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HPLC and SFC Enantioseparation of (±)-*Trans*-β-Lactam Ureas on Immobilized Polysaccharide-Based Chiral Stationary Phases—The Introduction of Dimethyl Carbonate as an Organic Modifier in SFC

Mladenka Jurin * , **Darko Kontrec and Marin Roje** *

Laboratory for Chiral Technologies, Division of Organic Chemistry and Biochemistry, Ruđer Bošković Institute, Bijenička cesta 54, 10 000 Zagreb, Croatia; darko.kontrec@irb.hr

* Correspondence: mladenka.jurin@irb.hr (M.J.); marin.roje@irb.hr (M.R.)

Abstract: A series of nine racemic *trans*-β-lactam ureas were analyzed for enantiomer separation by high-performance liquid chromatography (HPLC) and supercritical fluid chromatography (SFC). The separations were performed on three immobilized polysaccharide-based chiral analytical columns (CHIRAL ART Amylose-SA, CHIRAL ART Cellulose-SB and CHIRAL ART Cellulose-SC). In HPLC mode, a normal-phase consisting of *n*-hexane/2-PrOH (90/10, *v*/*v*), a polar organic mobile phase consisting of 100% MeOH or 100% EtOH, and a non-standard mobile phase consisting of 100% dimethyl carbonate (DMC) were investigated. In SFC mode, the mobile phases CO_2/alcohol (80/20, *v*/*v*) and CO_2/DMC/alcohol (MeOH or EtOH; 70/24/6, *v*/*v*/*v* or 60/32/8, *v*/*v*/*v*) were investigated. The best achieved enantioseparation of *trans*-β-lactam ureas was obtained with an Amylose-SA column. We have shown that the green solvent dimethyl carbonate (DMC) can be efficiently used as a mobile phase in HPLC mode as well as in SFC mode along with the addition of polar organic modifiers (MeOH or EtOH).

Keywords: *trans*-β-lactam ureas; enantioseparation; chiral chromatography; high-performance liquid chromatography (HPLC); supercritical fluid chromatography (SFC); dimethyl carbonate (DMC); immobilized polysaccharide-based CSPs; green solvents

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1. Introduction

Azetidin-2-ones, also known as β-lactams, are four-membered cyclic amides [1–4]. The β-lactam ring is the central structural element responsible for the antibacterial activity of β-lactam antibiotics, which are among the most commonly used types of antibiotics, such as penicillins, carbapenems, cephalosporins, nocardicins, and monobactams [5]. In addition, they show numerous other interesting pharmacological activities, such as cholesterol absorption inhibitors, human cytomegalovirus protease [6], tryptase and chymase inhibitors, thrombin inhibitors [5], and LHRH antagonists [7]. β-Lactams also have anticancer, antiviral [8], antitubercular, antifungal [6], anti-HIV, anti-inflammatory, and other biological activities [9]. β-Lactams can serve as building blocks for the synthesis of other compounds of biological and medicinal importance, such as β-amino acids, peptides, peptidomimetics, taxoids, alkaloids, and various heterocyclic molecules [7,10].

Chiral high-performance liquid chromatography (HPLC) on chiral stationary phases (CSPs) is one of the most commonly used analytical techniques for the enantioseparation of chiral β-lactams [11–20]. Pirkle et al. separated some β-lactam stereoisomers **1–7** (Figure 1) with aryl substituents at the C3 position and with alkyl or aryl substituents at the C4 position of the ring by HPLC on chiral stationary phase based on (*S*)-*N*-(3,5-dinitrobenzoyl)leucine, using *n*-hexane/2-propanol (80/20, *v*/*v*) as the mobile phase [11]. Lee et al. [12] separated the β-lactam stereoisomers with alkyl substituents in the 3-position and with aryl, furyl, or

styryl substituents **8–16** (Figure 1) in the 4-position of the β-lactam ring using an (*R*)-1-{1-naphthyl)ethylamine polymer chemically bonded to spherical silica (YMC A-K03 column). In this study, *n*-hexane/dichloromethane/ethanol (70/30/2, *v*/*v*/*v*) was used as the mobile phase. Cirilli et al. [13] reported the separation of stereoisomers of a C3, C4-substituted β-lactam cholesterol absorption inhibitor (*cis*-**17** and *trans*-**17**, Figure 1) on amylose-based chiral stationary phases (Chiralpak AD-H and Chiralpak AS-H columns) in the normal-phase mode using the different binary mixtures *n*-hexane/ethanol and *n*-hexane/2-propanol as the mobile phases. Among them, amylose *tris*[(*S*)-α-methylphenylcarbamate] CSP (Chiralpak AS-H) was more effective, and the resolutions were higher than those obtained with amylose *tris*(3,5-dimethylphenylcarbamate) CSP (Chiralpak AD-H).

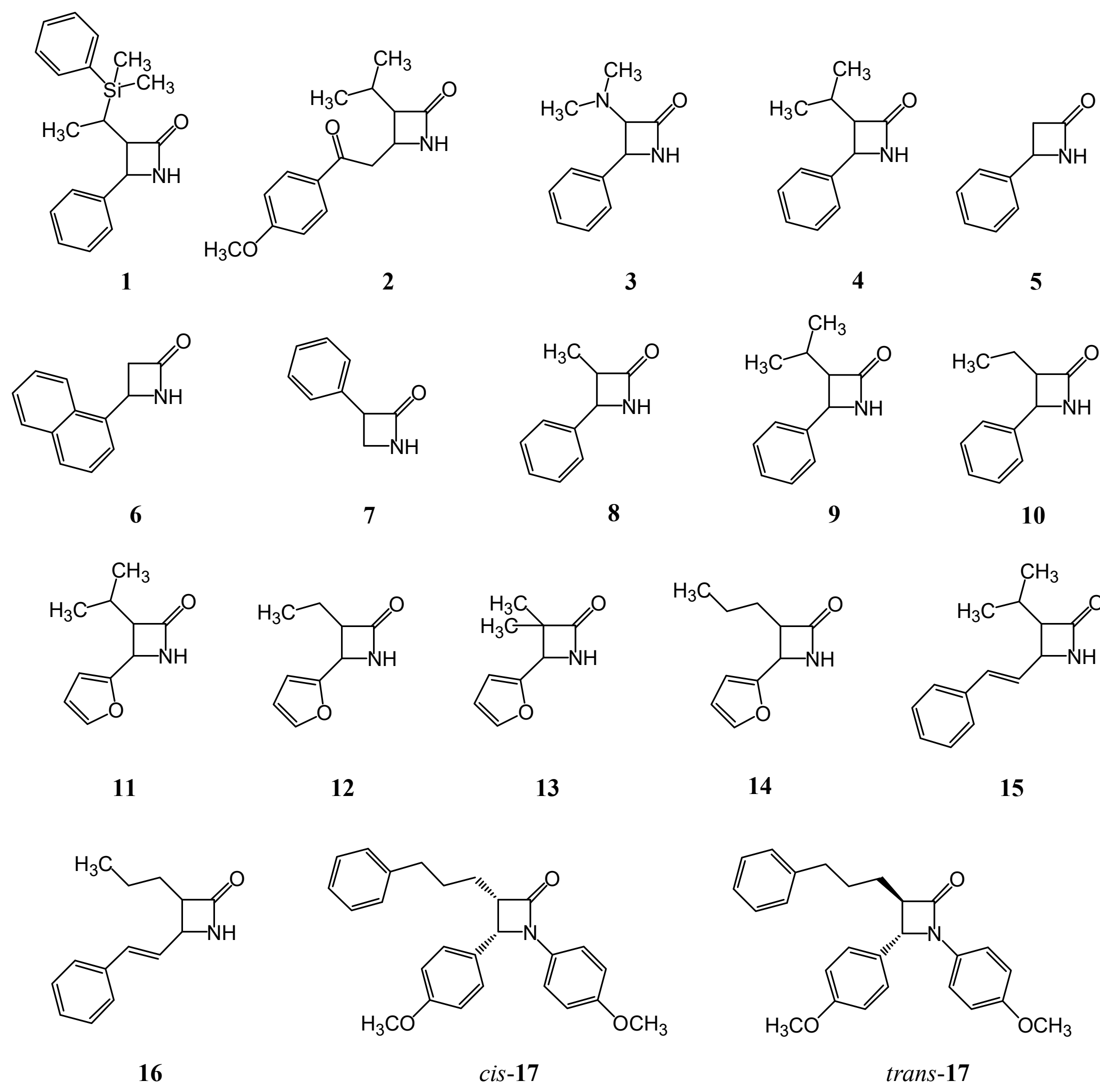

Figure 1. Chemical structures of β-lactam compounds separated by Pirkle et al. (**1**–**7**), Lee et al. (**8**–**16**), and Cirilli et al. (**17**).

Tris(phenylcarbamates) from amylose- or cellulose-based chiral stationary phases were used by Okamoto et al. [14] for the enantioseparation of various β-lactam compounds **18–35** (Figure 2). The authors used mobile phases consisting of mixtures of *n*-hexane and 2-propanol (80/20, *v*/*v* or 90/10, *v*/*v*). Most of the tested β-lactam compounds were completely resolved on the cellulose and/or amylose *tris*(phenylcarbamate) derivatives coated on the silica matrix. Pataj et al. [15] developed a direct HPLC method for the enantioseparation of nineteen racemic β-lactams **36–54** (Figure 3) on polysaccharide-based CSPs containing either amylose *tris*(3,5-dimethylphenylcarbamate) (Kromasil AmyCoat col-

umn) or cellulose *tris*(3,5-dimethylphenylcarbamate) (Kromasil CelluCoat column) as the chiral selectors. They analyzed these racemic β-lactams in normal-phase mode using mixtures of *n*-heptane with various amounts (2–10%) of polar alcoholic modifier (2-propanol). Reducing the alcohol content in the mobile phases led to better separation on the two columns tested. The amylose column Kromasil AmyCoat proved to be more suitable for the separation of the bi- and tricyclic β-lactams (all except compound **47**), whereas the 4-aryl-substituted β-lactams (all except compound **54**) were better separated on the cellulose column Kromasil CelluCoat.

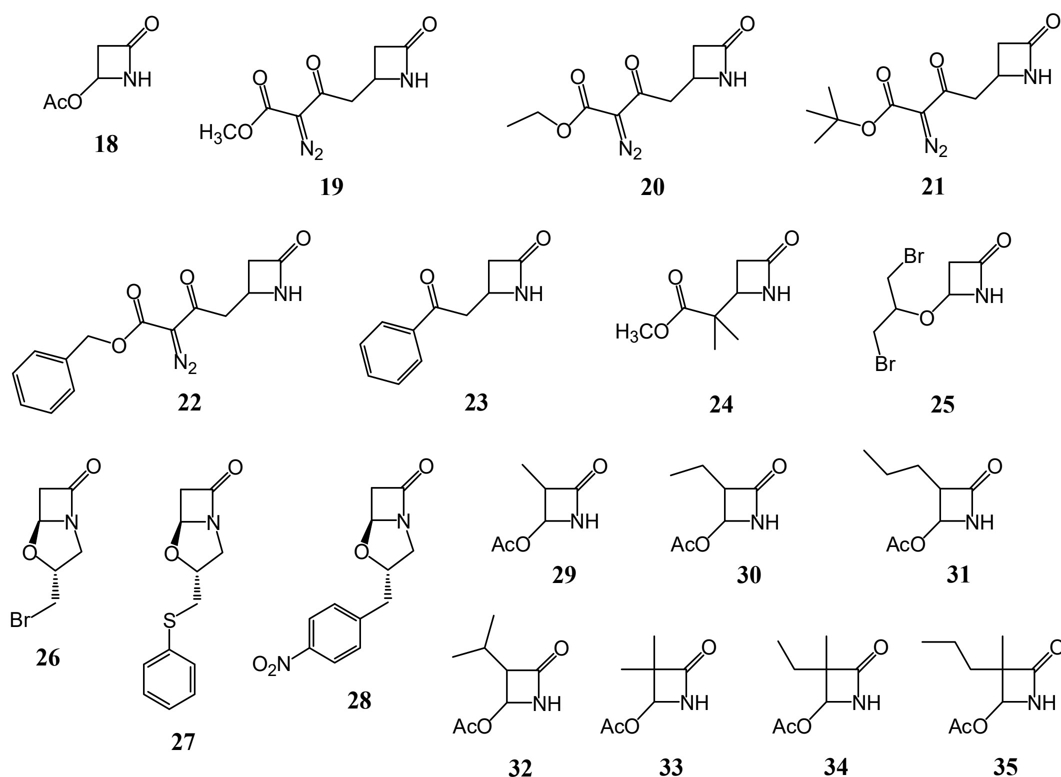

Figure 2. Chemical structures of β-lactam compounds **18**–**35** separated by Okamoto et al [14].

HPLC enantioseparation of the same twelve racemic bicyclic β-lactam compounds **36**–**47** (Figure 3) was investigated by Peter et al. [16] on two types of CSPs, one of which was cellulose *tris*(3,5-dimethylphenylcarbamate) (Chiralcel OD-RH and Chiralcel OD-H column) and the other containing a macrocyclic glycopeptide antibiotic teicoplanin (Chirobiotic T column) or teicoplanin aglycone (Chirobiotic TAG column) as a chiral selector. The authors investigated separation in different chromatographic modes. First, they investigated possibilities for HPLC separation on cellulose columns in the normal-phase mode (mixture of *n*-hexane and 2-propanol in different ration) and in the reversed-phase mode using water with different concentration of acetonitrile. In the next set of experiments, they investigated separation of compounds **36**–**47** on teicoplanin and teicoplanin aglycone CSPs in the polar organic mode (100% methanol or methanol/acetic acid/triethylamine, 100/0.01/0.01, *v*/*v*/*v*) and in the reversed-phase mode (water/methanol, 30/70 or 70/30, *v*/*v*; and 0.1% triethylammonium acetate pH = 4.1/methanol, 30/70 or 70/30, *v*/*v*). The result indicated that the aglycone alone was responsible for the enantioselective separation of bicyclic β-lactam compounds **36**–**47**. The resolution factors were higher for the aglycone CSP (Chirobiotic TAG column). Although the sugar units generally reduced the resolution of β-lactam enantiomers, they could contribute significantly to the resolution of some other compounds. The best enantioseparation of these β-lactams was obtained on the Chiralcel OD-H column in the normal-phase mode.

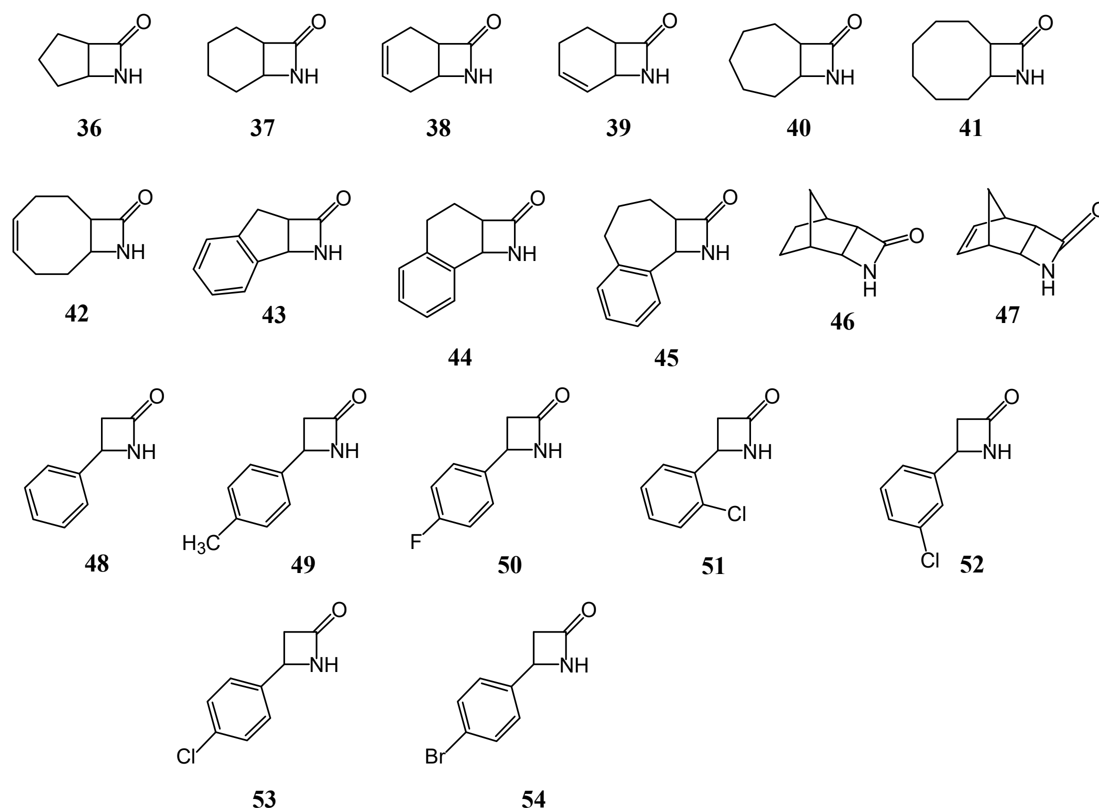

Figure 3. Chemical structures of bi- and tricyclic and 4-aryl-substituted β-lactams **36–54**.

The chiral recognition mechanisms for both polysaccharide and macrocyclic antibiotic CSP are not yet fully understood. There are interactions between enantiomers and CSP that are important for both general retention and enantioseparation. When considering retention, hydrophobic interactions (π-π interactions) are important in the reversed-phase mode, whereas hydrophilic interactions (hydrogen bonds) are important in the normal-phase mode and in the polar organic mode. However, several types of interactions can be considered in the case of enantioseparation.

Sun et al. [17] investigated the HPLC enantioseparation of the twelve β-lactam stereoisomers **36–47** (Figure 3) on three native cyclodextrin-based CSPs (α-, β-, and γ-) and on six derivatized β-cyclodextrins (acetylated, dimethylated, hydroxypropyl ether, dimethylphenyl carbamate, *S*-naphthylethyl carbamate, and *R*-naphthylethyl carbamate). On all cyclodextrin (CD) columns, the β-lactams were analyzed in the reversed-phase mode, on eight columns (except demethylated β-CD) in the polar organic mode, and on three aromatic derivatized β-cyclodextrin columns in the normal-phase mode. The dimethylphenyl carbamate β-CD proved to be the best CSP, separating eleven of twelve β-lactam compounds in the reversed-phase mode, whereas the dimethylated β-CD separated eight of twelve compounds. The other derivatized β-cyclodextrin CSPs and the native γ-cyclodextrin achieved enantioseparation for some β-lactams. The native α- and β-cyclodextrin CSPs did not separate any of the investigated β-lactams. As these β-lactams have no ionizable groups, the pH of the mobile phase has no major influence on the enantioseparation. When CD-based CSPs are used in polar organic or normal-phase media, the inner cavity is blocked by solvent molecules, which prevents the complexation of inclusions. Nevertheless, hydrophilic interactions can be enhanced in such media when solutes with hydrophilic groups bind to the polar surface of the CD. Derivatized CDs have been developed to allow additional intermolecular interactions, such as π–π interactions, hydrogen bonding, dipole–dipole interactions, and ion pairing, resulting in an improved ability for enantioseparation. In the reversed-phase mode, inclusion complexation is the dominant

retentive interaction, whereas CSPs form dipolar and π-complexes in the normal-phase mode. Hydrogen bonding interactions are the most important in the polar organic mode.

Berkecz et al. [18] used macrocyclic glycopeptide antibiotic teicoplanin (Chirobiotic T column) and its aglycone (Chirobiotic TAG column) as well as a dimethylphenyl carbamate-derivatized β-cyclodextrin (Cyclobond DMP column) as chiral selectors for enantioseparation of three tricyclic chiral β-lactams **43–45** (Figure 3). These compounds contain a five- **43**, six- **44**, or seven-membered **45** aliphatic ring fused to a four-membered β-lactam ring and a benzene ring. In this study, the authors investigated the separations of compounds **36–47** in the normal-phase, polar organic, and reversed-phase modes. The size of the aliphatic ring, the nature of the CSPs, and the composition of the mobile phase influence the chiral recognition mechanism.

For the enantioseparation of the β-lactams **36–48** (Figure 3), Fodor et al. [19] used CSPs based on β-cyclodextrin (Quest-C1, Quest-C2, and Quest-C3) with HPLC in the reversed-phase mode. The Quest-C1 column, containing permethyl-β-cyclodextrin as a chiral selector, proved to be the most effective for this group of β-lactam compounds. The native β-CD (Quest C3) and its derivative hydroxypropyl-β-CD (Quest C2) CSPs showed enantioselectivity for some β-lactams. Jiang et al. used capillary electrophoresis based on β-cyclodextrin for the enantioseparation of racemic β-lactams [20].

Recently, we reported on the SFC enantioseparation of seven racemic β-lactams, **55–61** (Figure 4), on a polysaccharide-based CSPs containing either amylose *tris*(3,5-dimethylphenylcarbamate) (Chiralpak AD and Chiralcel IA columns), cellulose *tris*(3,5-dimethylphenylcarbamate) (Chirapcel OD and Chiralcel IB columns), or cellulose *tris*(4-methylphenylcarbamate) (Chirallica PST-10 column) as the chiral selectors [21]. The effect of CSP type (coated or immobilized) on the enantioseparation of *trans*-β-lactam ureas was investigated on all five columns, whereas the effect of alcoholic modifiers (methanol, ethanol, or 2-propanol), additive (isopropylamine), temperature, and backpressure were investigated on the Chirallica PST-10 column. The article demonstrated that the Chiralcel OD and Chiralpak IB columns provided better baseline separation than their amylose analogs, the Chiralpak AD and Chiralpak IA columns. The Chirallica PST-10 column separated all seven compounds tested. The effects of the three other parameters investigated—temperature, addition of isopropylamine and backpressure—showed little or no influence on the separation factor and resolution.

In our recent work, we have shown that DMC, an environmentally friendly solvent, can be efficiently used as a mobile phase in HPLC for the enantioseparation of *syn*- and *anti*-3,5-disubstituted hydantoins on the immobilized polysaccharide-based chiral stationary phases [22]. The CHIRAL ART Amylose-SA column was the most effective stationary phase, separating fourteen out of eighteen substituted hydantoins in non-standard mobile phase mode. Of the cellulose-based columns, the CHIRAL ART Cellulose-SB column proved to be more suitable for the enantioseparation of *anti*-3,5-hydantoins than the CHIRAL ART Cellulose-SC column, whereas the two cellulose columns did not exhibit enantioselectivity of *syn*-hydantoins with DMC as mobile phase.

In this study, the enantioseparation of nine chiral *trans*-β-lactam ureas, **55**, **57**, **60**, and **62–67** (Figure 4), on three different polysaccharide-type CSPs was investigated in HPLC mode with the four mobile phases *n*-hexane/2-propanol (90/10, *v*/*v*), methanol (MeOH), ethanol (EtOH), and dimethyl carbonate (DMC) and in SFC mode with the solvent mixtures CO_2/alcohol (80/20, *v*/*v*), CO_2/DMC/alcohol (70/24/6, *v*/*v*/*v*), and CO_2/DMC/alcohol (60/32/8, *v*/*v*/*v*). The aim of the present study was to introduce DMC as an organic solvent in supercritical fluid chromatography. To our knowledge, no data are available in the literature for chiral separations of racemic compounds using DMC in the SFC.

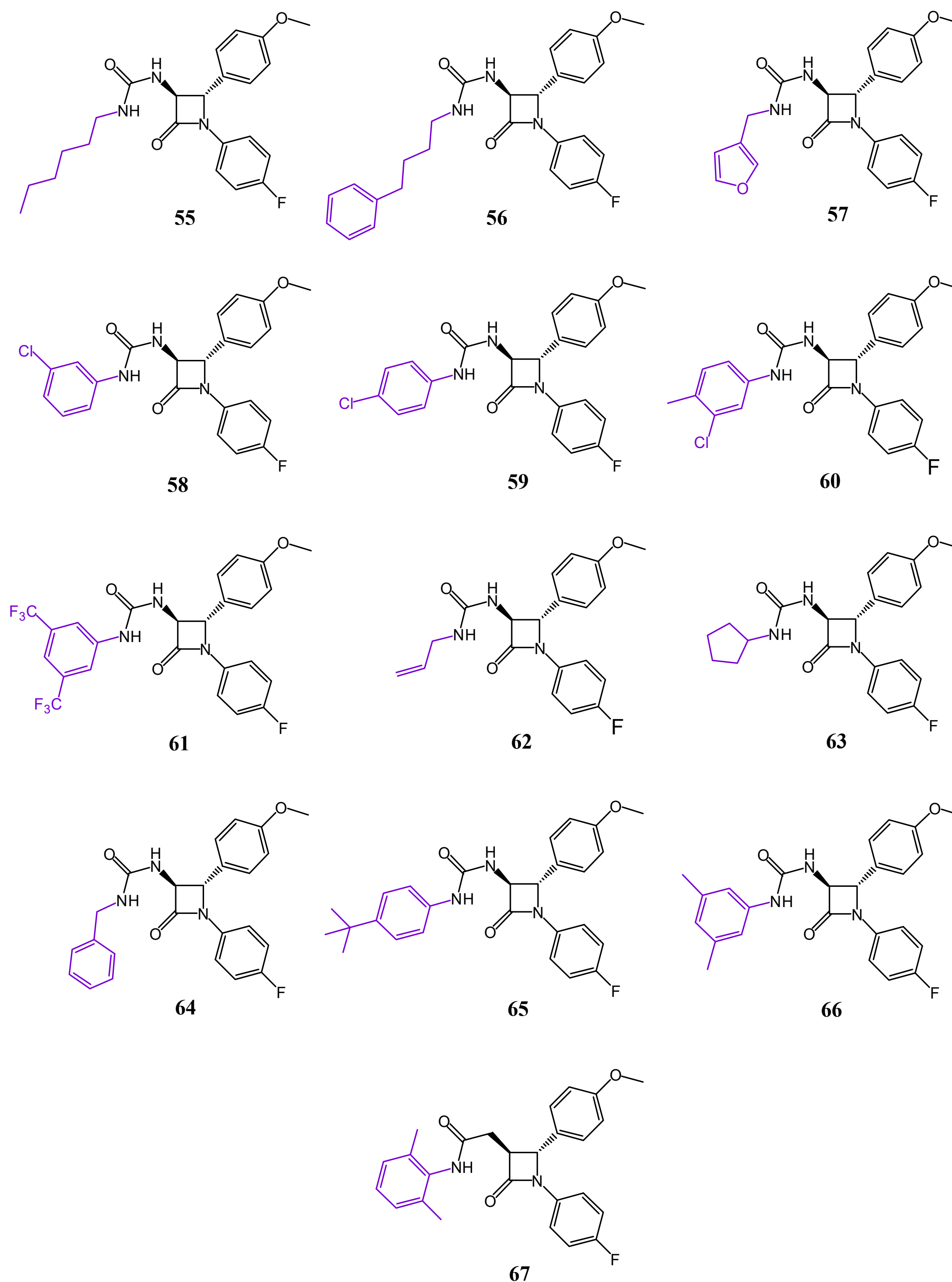

Figure 4. Chemical structures of (±)-*trans*-β-lactam ureas **55–67**.

2. Materials and Methods

The nine racemic β-lactam ureas were prepared in our laboratory by the addition of the corresponding isocyanate to (±)-*trans*-3-amino-β-lactam, which was prepared in three reaction steps [23].

HPLC-grade ethanol (EtOH), methanol (MeOH), 2-propanol (2-PrOH) and *n*-hexane were purchased from Honeywell (Seelze, Germany). Dimethyl carbonate (DMC) was purchased from Acros Organics (Geel, Belgium). Compressed CO_2 (class 4.5) was purchased from Messer (Zagreb, Croatia). The immobilized polysaccharide-based CSPs CHIRAL ART

Amylose-SA S-10 µm, CHIRAL ART Cellulose-SB S-10 µm, and CHIRAL ART Cellulose-SC S-10 µm were purchased in bulk from YMC (Kyoto, Japan). Empty stainless steel HPLC columns measuring 250 mm × 4.6 mm i.d. were purchased from Knauer GmbH (Berlin, Germany) and packed with the above CSPs.

HPLC analyses were performed using an Agilent 1200 Series system (Agilent Technologies GmbH, Waldbronn, Germany) consisting of a vacuum degasser, a quaternary pump, a thermostated column compartment, an autosampler, and a variable wavelength detector. The mobile phase was *n*-hexane/2-PrOH (90/10 *v*/*v*), 100% MeOH, 100% EtOH, or 100% DMC. All experiments in the normal-phase and polar and non-standard modes were performed under isocratic conditions at a flow rate of 1.0 mL min^{-1} and a column temperature of 35 °C. Detection was performed at 254 nm, and the injection volume was 20 µL. Data analysis and processing was performed using EZChrom Elite software version 3.1.7 (Agilent Technologies, Waldbronn, Germany).

The SFC analyses were performed with an Agilent 1260 Infinity II Hybrid SFC/UHPLC system (Agilent Technologies, Waldbronn, Germany). It consisted of an Infinity SFC binary pump, an Aurora A5 Fusion module, a degasser, an autosampler, a thermostated column compartment, a diode array detector, and a backpressure regulator. The system was controlled by the Open LAB CDS ChemStation Edition Rev. C01.08 software (Agilent Technologies, Waldbronn, Germany). The SFC was performed in isocratic mode at a flow rate of 4.0 mL min^{-1} and a column temperature of 35 °C in each case. The injection volume was 20 µL, and the outlet pressure was set to 15 MPa. Detection was performed at a wavelength of 254 nm using a diode array detector. The mobile phases used in the SFC consisted of liquid CO_2/alcohol (MeOH or EtOH) in a ratio of 80/20, *v*/*v* or CO_2/DMC/alcohol (MeOH or EtOH) in various ratios (70/24/6, *v*/*v*/*v* or 60/32/8, *v*/*v*/*v*). The sample solutions of the analytes were prepared by dissolving the β-lactam ureas **55**, **57**, **60** and **62–67** (Figure 4) in *n*-hexane/2-PrOH (90/10, *v*/*v*), 100% DMC, 100% MeOH, or 100% EtOH at a concentration of 0.5 mg mL^{-1} and filtered through a RC-45/25 Chromafil® Xtra 0.45 µm syringe filter (Macherey-Nagel GmbH & Co. KG, Düren, Germany).

The HPLC columns were packed with the immobilized chiral polysaccharide-based stationary phases from YMS CHIRAL ART Amylose-SA, CHIRAL ART Cellulose-SB, and CHIRAL ART Cellulose-SC. The size of the columns was 250 mm × 4.6 mm i.d., and the particle size was 10 µm. In the following text, these columns are referred to as Amylose-SA, Cellulose-SB, and Cellulose-SC. The chiral selectors in Amylose-SA, Cellulose-SB, and Cellulose-SC are amylose *tris*(3,5-dimethylphenylcarbamate), cellulose *tris*(3,5-dimethylphenylcarbamate), and cellulose *tris*(3,5-dichlorophenylcarbamate), respectively; all three are shown in Figure 5.

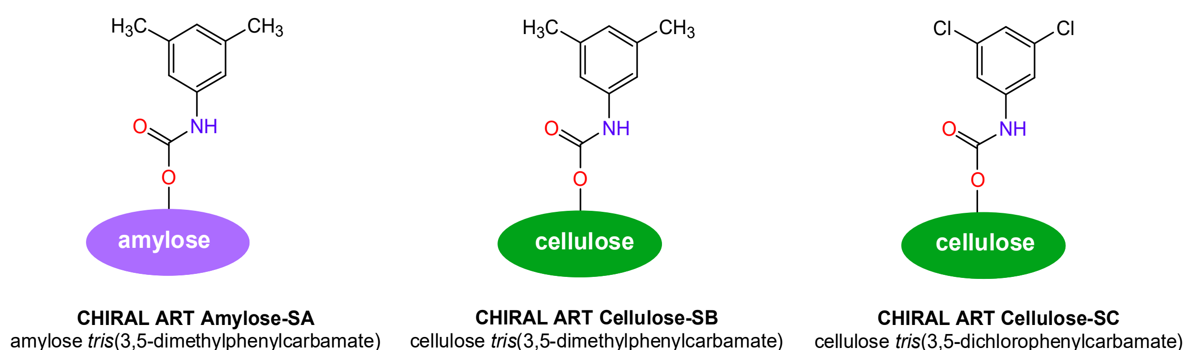

Figure 5. Chemical structure of the chiral selectors.

The retention factor (*k*) is a means of measuring the retention of an analyte on the chromatographic column [23]. It is calculated according to the following equation:

$$k = (t_R - t_0)/t_0, \quad (1)$$

where t_R and t_0 are the retention times of the analyte and the non-retained solute, respectively. The greatest gain in resolution is achieved when the k value is between 1 and 5. A high k value indicates that the sample is strongly retained and has interacted with the stationary phase for a considerable time.

The selectivity of the column is expressed by the enantioseparation factor (α). The enantioseparation factor is the ability of an HPLC method to separate two analytes from each other. It is calculated according to the following equation:

$$\alpha = k_2/k_1, \tag{2}$$

where k_1 and k_2 are the retention factors of the first and second eluted enantiomers, respectively. By definition, the selectivity is always greater than one—if α is equal to one, the two peaks are co-eluting (i.e., their retention factor values are identical). Larger selectivity values indicate better separation. As the selectivity of a separation depends on the chemistry of the analyte, the mobile phase composition, and the nature of the stationary phase, all of these factors can be altered to change or optimize the selectivity of an HPLC separation.

The resolution (R_s) indicates whether two peaks are separated from each other. It is calculated according to the following equation:

$$R_s = (t_{R2} - t_{R1})/(w_{b1} + w_{b2}), \tag{3}$$

where t_{R1} and t_{R2} are the retention times of the first and second eluted enantiomers, respectively, and w_{b1} and w_{b2} are the baseline peak widths of the first and second eluted enantiomers, respectively. According to the above definition, $R_s \geq 1.5$ means that the two peaks are baseline resolved. A higher resolution means that the peaks are well separated from each other.

In HPLC mode, the dead time (t_0), i.e., the retention time of a non-adsorbing component, was determined by injection of 1,3,5-tri-*tert*-butylbenzene, whereas in SFC mode the t_0 of the columns was determined at a first negative signal by injecting MeOH.

3. Results and Discussion

Polysaccharide-based CSPs are certainly the most dominant and widely used CSPs for the analytical and preparative separation of enantiomers in recent years due to their remarkable stability and loading capacity [24]. Polysaccharide CSPs are classified into two types, coated and immobilized, based on the chemistry of the application of the chiral selector on the chromatographic support matrix (usually silica). In the coated type, the polymeric chiral selector (amylose or cellulose derivatives) is physically coated by an adsorption process, whereas, in the immobilized type, the chiral selector is bound by a chemical process [25]. The coated polysaccharide CSPs have limited resistance to many solvents, whereas the immobilized CSPs are more robust, and can be used with non-standard solvents, such as acetone, chloroform, dichloromethane, ethyl acetate, tetrahydrofuran, etc. [26].

A screening for the enantioseparation of the nine β-lactam ureas **55**, **57**, **60**, and **62–67** using three different immobilized polysaccharide-type CSPs, including two cellulose-based columns, Cellulose-SB and Cellulose-SC, as well as one amylose-based column, Amylose-SA, have been performed by applying the HPLC and SFC modes. In the HPLC mode, either a standard mobile phase consisting of *n*-hexane/2-PrOH (90/10, v/v) or a polar organic mobile phase consisting of 100% alcohol (MeOH or EtOH) was used. The immobilized-type chiral columns contain a chiral selector covalently bound to the silica gel support, which enables the use of an extended range of the organic solvents [25,26], so, in this study, 100% DMC was used as a non-standard solvent in HPLC mode and in combination with an alcoholic modifier (DMC/alcohol; 80/20, v/v) in SFC mode. The effects of mobile phases: CO_2/alcohol (80/20, v/v), CO_2/DMC/alcohol (70/24/6, $v/v/v$), and CO_2/DMC/alcohol (60/32/8, $v/v/v$) on the separation were investigated in SFC mode. Chromatographic parameters such as retention factor of the first eluting enantiomer (k_1), separation factor (α), and resolution (R_s) are summarized in Table 1 for HPLC and Table 2 for SFC.

Table 1. HPLC chromatographic results of the enantioselective separations of (±)-*trans*-β-lactam ureas **55**, **57**, **60**, and **62–67** on Amylose-SA, Cellulose-SB, and Cellulose-SC columns.

Compound		Amylose-SA			Cellulose-SB			Cellulose-SC		
	Mobile Phase	k_1	α	R_s	k_1	α	R_s	k_1	α	R_s
55	A	7.99	1.00	–	9.53	1.81	3.22	21.58	1.96	3.86
	B	0.23	1.96	1.12	0.19	1.63	1.22	0.19	1.00	–
	C	0.40	2.13	2.00	0.23	1.00	–	0.24	1.00	–
	D	0.51	3.20	3.79	0.34	1.00	–	1.26	1.30	1.11
57	A	13.58	1.11	0.46	14.77	1.85	3.79	41.18	1.86	3.79
	B	0.16	2.88	1.77	0.18	1.44	0.82	0.16	1.00	–
	C	0.30	4.33	3.87	0.27	1.00	–	0.26	1.00	–
	D	0.35	2.29	2.28	0.22	1.00	–	0.75	1.16	0.53
60	A	10.78	1.34	1.58	11.33	2.36	4.59	13.52	1.38	1.02
	B	1.11	1.00	–	0.38	3.37	5.56	0.16	1.56	0.93
	C	0.90	1.00	–	0.31	2.55	3.95	0.13	1.62	0.58
	D	0.52	2.31	2.73	0.22	2.23	2.89	0.32	1.58	1.06
62	A	9.46	1.00	–	10.35	1.99	4.03	26.33	2.13	4.73
	B	0.09	2.89	1.21	0.12	1.67	0.90	0.13	1.00	–
	C	0.24	2.83	2.46	0.20	1.00	–	0.23	1.00	–
	D	0.46	2.43	2.92	0.31	1.00	–	1.12	1.10	0.44
63	A	2.41	2.90	1.26	8.61	1.66	2.68	18.54	1.70	3.02
	B	0.13	3.15	1.70	0.13	2.31	1.84	0.13	1.46	0.48
	C	0.22	3.18	2.58	0.14	1.64	1.19	0.16	1.00	–
	D	0.57	4.37	5.41	0.29	1.48	1.54	0.91	1.46	1.53
64	A	14.01	1.26	1.20	17.43	1.75	3.68	34.84	1.92	3.89
	B	0.24	2.96	2.16	0.30	1.33	0.97	0.39	1.00	–
	C	0.46	4.72	4.58	0.37	1.00	–	0.28	1.00	–
	D	0.47	2.49	2.97	0.30	1.00	–	0.91	1.07	0.29
65	A	12.36	1.00	–	9.39	1.94	3.58	16.13	1.39	1.78
	B	0.60	1.37	0.46	0.36	2.64	4.10	0.19	1.58	1.01
	C	0.96	1.73	1.88	0.30	2.10	2.94	0.18	1.56	0.76
	D	0.50	3.52	3.99	0.18	2.33	2.66	0.37	1.78	1.51
66	A	10.11	1.00	–	15.06	1.69	2.82	18.36	1.27	1.31
	B	0.51	1.98	1.50	0.34	3.68	5.72	0.38	1.21	0.56
	C	0.49	1.57	1.15	0.32	2.53	3.94	0.16	1.44	0.47
	D	0.55	4.19	4.91	0.22	2.04	2.59	0.37	1.73	1.55
67	A	10.24	2.17	3.67	6.81	1.09	0.38	15.18	1.38	1.74
	B	0.23	2.22	1.42	0.09	2.22	1.27	0.12	1.00	–
	C	0.27	7.04	3.82	0.15	1.47	0.92	0.17	1.00	–
	D	0.66	1.26	0.63	0.15	2.00	1.92	0.86	1.00	–

Note: k_1, retention factor of first eluting enantiomer; α, separation factor; R_s, resolution; "–", no separation; mobile phase, A, *n*-hexane/2-PrOH (90/10, v/v); B, MeOH; C, EtOH; D, DMC; flow rate, 1 mL min^{-1}; detection, 254 nm; column temperature, 35 °C.

Table 2. SFC chromatographic results of the enantioselective separations of (±)-*trans*-β-lactam ureas **55**, **57**, **60**, and **62–67** on Amylose-SA, Cellulose-SB, and Cellulose-SC columns.

Compound		Amylose-SA			Cellulose-SB			Cellulose-SC		
	Mobile Phase	k_1	α	R_s	k_1	α	R_s	k_1	α	R_s
55	A	3.40	1.54	1.65	2.04	1.80	4.04	2.50	1.00	–
	B	4.01	1.51	1.79	2.64	1.35	2.15	2.73	1.39	1.63
	C	6.82	1.68	2.10	3.43	1.32	1.95	4.96	1.22	1.00
	D	2.31	1.79	1.95	1.47	1.30	1.13	1.66	1.16	0.28
	E	9.85	2.05	3.01	5.97	1.00	–	7.55	1.52	2.32
	F	3.60	2.07	2.61	2.14	1.00	–	2.66	1.46	1.58

Table 2. *Cont.*

Compound	Mobile Phase	Amylose-SA			Cellulose-SB			Cellulose-SC		
		k_1	α	R_s	k_1	α	R_s	k_1	α	R_s
57	A	3.21	1.95	3.01	2.83	1.42	2.79	3.16	1.00	–
	B	3.79	2.08	3.85	3.59	1.13	0.91	3.43	1.25	1.18
	C	4.58	1.93	2.95	3.47	1.24	1.57	4.89	1.13	0.54
	D	1.52	1.96	2.25	1.42	1.23	0.84	1.70	1.00	–
	E	6.58	1.80	2.88	5.26	1.00	–	6.90	1.42	1.97
	F	2.30	1.83	2.40	1.87	1.00	–	2.32	1.37	1.24
60	A	10.06	1.17	0.70	6.62	3.52	11.78	3.13	1.85	3.50
	B	8.92	1.25	1.21	5.65	3.23	10.71	2.87	1.66	2.59
	C	11.06	1.19	0.81	5.39	3.35	10.99	3.56	1.74	2.76
	D	3.50	1.26	0.88	1.99	3.27	7.82	1.04	1.77	1.67
	E	14.24	1.35	1.50	6.48	3.07	9.89	4.80	1.55	2.16
	F	4.63	1.40	1.51	2.13	2.90	6.99	1.42	1.58	1.47
62	A	2.17	1.57	1.70	1.66	1.62	2.92	2.20	1.00	–
	B	2.67	1.69	2.35	2.22	1.26	1.52	2.38	1.46	1.82
	C	4.20	1.60	1.91	2.75	1.27	1.48	4.21	1.27	1.22
	D	1.43	1.69	1.56	1.20	1.29	0.83	1.46	1.21	0.46
	E	6.20	1.74	2.35	4.59	1.00	–	6.09	1.62	2.62
	F	2.32	1.78	2.09	1.65	1.00	–	2.18	1.55	1.70
63	A	2.80	2.30	3.22	1.92	2.16	5.33	1.85	1.34	1.26
	B	3.11	2.55	4.14	1.38	3.09	4.24	2.51	1.00	–
	C	5.16	2.67	4.05	3.35	1.63	3.58	3.72	1.27	1.13
	D	1.76	2.77	3.34	1.42	1.62	2.25	1.21	1.28	0.57
	E	6.74	3.09	4.69	5.27	1.39	2.69	6.44	1.09	0.16
	F	2.78	3.19	4.36	1.86	1.39	1.70	2.31	1.00	–
64	A	5.28	6.65	4.02	4.17	1.40	3.05	4.15	1.00	–
	B	5.90	2.41	4.74	4.93	1.14	1.32	4.45	1.28	1.43
	C	7.50	2.23	3.87	4.94	1.17	1.30	6.24	1.13	0.64
	D	2.40	2.26	3.18	1.97	1.14	0.55	2.15	1.00	–
	E	10.37	2.09	3.93	7.38	1.00	–	8.83	1.39	1.94
	F	3.47	2.11	3.16	2.59	1.00	–	2.86	1.35	1.28
65	A	6.73	3.11	1.61	6.65	2.46	8.30	2.96	2.00	3.80
	B	7.49	1.33	1.54	6.29	2.23	7.50	3.01	1.82	3.05
	C	7.91	1.67	2.21	6.73	2.22	7.12	3.75	1.98	3.37
	D	2.70	1.73	2.07	2.63	2.21	5.48	1.14	1.98	2.15
	E	10.14	2.01	3.35	7.74	2.20	7.18	5.43	1.77	2.88
	F	3.97	2.09	3.18	2.64	2.19	5.43	1.67	1.76	1.95
66	A	5.80	1.69	2.26	4.76	3.66	11.48	3.11	1.67	2.86
	B	5.61	1.78	2.90	4.44	3.20	10.30	3.18	1.51	2.13
	C	7.58	1.87	2.74	4.10	3.28	9.85	4.07	1.64	2.52
	D	2.54	1.97	2.55	1.57	3.22	7.03	1.21	1.67	1.59
	E	9.39	2.40	4.48	5.59	2.80	8.81	5.88	1.48	2.01
	F	3.68	2.49	3.93	1.91	2.65	6.07	1.75	1.52	1.44
67	A	3.17	2.20	3.00	1.51	1.79	3.39	2.16	1.06	0.33
	B	3.54	3.84	4.94	1.66	1.55	2.56	2.43	1.00	–
	C	4.01	2.39	3.45	1.53	1.25	0.98	3.07	1.06	0.57
	D	1.41	2.42	2.78	0.73	1.08	0.30	1.08	1.00	–
	E	6.29	2.19	3.45	2.27	1.00	–	4.46	1.00	–
	F	2.31	2.26	3.13	0.87	1.00	–	1.56	1.00	–

Note: k_1, retention factor of first eluting enantiomer; α, separation factor; R_s, resolution; "–", no separation; mobile phase, A, CO_2/MeOH (80/20, v/v); B, CO_2/EtOH (80/20, v/v); C, CO_2/DMC/MeOH (70/24/6, $v/v/v$); D, CO_2/DMC/MeOH (60/32/8, $v/v/v$); E, CO_2/DMC/EtOH (70/24/6, $v/v/v$); F, CO_2/DMC/EtOH (60/32/8, $v/v/v$); flow rate, 4 mL min^{-1}; backpressure, 15 MPa; detection, 254 nm; column temperature, 35 °C.

The analyzed racemic *trans*-β-lactam ureas **55**, **57**, **60**, and **62–67** (Figure 4) have the same β-lactam ring with two stereogenic centers at the C3 and C4 positions of the β-lactam ring. They contain various substituents on the ureido group attached to the C3 position of the ring, such as alkyl, hexyl, cyclohexyl, furfuryl, and various substituted phenyls. The type of the substituent in the structure of the analyte and the type of the polysaccharide selector significantly influence chiral recognition through multiple interactions. It is noted that chiral recognition of racemic solutes on polysaccharide CSPs is achieved through various types of bonding within the chiral helical grooves of the chiral selector (which form the chiral pocket), in particular through H-bonding, dipole–dipole, and π–π interactions, as well as steric effects.

3.1. HPLC Enantioseparation of (±)-Trans-β-Lactam Ureas 55, 57, 60, 62–67

Baseline separations were observed for eight compounds (except **67**) on the Cellulose-SB column with *n*-hexane/2-PrOH (9:1, *v*/*v*) as mobile phase, on the Cellulose-SC column for seven compounds (except compounds **60** and **66**) with the same mobile phase (Table 1). Comparing the data obtained with Cellulose-SB and Cellulose-SC, clearly higher retentions can be observed for all analytes on CSPs with *tris*(3,5-dichlorophenylcarbamate) moiety. The higher α and R_S values (except compounds **60**, **65** and **66**) generally observed on CSPs with *tris*(3,5-dichlorophenylcarbamate) indicate more pronounced chiral selector-chiral analyte interactions of the analytes investigated.

A higher separation factor was obtained for Cellulose-SB, which proved to be a better choice than Amylose-SA for the separation of most β-lactam ureas. According to the data in Table 1, structural variations can significantly affect the retention factors. For example, on the Amylose-SA column, the k_1 of the analytes **57**, **60**, **62**, and **64–67** are higher than those of the other analytes. The possible reason for this could be the presence of the allyl, furfuryl, or phenyl ring in the *N*-position of the ureido moiety, which could cause additional π–π interactions between the CSP and the analytes. On the other hand, analytes **55** and **63** always have the lowest k_1 values, indicating that the interaction between these compounds and the CSP is very weak, possibly due to the long alkyl or cycloalkyl substitution in the *N*-position of the ureido group. On Cellulose-SB, the k_1 values for the ureas **63** with cyclopentyl group and for **67** with 2,6-dimethylphenyl group are the lowest than the others tested, and the possible reason for this could be the rigidity of the cyclopentyl ring or the steric effect of two methyl groups in the *ortho*-position of the phenyl ring. These results indicate that the different structural features of the CSP in combination with the mobile phase *n*-hexane/2-PrOH ultimately lead to a different stereo-environment of the chiral cavities in the CSP, resulting in different chiral selectivities.

Next, dimethyl carbonate (DMC) was investigated as a mobile phase for the separation of racemic β-lactam ureas **55**, **57**, **60** and **62–67**. DMC has been classified as one of the most environmentally friendly solvents, in the same class as water, short-chain alcohols and ethyl acetate [27–29]. DMC also degrades rapidly in the atmosphere/environment (over 90% in 28 days) and can therefore be considered non-toxic [27,30]. However, the applications of DMC in analytical chemistry appear to be very limited. DMC has been used as a mobile phase modifier in reversed-phase liquid chromatography (RPLC) with ICP-MS detection [31] and for normal-phase liquid chromatography (NPLC) and hydrophilic interaction liquid chromatography (HILIC) [32]. So far, there are only two examples of the use of DMC in chiral HPLC chromatography, and both were carried out in our laboratory [23,33]. For chiral separation of *anti*- and *syn*-hydantoins 100% DMC was used [23], and DMC with 10% alcoholic modifier (MeOH or EtOH) was used for marinoepoxides [33].

When DMC was used, baseline separation was achieved for eight compounds (except **67**) on the Amylose-SA column and for the five compounds **60**, **63**, and **65–67** on the Cellulose-SB column. For the other compounds tested, only partial resolution was observed on these two columns. For the Amylose-SA and Cellulose-SC columns, the retention factors for the most compounds were higher when pure DMC was used as mobile phase compared to MeOH or EtOH, and this was accompanied by the higher resolution values

in almost all cases. Out of the nine β-lactam ureas used in this study, ureas **55**, **57**, and **62–66** achieved the higher R_s and α-values under DMC conditions on the Amylose-SA column compared to Cellulose-SB. In a few cases, such as with the compounds **55**, **63**, **65**, and **66**, the use of DMC as the mobile phase afforded superior R_s and α on the Amylose-SA. Notably, the Cellulose-SB showed significantly higher enentioselectivity values for the ureas **60** and **65–67** compared to Cellulose-SC, which contains the cellulose *tris*(3,5-dichlorophenylcarbamate) as the chiral selector. This column was able to baseline separate only three compounds, **63**, **65**, and **66**, whereas the others were partially separated or not separated at all with DMC. The nature of DMC, the structures of the analytes, and their interactions with the CSP all play a role in enantioselectivity.

Some interesting observations resulted from the comparison of the separation systems with different alcoholic modifiers. Interestingly, higher resolution values were obtained with EtOH compared to MeOH (except **65**) on Amylose-SA, indicating that EtOH may be an advantageous alternative mobile phase for MeOH. An additional example that shows the difference in using MeOH and EtOH as a pure mobile phase is that compounds **55**, **57**, **62**, and **64** on Cellulose-SB were partially separated using MeOH, but, by changing the mobile phase to EtOH, these compounds were not separated. In the case of compounds **55**, **57**, **62**, **64**, and **67**, enantioseparation was not achieved with either MeOH or EtOH when the Cellulose-SC column was used. The other tested compounds **60**, **65**, and **66** were partially separated with both alcohol modifiers, but higher R_s were obtained for these compounds when MeOH was used as mobile phase. On the Amylose-SA column, $R_s > 1.5$ were obtained for compounds **57**, **63**, **64**, and **66** with MeOH and for compounds **55**, **57**, **62–65**, and **67** with EtOH, but better resolution and separation values were obtained with 100% EtOH (all except compound **66**).

The success rate on each immobilized stationary phase is slightly different for each mobile phase. The success rates were expressed in terms of baseline-, partially, and not-separated compounds (Table 1). On Cellulose-SC, it is not possible to separate more than three of nine tested compounds (33%) with EtOH and four compounds with MeOH (44%) (Table 1). The mobile phase *n*-hexane/2-PrOH (90/10, *v*/*v*) is the most successful and separates all nine compounds, whereas eight compounds (89%) are separated with DMC. On Amylose-SA, the traditional mobile phase *n*-hexane/2-PrOH (90/10, *v*/*v*) performs slightly worse (55%) than the polar organic (89% with MeOH and EtOH) and non-standard DMC (100%) mobile phases. The Cellulose-SB column performs worse than the other two columns, providing the separation for only five racemates (55%) with 100% DMC. The mobile phases *n*-hexane/2-PrOH and MeOH are the most successful and separate all nine compounds. On this CSP, the polar organic mobile phase (EtOH) and atypical modifier (DMC) provide the same success rates (55%). For Cellulose-SB, MeOH yields slightly more baseline and partial enantioseparations than EtOH. For Cellulose-SC, the situation is the same, i.e., MeOH yields somewhat more separations. For Amylose-SA, MeOH and EtOH yield a similar number of separations, which is significantly higher than for Cellulose-SC. It is known from the literature that alcohols of different size and bulkiness can be incorporated into the CSP structures and can also cause conformational changes in the helical structure of the chiral selectors of amylose or cellulose, which result in different stereo-environments [34–36].

Some representative HPLC chromatograms are depicted in Figure 6.

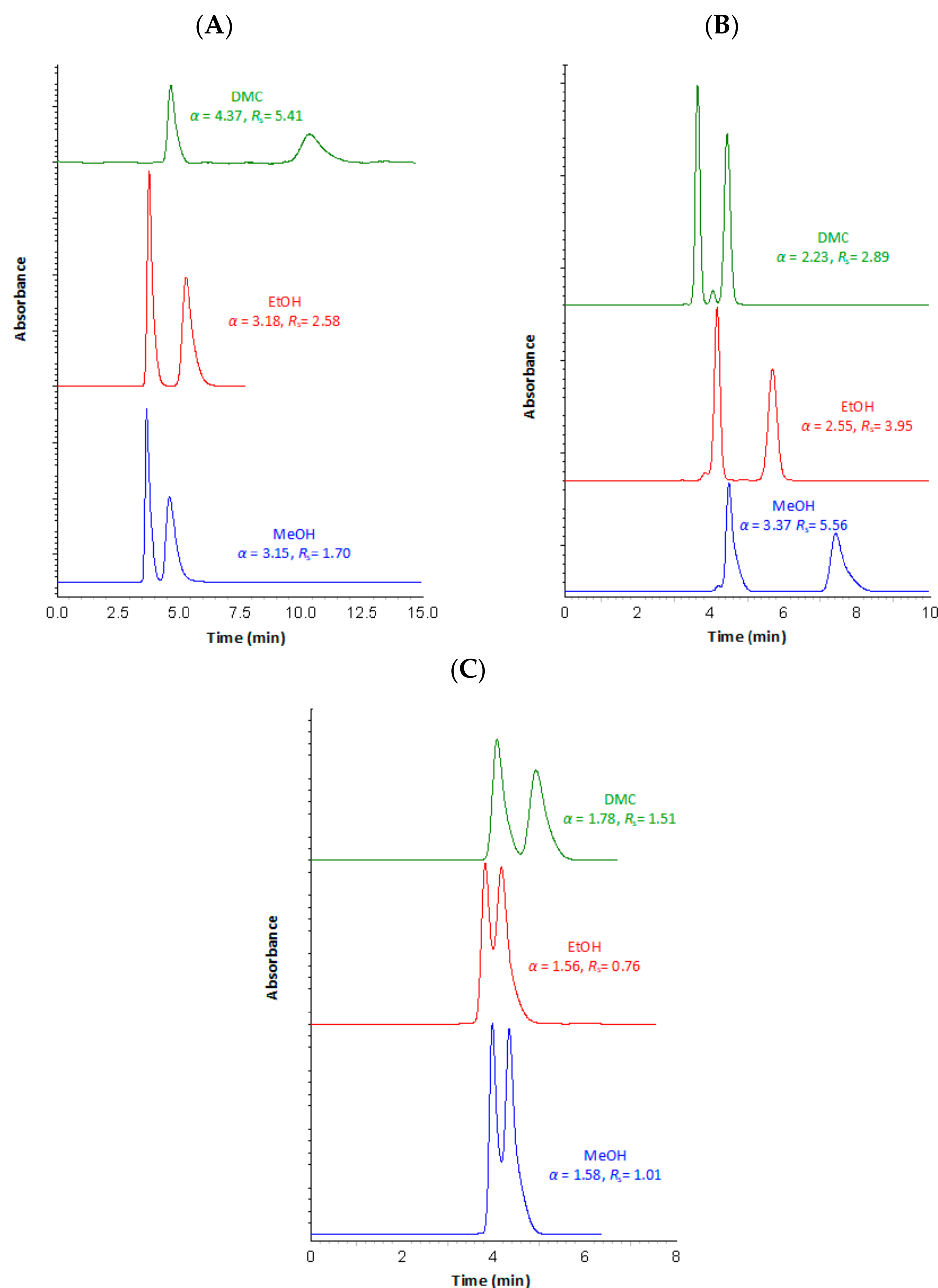

Figure 6. Effect of mobile phase composition on enantioseparation of cyclopentyl urea **63** on Amylose-SA (**A**), 3-chloro-4-methyl urea **60** on Cellulose-SB (**B**), and 4-*tert*-butylphenyl urea **65** on Celulose-SC (**C**) under HPLC conditions. Chromatographic conditions: mobile phase, MeOH (—), EtOH (—), and DMC (—); flow rate, 1 mL min^{-1}; detection, 254 nm; temperature, 35 °C.

3.2. SFC Enantioseparation of (±)-Trans-β-Lactam Ureas 55, 57, 60, and 62–67

The enantioseparation of (±)-*trans*-β-lactam ureas **55**, **57**, **60**, and **62–67** on polysaccharide CSPs in SFC mode was investigated under typical supercritical chromatography conditions, i.e., CO_2 with an alcoholic modifier, and under atypical conditions with a non-standard modifier, in this case with DMC.

The effect of the polar modifiers MeOH and EtOH in the mobile phase CO_2/alcohol on the enantioresolution for the selected (±)-*trans*-β-lactam ureas **55**, **57**, **60**, and **62–67** was investigated for all three immobilized CSPs.

The use of Amylose-SA for the enantioseparation of nine racemates showed no clear preference for the modifier. Eight compounds (except **67**) were baseline resolved with

MeOH, and the same number were baseline separated using EtOH as modifier. Herein, with EtOH as a bulk solvent component, retention values were higher in most cases on all used polysaccharide columns compared to the with the shorter alcohol MeOH. In general, better selectivity and resolution on Amylose-SA were achieved in most cases when EtOH was used as a polar modifier. The size of the alcoholic modifier seems to affect the chiral recognition mechanisms of the studied compounds of this type of polysaccharide CSP. On the Cellulose-SB column, nine analytes were baseline separated with MeOH and seven racemates with EtOH as mobile phase modifier. The Cellulose-SC column baseline separated three racemates with MeOH and five racemates with EtOH as the modifier. No separation was observed on Cellulose-SC for compounds **55**, **57**, **62**, and **64** with CO_2/MeOH (80/20, v/v) and for compounds **63** and **67** with CO_2/EtOH (80/20, v/v).

The type of alcoholic modifier can also influence retention and enantioseparation [37]. An NMR study on the effects of alcoholic modifiers on the structure and chiral selectivity of amylose-based CSP showed that the use of alcohols with different volume and concentration in the eluent can lead to differences in the chiral recognition ability of the polymer CSP as a consequence of its structural changes (e.g., crystallinity of the polymer, side chain mobility, and conformation) after incorporation of the alcohol into the CSP [34,38].

It can be concluded that both chiral stationary phases based on *tris*(3,5-dimethylphenyl carbamate) cellulose and amylose, Cellulose-SB and Amylose-SA, are suitable for the enantioseparation of the investigated β-lactam ureas **55**, **57**, **60**, and **62–67**. On the other hand, cellulose *tris*(3,5-dichlorophenylcarbamate), Cellulose-SC, showed a relatively lower separation performance. Interestingly, the chiral recognition abilities of Amylose-SA and Cellulose-SB are comparable, although they contain different polysaccharide chiral selectors.

When DMC was used as a co-solvent in the SFC, the enantiomers of compounds **55** and **62** were not eluted from the tested immobilized columns within one hour. It is important to note that, in this study, 10–40% volume percent of DMC was used under SFC conditions. Preliminary tests showed that up to a ratio of 60/40 (v/v), CO_2/DMC, the mobile phase strength was insufficient to elute these racemates. The alcohol content and type (MeOH, EtOH, and 2-PrOH) can be used to modulate retention and chiral recognition [37]. So, the alcoholic modifier (MeOH and EtOH) was added in 20% to DCM to increase elution strength for not-eluted compounds.

For the same modifier (DMC/MeOH or DMC/EtOH), as shown in Table 2, k_1 decreased with increasing DMC/alcohol volume fraction (from 24/6 to 32/8). This shows that increasing the volume fraction of the alcoholic modifier accelerates the elution rate and shortens the retention time. MeOH is a protic solvent that is also a proton donor and proton acceptor. It can form hydrogen-bonds with the β-lactam urea compounds and with the chiral stationary phase, thus competing with the compounds for hydrogen bonds. Increasing the volume fraction of MeOH (from 6% to 8%) increases this competition, reduces the interaction between the compound and the CSP, and shortens the retention time. MeOH competes with the analytes for the hydrogen bonding sites of the CSPs, whereas DMC as an aprotic solvent interacts significantly with the polymer through dipole–dipole interaction. The functional groups of the alcohols form strong H-bond complexes with the C=O and NH functional groups of the polysaccharide polymer [39]. When EtOH is used as a co-solvent instead of MeOH, the polarity of the mobile phase is reduced, the hydrogen bonding of EtOH is weaker, and the elution rate is much slower. Overall, the separation factor and resolution decreased with the increase in the volume fraction of mobile phase B, DMC/MeOH, or DMC/EtOH.

On Amylose-SA, nine separations are achieved with all four mobile phases CO_2/DMC/alcohol modifier (mobile phases C−F). For Cellulose-SB, nine separations are generated by CO_2/DMC/MeOH (mobile phases C and D) and four such separations CO_2/DMC/EtOH (mobile phases E and F), respectively, whereas Cellulose-SC generates a similar number of separations. The effects of the modifiers were rather unpredictable, as is generally the case for chiral separations. In some cases, the number of enantioseparations with all modifiers was quite similar, in other cases they differed significantly, as can be seen

in Table 2. The first SFC separations with CO_2/DMC/alcoholic modifier on immobilized amylose and cellulose columns are shown, providing a new approach for the supercritical separation of the (±)-*trans*-β-lactam ureas **55**, **57**, **60**, and **62–67**.

The most successful composition of the mobile phase is different for each stationary phase. For Cellulose-SB, the mobile phases CO_2 with MeOH as the organic modifier perform slightly better than those with only EtOH or with alcohol modifier/atypical solvent mixture (MeOH/DMC or EtOH/DMC). For Amylose-SA and Cellulose-SC, the mobile phases with CO_2/EtOH and CO_2/EtOH/DMC provide better separation than the other mobile phases used. Overall, as described above, Amylose-SA provides the most baseline and partial enantioseparations and thus has the highest success rate under these conditions.

Some representative HPLC chromatograms are depicted in Figure 7.

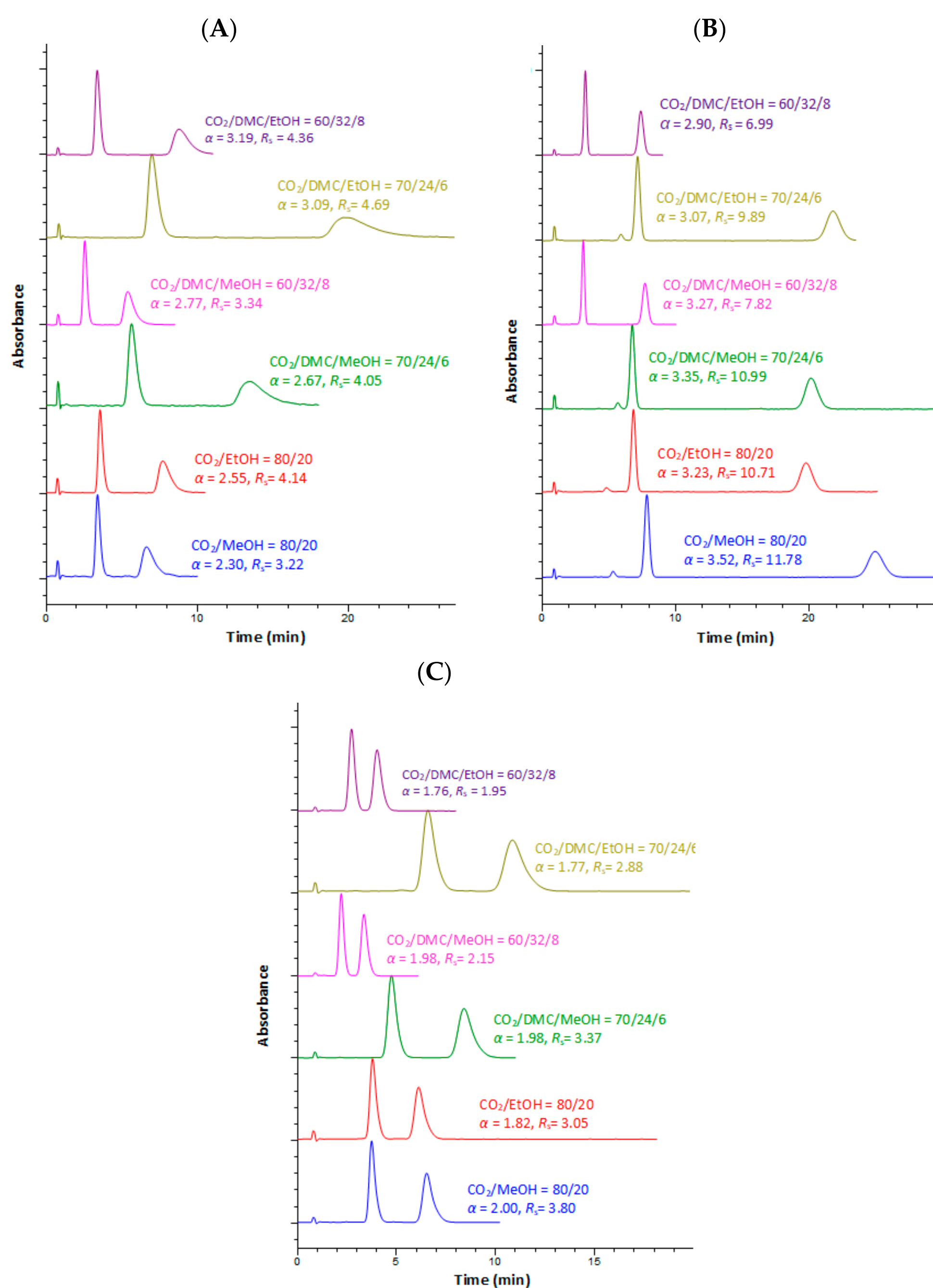

Figure 7. Effect of mobile phase composition on enantioseparation of cyclopentyl urea **63** on Amylose-SA (**A**), 3-chloro-4-methyl urea **60** on Cellulose-SB (**B**), and 4-*tert*-butylphenyl urea **65** on

Celulose-SC (**C**) under SFC conditions. Chromatographic conditions: mobile phase, CO_2/MeOH (80/20 *v*/*v*) (—), CO_2/EtOH (80/20 *v*/*v*) (—), CO_2/DMC/MeOH (70/24/6 *v*/*v*/*v*) (—), CO_2/DMC/MeOH (60/32/8 *v*/*v*/*v*) (—), CO_2/DMC/EtOH (70/24/6 *v*/*v*/*v*) (—), and CO_2/DMC/EtOH (60/32/8 *v*/*v*/*v*) (—); flow rate, 4 mL min^{-1}; detection, 254 nm; temperature, 35 °C.

4. Conclusions

In this work, the enantioseparation of nine (±)-*trans*-β-lactam ureas was performed by HPLC and SFC on three immobilized polysaccharide-based CSPs containing amylose *tris*(3,5-dimethylphenyl)carbamate (CHIRAL ART Amylose-SA), cellulose *tris*(3,5-dimethylphenyl)carbamate (CHIRAL ART Cellulose-SB), and cellulose *tris*(3,5-dichlorophenyl)carbamate (CHIRAL ART Cellulose-SC) as chiral selector.

In HPLC mode, the Cellulose-SB column is the only chiral stationary phase in this study that was able to separate all nine compounds tested with the mobile phases *n*-hexane/2-PrOH (90/10, *v*/*v*) and 100% MeOH. Of the three polysaccharide columns, Amylose-SA showed better recognition ability for the tested compounds with 100% DCM than Cellulose-SB and Cellulose-SC.

In the present study, DMC was used as an organic modifier in SFC for the first time. The elution strength of DMC was increased by adding a more polar solvent, MeOH or EtOH. Among the CSPs tested, both the Amylose-SA and Cellulose-SB were able to resolve all nine urea compounds to baseline or partially.

We have shown that the environmentally friendly solvent dimethyl carbonate (DMC) can be used efficiently as a mobile phase in HPLC mode, but it can also be used in SFC mode with the addition of the alcoholic modifiers MeOH or EtOH. The chiral stationary phase Amylose-SA provides the most baseline and partial enantioseparations and thus has the highest success rate with the mobile phase CO_2/DMC/alcohol.

Author Contributions: M.J. performed synthesis, chromatographic analyzes, literature search, and writing. D.K. and M.R. performed the study design, data analysis, revision, final approval, and handled the accountability of all aspects of the work. All authors contributed to the article and approved the submitted version. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: Data are contained within the article.

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Conflicts of Interest: The authors declare no conflicts of interest.

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