OL Organic Letters

pubs.acs.org/OrgLett Letter

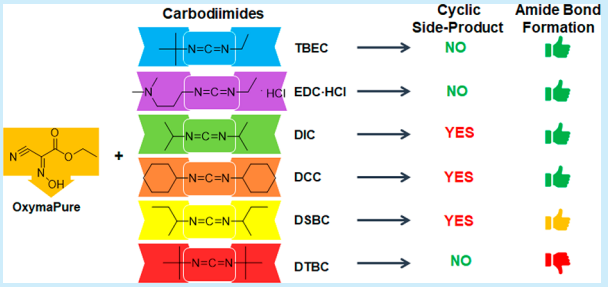
Amide Formation: Choosing the Safer Carbodiimide in Combination with OxymaPure to Avoid HCN Release

Srinivasa Rao Manne,[▽] Omar Luna,[▽] Gerardo A. Acosta, Miriam Royo, Ayman El-Faham, Gyorgy Orosz, Beatriz G. de la Torre,* and Fernando Albericio*

Cite This: *Org. Lett.* 2021, 23, 6900–6904 Read Online

ACCESS | Metrics & More | Article Recommendations | Supporting Information

ABSTRACT: It has been reported that DIC can react with OxymaPure to render an oxadiazole compound with the concomitant formation of HCN. Here we demonstrate that this reaction is not a feature of all carbodiimides but rather depends on the alkyl structure that flanks the two N atoms of the carbodiimide. Furthermore, we have identified two carbodiimides, TBEC and EDC·HCl, whose reaction with OxymaPure is exempt from HCN formation.

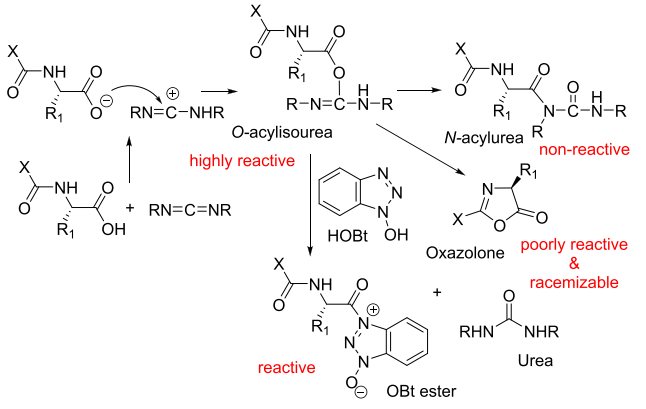


The amide bond is the most common bond not only in nature but also in synthetic organic chemistry, as reflected by independent studies carried out by both academia and industry.[1,2] The ACS Green Chemistry Institute (GCI) Pharmaceutical Roundtable (PR) has even included the amide bond as one of the 10 key green chemistry research areas,[3] thus highlighting its relevance. As in the field of protecting groups,[4,5] research on amide bond formation has been fueled by groups working in the peptide chemistry arena.[6] In this regard, the seminal work of Sheehan and Hess on the use of dicyclohexylcarbodiimide (DCC) for in situ activation of the carboxylic group opened up new avenues for peptide chemistry,[7] which crystallized a few years later with the advent of solid-phase peptide synthesis (SPPS) methodology proposed by Merrifield.[8] These discoveries laid the foundation of what is today peptide synthesis.[9]

The reaction of a carboxylic acid with a carbodiimide renders the *O*-acylisourea as the reactive species (Scheme 1). In the early 1970s, König and Geiger at Hoechst proposed adding 1-hydroxybenzotriazole (HOBt) to the carbodiimide-mediated coupling.[10] In that case, the active species was the OBt active ester. Although this ester was initially thought to be more reactive than the *O*-acylisourea, it is less reactive. However, in many cases this loss of reactivity is translated into greater efficacy because the OBt ester is free of rearrangement and formation of the oxazolone, which is a secondary reaction that takes place from the *O*-acylisourea, making the *O*-acylisourea less efficient than the OBt ester.[6]

Thus, the *O*-acylisourea rearrangement is a side reaction that renders the fully inactive *N*-acylurea. This reaction is important in dipolar aprotic solvents, such as *N*,*N*-dimethylformamide (DMF), which are to date the solvents of choice for the coupling reaction in SPPS.[5] The highly reactive *O*-acylisourea

Scheme 1. Mechanism by Which Carbodiimides Activate a Protected Amino Acid



can evolve to an oxazolone, which again is less reactive than the parent compound and, more importantly, highly prone to racemization.[6]

Because of the aforementioned considerations, almost all peptide couplings have been done in the presence of HOBt, which is shown in tautomer forms,[11,12] or its analogues, either as an additive to the carbodiimides or as part of stand-alone reagents, mainly iminium and phosphonium salts.[6] With the

Received: July 25, 2021
Published: August 23, 2021

https://doi.org/10.1021/acs.orglett.1c02466
Org. Lett. 2021, 23, 6900–6904

implementation of the 9-fluorenylmethoxycarbonyl (Fmoc) strategy instead of the *tert*-butoxycarbonyl (Boc) approach, DCC was substituted by *N*,*N*′-diisopropylcarbodiimide (DIC), whose urea is more soluble and therefore easier to wash out after the coupling.

After September 11, 2001, the potentially explosive nature of HOBt and its related triazole/triazine analogues was highlighted.[13] These compounds were recategorized into a Class 1 explosive category, thus jeopardizing their transportation.[13]

At that time, our group started a broad project to find a safe replacement for HOBt that would keep or even improve on its efficiency. In this regard, a few years later we presented ethyl 2-hydroxyimino-2-cyanoacetate (OxymaPure)[14] as a superior reagent to HOBt in terms of coupling yield, minimization of racemization, and safety.[15] Furthermore, we also proposed (1-cyano-2-ethoxy-2-oxoethylideneaminooxy)(dimethylamino)-(morpholino)carbenium hexafluorophosphate (COMU)[16] and (1-cyano-2-ethoxy-2-oxoethylideneaminooxy)tri-1-pyrrolidino-phosphonium hexafluorophosphate (PyOxim) as OxymaPure-based stand-alone derivatives (Figure 1).[17]

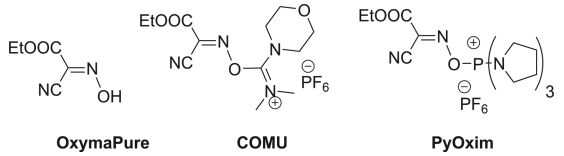


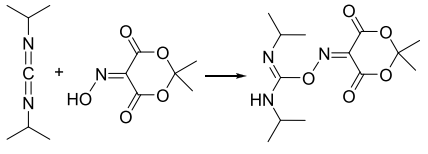
Figure 1. OxymaPure and OxymaPure-based stand-alone coupling reagents.

In spite of the excellent results given by OxymaPure derivatives as coupling additives/reagents, we continued our research to find more oxime derivatives to fulfill our motto "choosing the right peptide coupling reagent for each reaction".[18] In this regard, we were very interested in preparing and testing the oxime derivative of Meldrum's acid[19] because, although the pK_a of this derivative should be higher than that of OxymaPure, its rather rigid and planar structure with two carbonyl groups pointing out toward the potential oxime ester could favor an assisted basic catalysis similar to those described for 1-hydroxy-7-azabenzotriazole (HOAt), and *N*-ethoxycarbonyl-2-ethoxy-1,3-dihydroquinoline (EEDQ).[6]

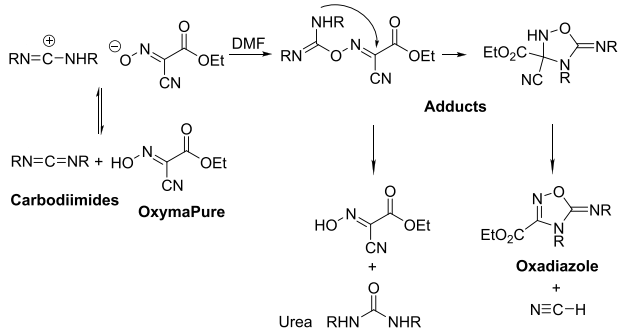
Surprisingly, although the onium derivative of Meldrum's acid gave excellent results similar to those achieved by COMU, the simple additive 5-(hydroxyimino)-2,2-dimethyl-1,3-dioxane-4,6-dione (HONM) in conjunction with DIC was inefficient, as reflected by the lack of full conversion even for easy couplings.[19] We demonstrated that the very poor performance of DIC and HONM was attributable to the efficient reaction of HONM with DIC to give the corresponding adduct, with the concurrent consumption of DIC and consequent inability to activate the carboxylic group (Scheme 2).[19]

Kolis and co-workers at Eli Lilly recently observed that OxymaPure also reacts with DIC.[20] In this case, although the formation of the adduct occurs to a much lesser extent than with HONM, it can cyclize to give an oxadiazole with the concomitant formation of HCN (Scheme 3).

Scheme 2. Reaction of HONM with DIC



Scheme 3. Reactions of Carbodiimides with OxymaPure[20]



These results were corroborated by Pawlas and co-workers at Polypeptide.[21] Those authors proposed a coupling reaction in the presence of dimethyl trisulfide (DMTS) as a HCN scavenger to further minimize its formation.

Herein we addressed the reaction of OxymaPure with various commercially available carbodiimides and their performance in SPPS. In addition to the most frequently used carbodiimides, we also studied DCC, DIC, and *N*-ethyl-*N*′-(3-(dimethylamino)propyl)carbodiimide hydrochloride (EDC or WSC), *N*,*N*′-di-*sec*-butylcarbodiimide (DSBC), *N*,*N*′-di-*tert*-butylcarbodiimide (DTBC), and *N*-*tert*-butyl-*N*′-ethylcarbodiimide (TBEC) (Figure 2).

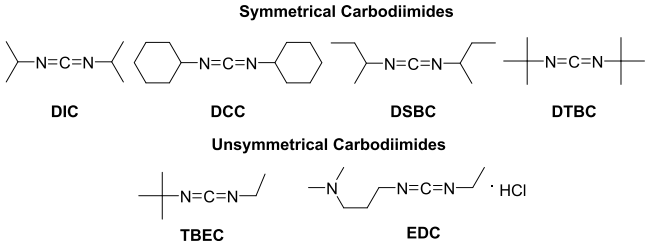


Figure 2. Structures of the carbodiimides used in this study.

All of the reactions were followed by liquid chromatography−mass spectrometry (LC−MS). It should be noted that the formation of the adduct does not involve the formation of HCN. This compound is formed only after the cyclization of the adduct to give the oxadiazole. Indeed, the formation of oxadiazole has been used as a marker for HCN release (Scheme 3).[21]

First, the six carbodiimides were dissolved in DMF together with OxymaPure at a ratio of 1:1. The absence of protected amino acid in the solution forces the side reaction to take place. One set of solutions were kept at 25 °C, and the course of the reaction was analyzed by LC−MS after 24 h and 4 days. A second set of solutions were kept at 60 °C and analyzed after 18 h.

The results are shown in Table 1. The results obtained with DIC are consistent with those reported in the literature by the groups at Eli Lilly and Polypeptide (entries 1, 8, and 15).[20,21] Similar results were obtained when the experiment was repeated with DCC (entries 2 vs 1, 9 vs 8, and 16 vs 15). In both cases, the amount of adduct was low, and even almost nonexistent in the case of DCC (entry 2). It is important to

https://doi.org/10.1021/acs.orglett.1c02466
Org. Lett. 2021, 23, 6900−6904

Table 1. LC−HRMS Analysis of the Reaction of OxymaPure with the Different Carbodiimides[a]

entry	time/temp	carbodiimide	OxymaPure (%)	adduct (%)	oxadiazole (%)
1	24 h/25 °C	DIC	88.9	3.5	7.6
2		DCC	92.3	0.5	7.2
3		EDC·HCl[b]	25.9	14.7	–
				57.8	
4		EDC·HCl/DIEA[b]	67.8	2.3	–
				12.8	
5		DTBC	100	–	–
6		DSBC	90.0	3.0	3.4
					3.6
7		TBEC	71.8	28.2	–
8	4 days/25 °C	DIC	87.7	2.6	9.7
9		DCC	85.4	0.6	14.0
10		EDC·HCl[b]	38.0	15.4	–
				44.4	
11		EDC·HCl/DIEA[b]	38.0	15.4	–
				22.7	
12		DTBC	100	–	–
13		DSBC	91.0	2.3	3.3
					3.4
14		TBEC	75.8	23.7	0.6
15	18 h/60 °C[c]	DIC	78.6	2.4	18.9
16		DCC	81.5	1.1	17.4
17		EDC·HCl[b]	45.9	13.2	–
				40.2	
18		EDC·HCl/DIEA[b]	76.9	9.5	–
				13.6	
19		DTBC	100	–	–
20		DSBC	76.0	1.5	10.9
					11.6
21		TBEC	83.5	10.4	6.1

[a]Reaction conditions: OxymaPure/carbodiimide (1:1) in DMF. [b]An extra peak with $[M + H]^+$ = 252.05 is observed. See ref 22 for a tentative mechanism and structure. [c]Oil bath.

highlight that from a structural perspective, DIC and DCC are similar in the sense that a secondary C atom is bound to each N atom of the carbodiimide.

When the reaction was repeated with DSBC, which is structurally similar to DIC and DCC, the reaction trend was the same as before in all cases (entries 6, 13, and 20). Interestingly, two peaks appeared with the same mass, which could be related to diastereomers.

The relevance of steric hindrance was confirmed when the reaction was studied with DTBC, in which the carbodiimide moiety is flanked by two *tert*-butyl groups. In this case, the carbodiimide was unaltered even after 4 days at 25 °C (entry 12) or 18 h at 60 °C (entry 19).

When the reaction was studied with the much less hindered EDC·HCl, an asymmetrical carbodiimide with primary carbons flanking the carbodiimide moiety, no formation of the oxadiazole was detected. However, this reaction yielded the greatest amount of the adduct of all the series (entries 3, 10, and 17). Because of the asymmetry of EDC·HCl, the chromatogram showed two peaks for the adduct (see the Supporting Information for structures). The reduced formation of the adduct after 4 days compared with 24 h (entry 10 vs 3) could be interpreted in terms of hydrolysis of the adduct, rendering again OxymaPure and the corresponding urea (in all cases, urea was observed by LC−MS) (Scheme 3). Same trend was observed when the reaction was studied with EDC·HCl in the presence of *N*,*N*-diisopropylethylamine (DIEA), which resulted in no formation of the oxadizole. Interestingly, in this case, in the absence and presence of DIEA, a new peak with $[M + H]^+$ = 252.05 is clearly observed. This could correspond to the six-membered-ring cyclic compound formed by the attack of the N atom to the carbonyl of the ethyl ester (see ref 22 for the mechanism and structure). This peak also was observed with EDC·HCl in the absence of DIEA, but in a very little extension. It is very important to note that this six-membered-ring compound contains the CN moiety. Therefore, its formation does not provoke the release of HCN and thus poses no risk.

Finally, TBEC, which could be considered a hybrid of DTBC (the *tert*-butyl part is rather unreactive) and EDC·HCl (the ethyl part renders only the adduct, with no progress to the cyclic structure) performed as expected on the basis of the results for DTBC and EDC·HCl separately, with no formation of the oxadiazole after 1 day and, importantly, no formation of HCN. Under the most energic conditions (18 h at 60 °C), some oxadiazole formed.

The performance of five carbodiimides in SPPS was studied using two model peptides. DCC was not included because the insolubility of the dicyclohexylurea in DMF precludes its use in Fmoc chemistry. Leu-enkephalin (H-Tyr-Gly-Gly-Phe-Leu-NH_2) and $Ile^{2,3}$-Leu-enkephalin (H-Tyr-Ile-Ile-Phe-Leu-NH_2), with two β-branched amino acids in a row (Ile-Ile), which is a demanding coupling, were synthesized manually on Rink-amide resin. The results, which are summarized in Table 2, are in full agreement with the previous ones presented in Table 1, which shows the reactivity of the carbodiimides with OxymaPure. Thus, both DIC (entry 1) and TBEC (entry 5) gave excellent results, showing no major differences in performance. On the other hand, the structurally similar DSBC gave a slightly poorer but acceptable performance, as did EDC·HCl. Finally, the performance of sterically hindered DTBC was much poorer, to the extent that it cannot be used in SPPS.

Table 2. Purities of the Peptides Synthesized Using Carbodiimide/OxymaPure[a] as determined by HPLC

entry	carbodiimide	Leu-enkephalin (%)	$Ile^{2,3}$-Leu-enkephalin (%)
1	DIC	>99.9	96.7
2	EDC·HCl	90.0	79.7
3	DTBC	72.5	39.8
4	DSBC	91.4	82.8
5	TBEC	98.4	96.6

[a]Couplings were performed using 5 equiv of reagents without preactivation (in situ activation) in DMF at 25 °C for 30 min.

Finally, given that EDC·HCl is a reagent commonly found in all chemistry laboratories and that its use is not accompanied by the formation of HCN, we addressed SPPS optimization using this carbodiimide. Although preactivation gave very good results for nonhindered protected amino acids (Leu-enkephalin; Table 3, entry 2), in situ activation rendered better results overall (Table 3, entry 1). Finally, the addition of an equimolar amount of DIEA to the in situ activation using EDC·HCl gave results similar to those found with DIC and TBEC (Table 3, entry 3, vs Table 2, entries 1 and 5).

https://doi.org/10.1021/acs.orglett.1c02466
Org. Lett. 2021, 23, 6900−6904

Table 3. Purities of the Peptides Synthesized Using EDC·HCl/OxymaPure[a] as Determined by HPLC

entry	conditions	Leu-enkephalin (%)	Ile[2,3]-Leu-enkephalin (%)
1	in situ activation[b]	90.0	79.7
2	preactivation[c]	>99.9	52.9
3	in situ activation[d]	98.3	92.8

[a]Couplings were performed for 30 min using 5 equiv of reagents in DMF at 25 °C for 30 min. [b]Table 2, entry 2. [c]Preactivation of the protected amino acid for 5 min. [d]Preactivation of the protected amino acid for 5 min in the presence of 5 equiv of DIEA.

In conclusion, we have demonstrated that not all carbodiimides have the same reactivity with OxymaPure. Their reactivity is modulated by the steric hindrance of the alkyl moieties that flank the carbodiimide backbone. Thus, tertiary carbon groups such as the *tert*-butyl groups in DTBC prevent the formation of the adduct, which is the first step of the secondary reaction. In contrast, primary substituents, such as those in EDC·HCl, enhance the formation of the adduct, but this compound does not evolve to the oxadiazole, and therefore, HCN does not form. Finally, in this structural analysis, the use of carbodiimides flanked by secondary substituents, namely, DIC, DCC, and DSBC, led to the formation of the oxadiazole with the formation of HCN. From the perspective of coupling efficiency, all of the carbodiimides except DTBC were found to be suitable for SPPS.

To avoid the formation of HCN, the use of the hybrid TBEC, which combines the good performance of the tertiary substituents (no reaction at all) and primary substituents (only formation of the adduct), or a carbodiimide with two primary substituents such as EDC·HCl would ensure a HCN-free reaction. Preliminary results carried out in our laboratory have shown that in a standard coupling, formation of the oxadiazole is not observed at all when the activation is carried out either with TBEC or EDC·HCl in the presence of DIEA. Furthermore, racemization using TBEC for protected His, which is the most demanding amino acid, is comparable to that observed with DIC (a complete study will be published elsewhere). To reinforce the validity of TBEC, Izdebski and Kunce reported that this carbodiimide (it was called BEC) showed performance similar to that of DIC in a model study.[23]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.orglett.1c02466.

Materials and methods, reactivity of carbodiimides with OxymaPure, solid-phase peptide synthesis, and LC−MS of the products of all reactions carried out and peptides synthesized (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

Fernando Albericio − *Peptide Science Laboratory, School of Chemistry and Physics, University of KwaZulu-Natal, Durban 4001, South Africa; Institute for Advanced Chemistry of Catalonia (IQAC−CSIC), 08034 Barcelona, Spain; CIBER-BBN, Networking Centre on Bioengineering, Biomaterials and Nanomedicine, and Department of Organic Chemistry, University of Barcelona, 08028 Barcelona, Spain;* orcid.org/0000-0002-8946-0462; Email: albericio@ukzn.ac.za

Beatriz G. de la Torre − *KwaZulu-Natal Research Innovation and Sequencing Platform (KRISP), School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, Durban 4041, South Africa;* orcid.org/0000-0001-8521-9172; Email: garciadelatorreb@ukzn.ac.za

Authors

Srinivasa Rao Manne − *Peptide Science Laboratory, School of Chemistry and Physics, University of KwaZulu-Natal, Durban 4001, South Africa*

Omar Luna − *Institute for Advanced Chemistry of Catalonia (IQAC−CSIC), 08034 Barcelona, Spain; CIBER-BBN, Networking Centre on Bioengineering, Biomaterials and Nanomedicine, and Department of Organic Chemistry, University of Barcelona, 08028 Barcelona, Spain*

Gerardo A. Acosta − *Institute for Advanced Chemistry of Catalonia (IQAC−CSIC), 08034 Barcelona, Spain; CIBER-BBN, Networking Centre on Bioengineering, Biomaterials and Nanomedicine, and Department of Organic Chemistry, University of Barcelona, 08028 Barcelona, Spain*

Miriam Royo − *Institute for Advanced Chemistry of Catalonia (IQAC−CSIC), 08034 Barcelona, Spain; CIBER-BBN, Networking Centre on Bioengineering, Biomaterials and Nanomedicine, and Department of Organic Chemistry, University of Barcelona, 08028 Barcelona, Spain;* orcid.org/0000-0001-5292-0819

Ayman El-Faham − *Department of Chemistry, Faculty of Science, Alexandria University, Alexandria 21321, Egypt;* orcid.org/0000-0002-3951-2754

Gyorgy Orosz − *Luxembourg Bio Technologies, Ness Ziona 7403631, Israel*

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.orglett.1c02466

Author Contributions

[▽]S.R.M. and O.L. contributed equally. The strategy was designed by all of the authors. The experiments were mainly carried out by S.R.M and O.L. All of the authors discussed the results and prepared the manuscript.

Notes

The authors declare no competing financial interest.

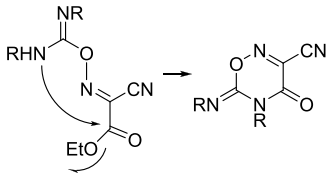
■ ACKNOWLEDGMENTS

We thank Yoav Luxembourg (Luxembourg Bio Technologies) for encouraging this work. This project received funding from the European Union's Horizon 2020 Research and Innovation Programme under Marie Skłodowska-Curie Grant Agreement 861190 (PAVE). O.L.'s work was funded by PAVE. The authors acknowledge an American Chemical Society (ACS) Green Chemistry Institute (GCI) Pharmaceutical Roundtable Ignition Grant awarded to S.R.M. The work carried out in South Africa was partially funded by the National Research Foundation (NRF) (Blue Sky's Research Programme 120386). Peptide preparation was performed at the NANBIOSIS−CIBER BBN Peptide Synthesis Unit (U3).

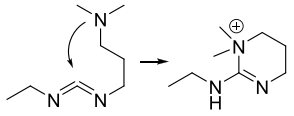
■ REFERENCES

(1) Roughley, S. D.; Jordan, A. M. *J. Med. Chem.* **2011**, *54*, 3451−3479.

(2) Cooper, T. W. J.; Campbell, I. B.; Macdonald, S. J. F. *Angew. Chem., Int. Ed.* **2010**, *49*, 8082−8091.
(3) Bryan, M. C.; Dunn, P. J.; Entwistle, D.; Gallou, F.; Koenig, S. G.; Hayler, J. D.; Hickey, M. R.; Hughes, S.; Kopach, M. E.; Moine, G.; Richardson, P.; Roschangar, F.; Steven, L. A.; Weiberth, F. J. *Green Chem.* **2018**, *20*, 5082−5103.
(4) Albericio, F. *Biopolymers* **2000**, *55*, 123−139.
(5) Isidro-Llobet, A.; Álvarez, M.; Albericio, F. *Chem. Rev.* **2009**, *109*, 2455−2504.
(6) El-Faham, A.; Albericio, F. *Chem. Rev.* **2011**, *111*, 6557−6602.
(7) Sheehan, J. C.; Hess, G. P. A. *J. Am. Chem. Soc.* **1955**, *77*, 1067−1068.
(8) Merrifield, R. B. *J. Am. Chem. Soc.* **1963**, *85*, 2149−2154.
(9) Jad, Y. E.; El-Faham, A.; De la Torre, B. G.; Albericio, F. In *Peptide-Based Drug Discovery: Challenges and Opportunities*; Srivastava, V., Ed.; Discovery Series, No. *59*; Royal Society of Chemistry: London, 2017; pp 518−550.
(10) König, W.; Geiger, R. *Chem. Ber.* **1970**, *103*, 788−798.
(11) Barlos, K.; Papaioannou, D.; Theodoropoulos, D. *Int. J. Pept. Protein Res.* **1984**, *23*, 300−305.
(12) Abdelmoty, I.; Albericio, F.; Carpino, L. A.; Foxman, B. F.; Kates, S. A. *Lett. Pept. Sci.* **1994**, *1*, 57−67.
(13) Wehrstedt, K. D.; Wandrey, P. A.; Heitkamp, D. *J. Hazard. Mater.* **2005**, *126*, 1−7.
(14) Subirós-Funosas, R.; Prohens, R.; Barbas, R.; El-Faham, A.; Albericio, F. *Chem. - Eur. J.* **2009**, *15*, 9394−9403.
(15) El-Faham, A.; Albericio, F. *J. Pept. Sci.* **2010**, *16*, 6−9.
(16) El-Faham, A.; Subirós-Funosas, R.; Prohens, R.; Albericio, F. *Chem. - Eur. J.* **2009**, *15*, 9404−9416.
(17) Subirós-Funosas, R.; El-Faham, A.; Albericio, F. *Org. Biomol. Chem.* **2010**, *8*, 3665−3673.
(18) Albericio, F.; El-Faham, A. *Org. Process Res. Dev.* **2018**, *22*, 760−772.
(19) El-Faham, A.; Subirós-Funosas, R.; Albericio, F. *Eur. J. Org. Chem.* **2010**, *2010*, 3641−3649.
(20) McFarland, A. D.; Buser, J. Y.; Embry, M. C.; Held, C. B.; Kolis, S. P. *Org. Process Res. Dev.* **2019**, *23*, 2099−2105.
(21) Erny, M.; Lundqvist, M.; Rasmussen, J. H.; Ludemann-Hombourger, O.; Bihel, F.; Pawlas, J. *Org. Process Res. Dev.* **2020**, *24*, 1341−1349.
(22) A tentative mechanism for the formation of the six-membered-ring cyclic compound from the intial adduct is shown below:For



unsymmetrical carbodiimides such as EDC·HCl, two isomers were observed. Interestingly, this product was not observed after reaction at 60 °C for 16 h, presumably because at this temperature the autoreaction of EDC to give its own adduct is favored:



(23) Izdebski, J.; Kunce, D. *J. Pept. Sci.* **1997**, *3*, 141−144.

https://doi.org/10.1021/acs.orglett.1c02466
Org. Lett. 2021, 23, 6900−6904