary>methyl.

Analyzing the preliminary data led to the isolation of a point (factor combination) with the best results (fig. 2)

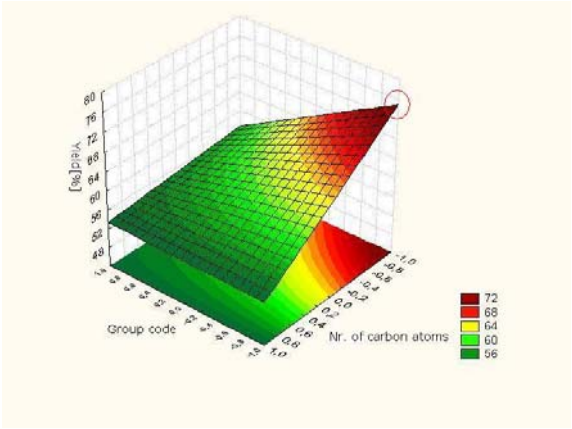

Fig. 2. Response surface revealing the influence of the factor groups

around which we can develop an additional analysis. A supplementary study investigated the effects of reaction time and catalyst quantity on the objective function to determine the possibility of further improving the reaction results. Based on this study, an optimized set of reaction conditions were proposed for maximum efficiency. However, IR monitoring of the reaction of *n*-butyl alcohol (R_{13}) with DSC was necessary to establish optimal conditions for the formation of the related succinimidyl derivative (fig. 3).

The reaction *N*-butylsuccinimidyl carbonate synthesis was performed in a heterogeneous medium at room temperature (29°C) in acetonitrile [23]. First, the IR spectrum of alcohol in acetonitrile was recorded, which was followed by the IR spectra of mixtures of alcohol, DSC and TEA in acetonitrile. The spectroscopic data obtained in the 2000-1620 cm^{-1} region (fig. 3) allowed for the following conclusion: in the first minutes after amine (TEA) was added to the reaction mixture (alcohol and DSC in ACN), a pronounced decrease in the intensity of the DSC absorption band at 1860 cm^{-1} was observed along with an increase in the 1840 cm^{-1} band. In addition, an intense band at 1870 cm^{-1}, which corresponded to the *N*-butyl-succinimidyl carbonate, appeared.

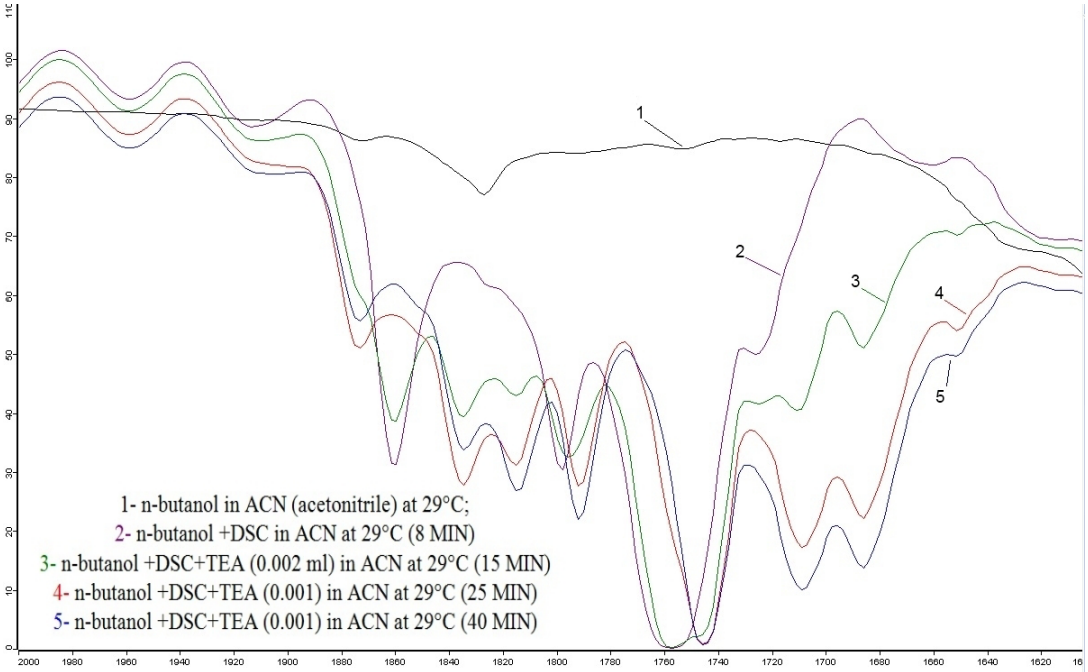

Fig. 3. IR spectrum of n-BuOH in acetonitrile, and the spectra of mixtures of n-BuOH, DSC and TEA in acetonitrile

The IR spectra of the reaction mixture (recorded at different times) revealed the disappearance of the valence vibrations of the carbonyl group of the symmetrical carbonate with increasing reaction time. The spectrum recorded at 40 min after the reaction was initiated confirmed the total decomposition of the symmetric carbonate and complete formation of the butylsuccinimidyl derivative (fig. 3). The described IR study indicated an ideal reaction time of 40 min.

Conclusions

Because of the lack of methods for the synthesis of mixed *N,N'*-disuccinimidyl-carbonate and *N,N'*-diphthalimidyl carbonate in the literature, a qualitative study of synthetic conditions was necessary. The results revealed that the yield of the mixed carbonates was influenced by the reactivity of the alcohol, steric factors and stability of the finite compound. In addition, the yield was lower for phthalimide when compared with succinimide, which was due to the lower reactivity of the *N,N'*-diphthalimidyl carbonate. Furthermore, quantitative elimination of *N*-hydroxy-phthalimide and unreacted *N,N'*-diphthalimidyl carbonate was difficult.

The improvement of the chemical processes is an issue of major importance in the current concept of green chemistry which require the energy and the raw materials savings and also the reduction of the dangerous reactants and co-products. Using traditional, empirical algorithms of laboratory experiments programming, without making use of a number of factors that may interfere in their number reduction, unnecessary lead to an unjustified consumption of energy and materials.

The systematic scientific planning of the laboratory experiments for the synthesis of the intermediates (carbonates), which can be extended to any type of experiments, has allowed the substantially reducing both of their number and involved cost and the improvement of the cost/ benefit ratio, by identifying discrete and continuous variables that influence the reaction process and by choosing the best combination between the reactants type, substrate and catalyst.

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