Alkyl succinimidyl carbonates undergo Lossen rearrangement in basic buffers

Samuel Zalipsky*

SEQUUS Pharmaceuticals, Inc., 960 Hamilton Court, Menlo Park, CA 94025, USA

A side reaction producing β-alanine and derivatives through Lossen rearrangement was found to accompany hydrolysis of alkyl succinimidyl carbonates in basic aqueous buffers.

Alkyl succinimidyl† carbonate (SC) derivatives are useful alkoxycarbonylating reagents. In particular, their reactivity with primary amines has found a wide use for the introduction of urethane protecting groups,[1] protein modification,[2–5] and covalent attachment of ligands to matrices for solid-phase synthesis[6] and affinity chromatography.[7] Often these carbamoylation reactions are carried out in aqueous or aqueous–organic medium at mildly basic pH. Such conditions are dictated by the solubility properties of the amino reactants, amino acids, aminosugars, proteins, *etc.* In recent years, we have used SC derivatives of poly(ethylene glycol) (SC-PEG) for attachment of the polymer residues to amino groups of proteins, lipids and various biologically-relevant ligands.[3–5,8,9] In the course of these studies it was noticed that occasionally upon completion of a protein modification reaction using an excess of SC-PEG, there were larger amounts of primary amino group-containing compounds in solution than originally present on the protein. Accurate determinations of the extent of protein modification were only realized following removal of low molecular weight amines by extensive diafiltration.[4] These observations lead to the belief that the low molecular weight amine originated from the SC reagent itself. In order to verify this hypothesis, several ^{1}H NMR experiments were performed with bis-SC-PEG-2000[3,9] and benzyl succinimidyl carbonate (Bn-SC) under conditions often used for protein modification. Herein I present the results of these experiments and their corroboration by amino acid analysis (AAA), all of which indicate that SC derivatives undergo Lossen-type rearrangement producing β-Ala and its derivatives.

Table 1 Quantities of β-Ala formed after dissolution of bis-SC-PEG (10 mg, 8.4×10^{-4} equiv. SC per g) in tetraborate or carbonate buffers (0.1 M, pH 9.3, 0.65 ml) and incubation at 23 °C for 24 h. Aliquots taken from the reaction solutions were subjected to AAA, both directly and after hydrolysis (6 M HCl, 24 h, 110 °C). Amino groups were determined by the 2,4,6-trinitrobenzene sulfonate assay[17] using β-Ala for calibration

	β-Ala/10^{-5} mol g^{-1} [% of SC group]		
Buffer type	Total (HCl hydrolysis)	Free amino acid	Amino groups/ 10^{-5} mol g^{-1}
Tetraborate	15.8 [19]	4.6 [5.5]	8.6
Carbonate	20.6 [24]	6.6 [7.8]	10.3

Hydrolysis of bis-SC-PEG in D_2O [δ 2.96 (s, Su of SC, 8H), 3.71 (s, PEG, 180H), 4.58 (m, CH_2-O$_2$COSu, 4H)] proceeded slowly over 24 h without any side reactions, yielding *N*-hydroxysuccinimide [HOSu, δ 2.77 (s)] quantitatively. Next, the degradation of SC-PEG was examined in D_2O-based potassium tetraborate buffer and monitored by ^{1}H NMR

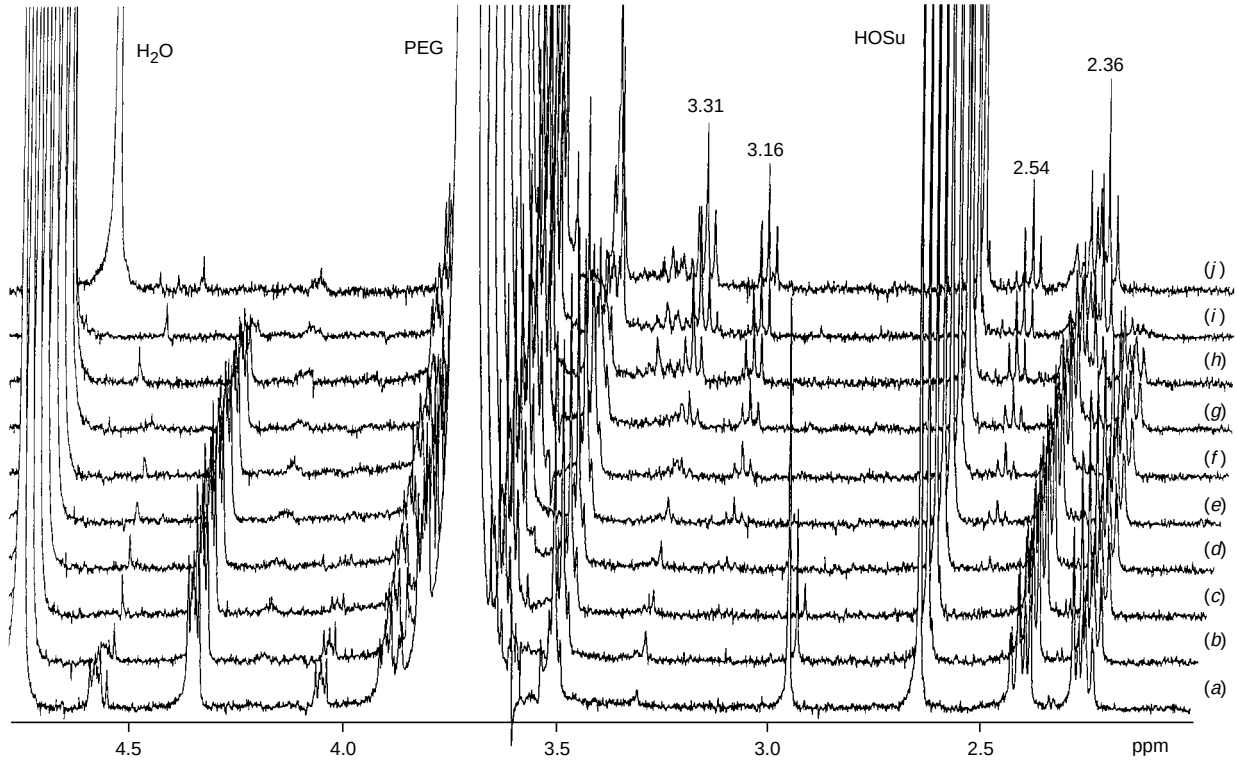
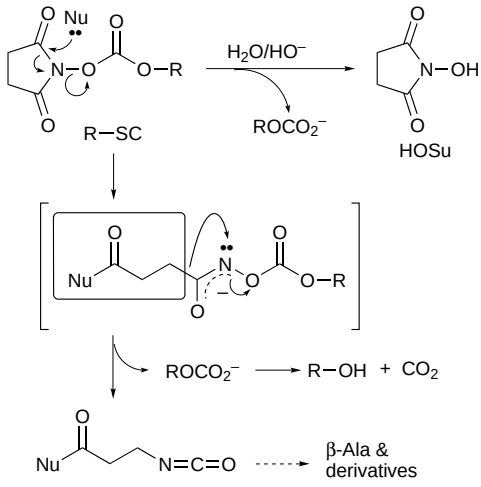


Fig. 1 Progress of the bis-SC-PEG (10 mg) decomposition monitored by 360 MHz ^{1}H NMR spectroscopy in D_2O–tetraborate buffer (0.1 M, pH 9.3, 0.65 ml). Spectra were acquired at (*a*) 3 min, (*b*) 5 min, (*c*) 8 min, (*d*) 11 min, (*e*) 16 min, (*f*) 30 min, (*g*) 45 min, (*h*) 2 h, (*i*) 4 h and (*j*) 20 h.



R = $–CH_2CH_2O(CH_2CH_2O)_{43}CH_2CH_2–$, $–CH_2C_6H_5$
Nu = ^-OH, ^-OSu

Scheme 1 Reaction pathways of alkyl-SC degradation through hydrolysis and through Lossen rearrangement

spectroscopy (Fig. 1). Although the disappearance of SC groups [δ 2.96 (s), 4.58 (m)] was complete within 10 min, and most of the SC was hydrolyzed to −OSu [δ 2.65 (s)], additional reactions have also taken place. Appearance of a new signal [δ 4.35 (m)] of the terminal methylene group of PEG and two symmetrical sets of peaks of type X–CH_2–CH_2–Y at δ 2.3 and 2.4 suggest that a substantial amount of the starting SC-PEG has been converted into a new derivative resulting from the succinimide (Su) ring opening. Within 16 min two triplets at δ 2.54 and 3.16, representing free β-Ala, have appeared and increased in size for the next few hours. At the end of the experiment, in addition to the β-Ala peaks, signals clustering around δ 2.3–2.5 and 3.2–3.4 with two major triplets at δ 2.36 and 3.31 were apparent. These chemical shifts are in the areas where various acylated derivatives of β-Ala yield NMR peaks.[10,11] Similar observations in the ^{1}H NMR spectra were made by following the decomposition of SC-PEG in a D_2O–carbonate buffer.

Quantitation of β-Ala in both buffer solutions by AAA (Table 1), either directly or after extensive hydrolysis, revealed that 20–25% of the original SC groups underwent conversion into β-Ala. Approximately one third of the total β-Ala was found in the free amino acid form. Amounts of primary amine somewhat higher than of free β-Ala were detected, suggesting that some of the amines were present in the form of β-Ala derivatives, *e.g.* β-Ala-β-Ala. Due to its low water solubility, Bn-SC was first dissolved in CD_3CN and then diluted ten-fold with D_2O–tetraborate buffer. ^{1}H NMR spectra again showed decomposition of the SC groups proceeded as in the case of SC-PEG yielding β-Ala and derivatives. This experiment confirmed that the observed reaction is more general, and is not exclusive to SC-PEG. The reaction pathways shown in Scheme 1 are consistent with the above observations. Opening of the succinimide ring by a nucleophile, Nu, leads to a Lossen-type hydroxamic acid intermediate, which rearranges into isocyanate *via* a scission of the N–O bond with the concomitant departure of the alkoxycarboxylate, and 1,2-migration of the outlined residue.[12] The isocyanate is expected either to hydrolyze, or to react with various nucleophilic groups (amino, carboxylate, hydroxy), yielding free β-Ala as well as its urea, amide and urethane derivatives. Specifically, β-isocyanatopropionate (Nu = OH) is known to produce 3,3′-ureylenedipropionic acid and oligo(β-Ala), among other β-Ala acylates.[10] This explains why most of the β-Ala was found in the form of acylated derivatives as revealed by AAA after hydrolysis (Table 1).

The tendency of Su-OX derivatives, where OX constitutes a good leaving group, to undergo Lossen rearrangement initiated by a nucleophilic attack on the succinimide ring by either −OH, −OSu, or even amine, is known.[12–16] Although alkoxycarboxylates are recognized as very effective leaving groups, this is the first time that alkyl-SC (Su-OCO_2R) derivatives are implicated in this process. It is pertinent to note that nucleophilic attack by an amino group on the exocyclic carbonyl of SC-derivatives, occurring during carbamoylation reactions, is a lot faster than the side reactions described here. However, the reactions described here can potentially be a source of undesirable by-products, particularly in protein modifications with an excess of alkyl-SC. Preliminary observations in our laboratory suggest that this rearrangement can be measurably suppressed by lowering the pH or the temperature of the solution.

Footnotes and References

* E-mail: SamuelZ@SEQUUS.com
† IUPAC: succinimido.

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