Chapter 3
Professor Casu and Cyclodextrins

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Abstract Three years ago, Professor Benito Casu, G. Ronzoni Institute for Chemical and Biochemical Research, passed away on November 11, 2016, shortly before his 90th birthday. Professor Casu was an eminent scientist in the area of chemistry and biochemistry of polysaccharides and glycosaminoglycans.

Casu's scientific career started in the 1950s with the study of carbohydrates by innovative spectrometric techniques. Professor Casu was on the list of prestigious researchers who contributed in the 1960s to the development of cyclodextrins. At that time, there was a lack of sufficient knowledge about these molecules. Few researchers believed in the potential that these new molecules had. In the mid-1960s, Professor Casu was the first to demonstrate that infrared spectroscopy and proton

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nuclear magnetic resonance were powerful methods to study the structure and conformations of cyclodextrins. He suggested a common shape of the glucopyranose rings in the chair C-1 and the presence of hydrogen bonds between the hydroxyl groups. These conclusions greatly advanced the understanding of the structure and chemistry of cyclodextrins and their properties. In 1967, Professor Casu published the first perspective view of 2,6-di-O-methylated cyclodextrin and, 1 year later, prepared cyclodextrin derivatives such as methylated and acetylated products. At the end of the 1960s, Professor Casu was among the first to verify the idea that if the guest molecule is accommodated in the cyclodextrin cavity, then the hydrogen atoms located in the interior of the cavity would be significantly shielded by the guest, whereas the hydrogen atoms on the outer surface would not be affected by the formation of the inclusion complex. In 1969, his interest extended to biological active substances such as heparin with a visit to Department of Chemistry of McGill University of Montreal, Canada, guest of Professor Arthur S. Perlin. Nevertheless, Professor Casu has continued to work on cyclodextrin, publishing important contributions on the use of methylated cyclodextrins as versatile complexing agents for the complexation of some n-alkanes and dyes, and on the interactions of cyclodextrins with glycolipids. He also studied inclusion properties of methylated cycloamyloses, demonstrating that the inclusion complexes of methylated derivatives were more stable than the corresponding complexes with the parent cyclodextrins. Professor Casu was among the first to publish general notes and reviews on cyclodextrins for a broader public. He was a prolific publisher on topics related to cyclodextrin and heparin, an enthusiastic teacher of many doctoral students and postdoctoral fellows, a genial host to visitors, and a truly caring person.

In this chapter, we would like to pay a posthumous tribute to Professor Casu, one of the pioneers in the dissemination of the knowledge of cyclodextrins in the 1960–1970s and later of heparin.

Keywords Tribute · Professor Casu · Polysaccharides · Structure · Cyclodextrin derivatives · Nuclear magnetic resonance · Infra-red spectroscopy · Awards and distinctions

3.1 Introduction

On November 11, 2016, the glycoscience community lost one of its leading figures, Professor Benito Casu (Fig. 3.1), G. Ronzoni Institute for Chemical and Biochemical Research, at the age of 89. In him, we have lost an eminent and passionate scientist, a visionary, and a colleague of great merit, particularly in the area of chemistry and biochemistry of natural substances (Crini 2014; Crini and Torri 2017; Torri and Cassinelli 2018). Gentleman Professor Casu has been a friendly and generous

Fig. 3.1 Professor Benito Casu in 2012. (Image credit: G. Torri)

communicator, an enthusiastic teacher of many doctoral students and postdoctoral fellows, a genial host to visitors, and a truly caring person.

Casu's scientific career was based on two main research axes: the first focused on oligosaccharides and polysaccharides developed essentially during the years between 1951 and 1980 and the second on biological substances during 1969–2014.

Professor Casu had many varied scientific interests: chemistry and biochemistry of starch, linear and cyclic dextrins, glycosaminoglycans, mechanism of action of biologically active substances, structural analysis of carbohydrates, and their interactions with proteins, enzymology, analytical chemistry, surfactant chemistry, etc. Professor Casu has also made a significant contribution to the development of structural modelling and NMR, infrared, and Raman techniques applied to carbohydrates (Crini 2014; Crini and Torri 2017; Torri and Cassinelli 2018). All this research was carried out, thanks to multidisciplinary and transversal collaborations developed and thanks to his willingness to involve both academic and industrial colleagues, both national and especially international. Its activities have also focused on scientific fields with direct social implications, in accordance with the principles dictated by the founder of the Ronzoni Institute.

At the end of the 1940s, the young student Casu first concentrated on the field of carbohydrate chemistry, e.g., investigating the structure of glucose and its chemical modification, at the University of Pavia, Italy, where he presented his PhD in 1950 (Crini 2014). In the early 1950s, Dr. Casu began his scientific career at the G. Ronzoni Institute, as research fellow in 1951, and research assistant in 1954, studying the structure of disaccharides such as maltose, oligosaccharides, e.g.,

linear and cyclic dextrins, and polysaccharides such as starch, using the then highly innovative spectrometric techniques (Reggiani and Casu 1957; Crini 2014).

In the mid-1950s, Professor Casu investigated in details the structure and conformation of the two components of starch, i.e., amylose and amylopectin. At that time, the studies on amylose conformation were even marred by hot debate between the different laboratories. At the same time, Professor Casu studied the structure, chemistry, and inclusion properties of cycloamyloses-cyclodextrins, mainly α-cyclodextrin and β-cyclodextrin and their methylated and acetylated derivatives. Indeed, there was a lack of sufficient knowledge about their structure (Crini 2014; Crini et al. 2018). In the mid-1960s, the works of Professor Casu on the structure and the conformations of amylose and cycloamyloses, using infrared and nuclear magnetic resonance techniques, were acknowledged to have made an important contribution (Crini 2014). Significant collaborations have been put in place by Professor Casu with American, e.g., Professor Dexter French; Hungarian, e.g., Professor József Szejtli; and French, e.g., Professor Michel Morcellet, groups in the 1960s, 1970s, and 1990s, respectively.

During more than 30 years, Professor Casu occupied a central place in the scientific knowledge of cyclodextrins. His first article on cyclodextrins entitled "Infrared spectra of amylose and its oligomers" was published in 1964 in Journal of Polymer Science (Casu and Reggiani 1964). This work, supported by a grant from the US Department of Agriculture, was presented in preliminary form at the International Conference on the IR Spectra of High Polymers in July 1963 (Milan, Italy). In 1965, Professor Casu gave an invited lecture on this work at the Starch Round Table Conference, Pocono Manor, Pa., USA. From the 1970s, as Director of the Ronzoni Institute, Professor Casu mainly focused the research on heparin. However, he continued to publish several articles and reviews on cyclodextrins and worked on several International, European, and National projects related to cyclodextrins. His last article entitled "Disruption of micellar aggregates of ganglioside GM-1 by complexation with alpha-cyclodextrin" was published in 1994 in the International Journal of Pharmaceutics (Ahmed et al. 1994).

Throughout his scientific career, Professor Casu has given numerous conferences around the world, particularly in North America. He has published an impressive number of articles and patents, and his name is very often cited in the bibliographic references of articles on these topics. Until his death, Professor Casu was still Scientific Consultant of the President of the G. Ronzoni Institute (Crini and Torri 2017; Torri and Cassinelli 2018).

This chapter is a tribute to his scientific oeuvre. First, it gives an overview of his exceptional scientific career. Next, we highlight some important work on cyclodextrins published by Professor Casu.

3.2 Benito Casu, 1927–2016

3.2.1 Early Years

Benito Casu was born on March 27, 1927, in Brescia, Italy, the son of Luigi Casu and Angela Porcu. He studied chemistry at the University of Pavia where, in 1950, he earned his doctorate in carbohydrate chemistry, studying the structure of glucose and some related carbohydrates using spectrometric techniques.

In 1951, as research fellow, Dr. Casu started his scientific career with research on starch and cyclodextrins at the Physical-Chemistry Unit of the G. Ronzoni Institute, a nonprofit research foundation for chemical and biochemical research under the direction of Professor Alfredo Dansi. Two years later, Dr. Casu became a research assistant in this institute. In May 6, 1954, Benito Casu married Marta Mancini, and they have two children.

During more than 30 years, Professor Casu published several significant papers on the structure of native and modified cyclodextrins using infrared and nuclear magnetic resonance (NMR) techniques (Crini 2014).

In early 1968, Dr. Casu obtained the professorship in chemical spectroscopy. One year later, he was a Harold Hibbert Memorial Fellow (1968–1969) at the Department of Chemistry of McGill University of Montreal, Canada, guest of Professor Arthur S. Perlin, where they forged a lifelong friendship.

During this stage, Professor Casu studied polysaccharides and biological substances using innovative NMR techniques. He also had the opportunity to present several conferences in the USA, where he met Professor Dexter French (Iowa State University), one of the prestigious researchers who have contributed to the development of cyclodextrins. The two research groups then collaborated for about 10 years (Crini and Torri 2017; Torri and Cassinelli 2018). During this "American period," Professor Casu also had friendly contacts and interactions with the Professors R.H. Marchesseault, C. Dietrich, and A. Gorin.

3.2.2 Professor Casu: Director of the G. Ronzoni Institute

Professor Casu returned to Milan in 1970 and focused his work on heparin. In 1973, he became the Director of the *Istituto di Ricerche Chimiche e Biochimiche Giuliana Ronzoni* and served in this position until 1992 (Crini and Torri 2017). Professor Casu remained the scientific consultant of the board of directors of Ronzoni Institute until to 2015.

For over 20 years, under his direction, the institute underwent significant economic and scientific development. In the mid-1970s, the pioneer NMR studies on the structure and conformational flexibility of heparin as well as of the heparin active sites to antithrombin provided him an international notoriety. Through interdisciplinary and international networks and collaborations, the institute significantly

contributed to the development of both new analytical methodologies and novel heparin derivatives (Torri and Cassinelli 2018). Pleiotropic activities of heparins have been studied through the pioneering development of non-anticoagulant/anti-thrombotic heparins.

The institute also participated to numerous National, European, and International cyclodextrin-based projects, e.g., the European FAIR program 1995–1999 "Development from cyclodextrin derivatives to polymeric materials for selective transport, separation and detection of active substances" (European Commission DGXII, contract no. CT 95-0300). This project, coordinated by Professor Gerhard Wenz, also involved Wilfried König, Michel Morcellet, Bruno Perly, Jacques Defaye, David Reinhoudt, Giangiacomo Torri, Annamaria Naggi, Carmen Vecchi, Bernard Martel and Grégorio Crini, among others.

3.2.3 Professor Casu: A Friendly and Generous Communicator

Professor Casu was a warm, friendly, and generous communicator and a gifted and dedicated teacher. He was lecturer on biopolymers from 1970 to 1982 at the University of Pavia, Italy. In order to promote the exchange of scientific information, Professor Casu organized numerous symposia and workshops and encouraged collaborative works with foreign universities and laboratories and the exchange of researchers and students (Crini and Torri 2017; Torri and Cassinelli 2018). In 1996, Professor Casu organized the 18th International Carbohydrate Symposium (July 21–26, Milan, Italy) (Fig. 3.2). The main talks were published in the journal *Pure and Applied Chemistry* (volume 69, issue 9, 1997).

Professor Casu also trained numerous students, PhD, and postdoctoral students. He has always been a teacher of the highest quality and a gentleman professor who has inspired enthusiasm for research among his students and Italian and foreign colleagues, and his hospitality to visitors has been generous. His talent was also applied in academic affairs.

3.2.4 Membership

Professor Casu was a member of the Italian Chemical Society (Carbohydrate Group Coordinator, 1984–1996) and of the International Carbohydrate Organization (National Representative since 1976, President 1996–1998). Professor Casu was a member of the International Advisory Board of Carbohydrate Research (1970–1985) and Biochemical Journal (since 1973). He also served as a member of Scientific Advisory Board at Momenta Pharmaceuticals Inc. and Endotis Pharma SA.

Fig. 3.2 Eighteenth International Carbohydrate Symposium organized by Professor Casu in 1996. (Image credit: G. Torri)

3.2.5 Awards and Distinctions

Professor Casu received many distinctions and prizes, most notably the degree of Doctor in Medicine *Honoris causa* at the University of Uppsala, Sweden (1998), the gold medals of the Italian Chemical Society (1998) and of the Italian Carbohydrate Group (2003), and recognition plaques from the Italian Cyclodextrin Group (2009) and the International Union of Angiology (1990 and 2012).

3.3 Casu's Scientific Achievements

3.3.1 Professor Casu and Polysaccharides

In the early 1950s, Dr. Casu began working on carbohydrate structure and conformation using the most innovative spectrometric techniques of the time (Reggiani and Casu 1957; Crini and Torri 2017). He was the first to demonstrate that infrared spectroscopy (IR) and proton nuclear magnetic resonance (^{1}H-NMR) were powerful methods to study the structure and conformations of disaccharides, oligosaccharides, and polysaccharides, including starch and its linear and cyclic derivatives (Crini 2014; Crini and Torri 2017). All his results were summarized in the 1980s in two comprehensive reviews (Casu 1982, 1985a).

In 1964, Professor Casu studied infrared spectra of starch derivatives. This work was supported by a grant from the US Department of Agriculture. Professor Casu reported infrared spectra of amylose, cycloamyloses, and amylodextrin and assigned

the main individual bands (Casu and Reggiani 1964). The IR spectra were taken with a Perkin-Elmer model 125 spectrophotometer. In this paper, he suggested for the first time a common shape of the glucopyranose rings, in the chair C-1, in all the starch derivatives studied, and the presence of hydrogen bonds between the hydroxyl groups (Crini 2014). The same year, Professor Casu also published hydroxyl proton resonances of sugars in water and dimethyl sulfoxide solutions in order to characterize the structural factors which affected ring vibrations in infrared data and to clarify the significance of their shifts ongoing from cyclic to linear structures (Casu 1964; Casu et al. 1964). Later, he detailed NMR spectra and conformation of glucose and some related carbohydrates in the same conditions (Casu et al. 1965b, 1966, 1967, 1968b).

In 1965, an IR spectrophotometric procedure was developed for determining water in carbohydrates (Casu et al. 1965a). The method was based on the measurement of the intensity of 1655 cm^{-1} band of water in a solution of the hydrated sugar in dimethyl sulfoxide. This method was particularly interesting when unsubstituted carbohydrates contained crystallized water. The average percent difference between the results obtained by the IR versus the usual oven-drying method was find to be about ±1%. One year later, his results clearly demonstrated that the amylose macromolecule had a flexible structure, with a helical pattern, which could take various conformations through rotation of the monomeric blocks around the glucosidic linkages (Casu and Reggiani 1966). Both IR and NMR spectra showed that the C1-H bond was equatorial and C1-O axial, also confirming the C-1 chair conformation of the glucopyranose units (Casu and Reggiani 1964, 1966). Professor Casu also showed the existence of intermolecular hydrogen bonds contributing to the stabilization of the helical structures. At that time, these works were acknowledged to have made an important contribution to this topic (Crini and Torri 2017; Crini et al. 2018). Professor Casu obtained similar results when studying the native cycloamylose molecules, particularly cyclohexaamylose or α-cyclodextrin, with the first IR and ^{1}H-NMR spectroscopic studies (Casu and Reggiani 1964, 1966). The cycloamylose samples were prepared by Professor French.

3.3.2 Professor Casu and Cyclodextrins

Professor Casu was also on the list of prestigious researchers, such as Professors Dexter French, Friedrich Cramer, Myron Lee bender, József Szejtli, and Wolfram Saenger, who contributed in the 1960–1970s to the development of cyclodextrins (Crini 2014). Few researchers at that time believed in the potential that these new molecules had (Szejtli 1982, 1988; Crini 2014; Crini et al. 2018).

Professor Casu showed that IR and ^{1}H-NMR spectroscopy were powerful methods to study the conformations of not only amylose and linear dextrins but also cyclodextrins. In the middle of the 1960s, he investigated for the first time the NMR spectra of cyclodextrins (Casu 1964, 1968a, b), and his conclusions greatly advanced the understanding of the structure and chemistry of cyclodextrins and their

properties (Crini 2014; Crini et al. 2018). For instance, Professor Casu demonstrated three important characteristics (Crini 2014): (1) all secondary hydroxyl groups were situated on one of the two edges of the cyclodextrin ring, whereas all the primary ones were placed on the other edge; (2) the cavity was lined by the hydrogen atoms and the glycosidic oxygen bridges, respectively; and (3) the C-2 and C-3 hydroxyl groups of the adjacent glucopyranose units formed hydrogen bonds which stabilized the shape of the cyclodextrin molecule. Later, Professor Szejtli has shown that these characteristics had a significant influence on the solubility of cyclodextrins in water (Szejtli 1982, 1988).

Professor Casu reported the infrared spectra of several cycloamyloses (Fig. 3.3) and assigned the main individual bands (Fig. 3.4). He detailed the spectra of hydrates of cyclohexaamylose where he observed the reduced splitting and the broadening of the bands in the dehydrated samples with respect to the hydrated forms (Casu and Reggiani 1964; Casu et al. 1965a). Professor Casu concluded that "it was not easy to explain the shift of the frequencies of the ring vibrations on going from cyclohexaamylose to cycloheptaamylose to amylodextrin and amylose in terms of the conformation of glucopyranose rings" (Casu et al. 1965a). He suggested that "the configuration of the ring sequences and the bulkiness of the chains certainly affected ring vibrations" (Crini 2014). He also suggested the existence in water of hydrogen bonds between the secondary hydroxyl functions (Casu et al. 1965a).

In 1966, Professor Casu, studying the IR spectra of cycloamyloses in the amorphous solid phase and in aqueous and dimethyl sulfoxide solutions (Fig. 3.5), showed that the C1-H bond was equatorial and the C1-O bond axial, and this was consistent with the C-1 conformation of the glucopyranose (Casu and Reggiani 1966). The same year, Professor Casu published the first NMR spectra of cycloamyloses (Fig. 3.6). Comparing the spectra of maltose and cycloamyloses, "an interesting and curious downfield displacement of the non-anomeric hydroxyl signal below 5" was evident (Casu et al. 1966). Professor Casu also showed that ^{1}H-NMR spectroscopy was applicable to quantitative analysis of concentrated solutions of mixtures of cyclodextrins in dimethyl sulfoxide (Casu 1966, 1967; Casu and Reggiani 1966; Casu et al. 1966).

Using NMR of α-cyclodextrin in DMSO-d_6 through hydrogen-deuterium exchanges of cyclodextrins, Professor Casu also demonstrated that the D-glucopyranose units in cyclodextrins were in the C-1 chair conformation and the primary and secondary hydroxyl groups had similar conformation to those in the crystalline state (Casu 1966; Casu and Reggiani 1966). Both NMR and IR spectra through hydrogen-deuterium exchanges of cyclodextrins also first showed the existence in water of hydrogen bonds between the secondary hydroxyl functions, which brought about a slight chemical shift in the protons of these functions (Casu 1966; Casu and Reggiani 1966). Later, Professor Casu determined the value of the deuteration equilibrium constant of the same functions (Casu 1967; Casu et al. 1968a). The equilibrium constant for the secondary hydroxyl groups was 0.75 in α-cyclodextrin and 0.65 in β-cyclodextrin, both much less than the corresponding value for amylose, i.e., 0.85. This clearly indicated that intramolecular hydrogen bonding rendered the secondary hydroxyl groups in cyclodextrins more resistant to

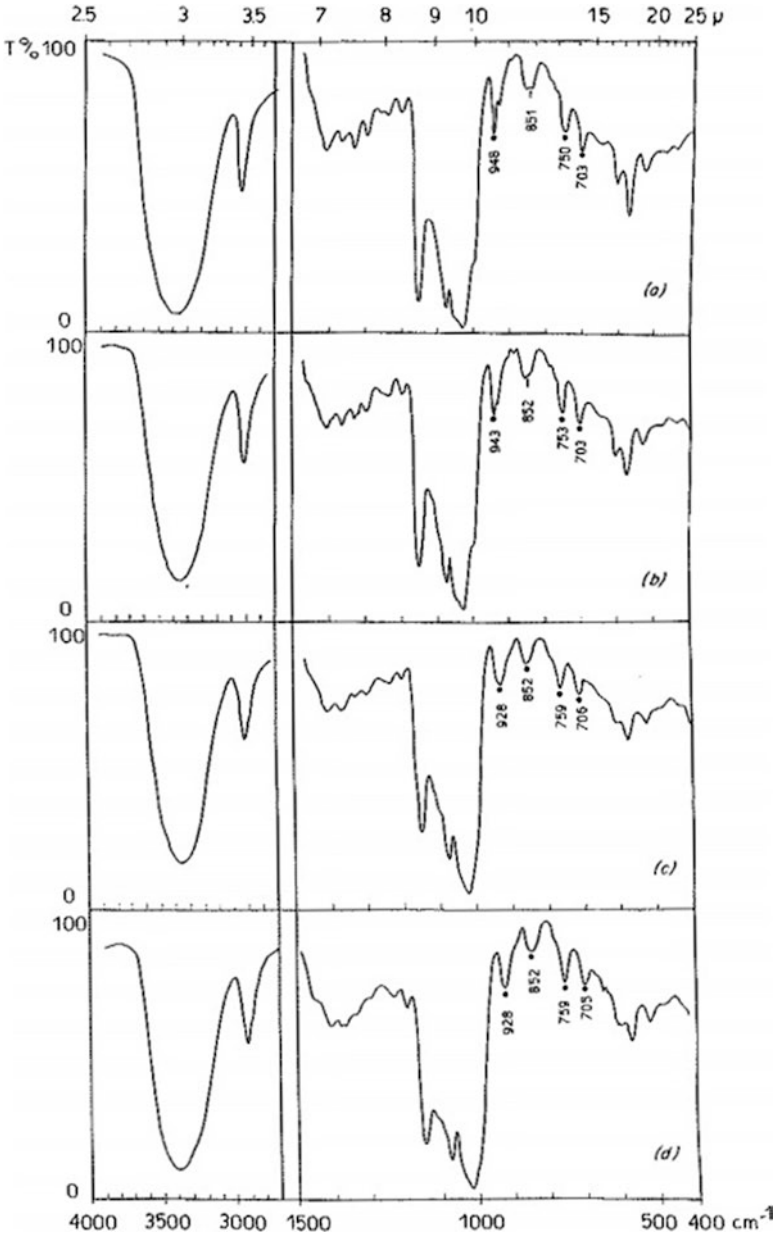


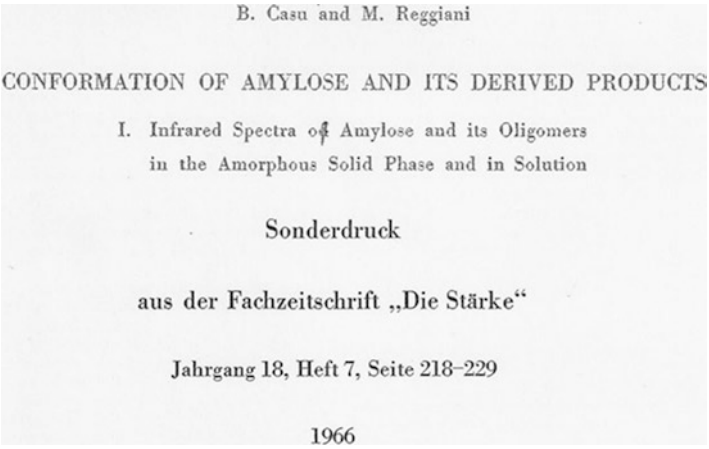
Fig. 3.3 Infrared spectra of amorphous samples performed by Professor Casu in 1964: (**a**) cyclohexaamylose, (**b**) cycloheptaamylose, (**c**) amylodextrin, and (**d**) amylose. (Source: Ronzoni Institute archives)

Frequency, cm.$^{-1}$				
Cyclo-hexa-amylose	Cyclo-hepta-amylose	Amylo-dextrin	Amylose	Tentative assignment
1445	1445	1450	1450	CH_2 bending
1405	1408	1410	1409	O—H in-plane bending
1363	1364	1370	1368	C—H bending
1330	1329	1330	1335	C—H bending
1293	1297	1300	1300	C—H bending
1260	1258			O—H in-plane bending
1237	1236	1234	1234	O—H in-plane bending
1199	1199	1200	1197	O—H in-plane bending
1151	1152	1150	1148	Coupled C—O (bridge) as stretching/O—H bending
1074	1074	1076	1075	C—O stretching/C—C stretching
1028	1025	1019	1018	C—O stretching/C—C stretching
948	943	928	928	Ring vibration
934	936			Ring vibration
856	856	852	852	C_1 group vibration
842	845			C_1 group vibration
750	753	759	759	Ring breathing vibration
703	703	706	705	Ring vibration
650	650	650	650	O—H out-of-plane bending
605	605	605	605	Ring vibration
570	574	575	573	Ring vibration
527	527	525	525	Ring vibration

Fig. 3.4 Infrared frequencies of amorphous cyclohexaamylose, cycloheptaamylose, amylodextrin, and amylose in the 1500–400 cm^{-1} range assigned by Professor Casu in 1964. (Source: Ronzoni Institute archives)

hydrogen exchange. All these results, presented for the first time at a conference invited to the 10th Congress of the Italian Chemical Society (Padova, Italy, June 1968), were "relevant" according to Professor Szejtli, since most of the reactions in which cyclodextrins were involved were carried out in solution, mostly in water (Szejtli 1982, 1988).

Another interesting fact was that the hydrogen bonds in β-cyclodextrin were also stronger than in α-cyclodextrin (Casu 1966; Casu et al. 1968a, b). On raising the temperature, the hydroxyl signals shifted to higher fields, which was indicative of a weakening of the hydrogen bonds. At the same time, the signals for the anomeric protons remained practically unchanged. These results were in accordance with the presence of intramolecular hydrogen bonding in cyclodextrins previously proposed by Hybl et al. (1965), on the basis of X-ray crystallography data. Two years later, Cramer's group also published similar results using optical rotator dispersion spectroscopy (Cramer and Hettler 1967; Cramer et al. 1967). Later, Takeo and Kuge (1969), investigating the NMR spectra of β-cyclodextrin and γ-cyclodextrin (DMSO-d_6 at 25 °C and 80 °C), showed that hydrogen bonds were stronger in γ-cyclodextrin than in β-cyclodextrin, in agreement with Casu's results.



B. Casu and M. Reggiani

CONFORMATION OF AMYLOSE AND ITS DERIVED PRODUCTS

I. Infrared Spectra of Amylose and its Oligomers in the Amorphous Solid Phase and in Solution

Sonderdruck

aus der Fachzeitschrift „Die Stärke"

Jahrgang 18, Heft 7, Seite 218–229

1966

Fig. 3.5 First page of the article of Professor Casu published in 1966 where he described the infrared spectra of cyclodextrin in the amorphous solid phase and in water and dimethyl sulfoxide solutions. (Source: Wissenschaftliche Verlagsgesellschaft MBH., Stuttgart, Germany; with permission of Pergamon Press Ltd.)

In the mid-1960s, Professor Casu also studied the formation of inclusion complexes using NMR, infrared, and UV techniques (Casu 1966, 1968a; Casu and Rava 1966). These results were presented at the *Consiglio Nazionale delle Ricerche*, Rome, Italy, in July 1966. At that time, NMR investigations of the complexes were technically difficult due to the low solubility of the products in D_2O (Higuchi and Connors 1965; Demarco and Thakkar 1970; Wood et al. 1977; Szejtli 1982, 1988; Crini 2014). Professor Casu was among the first to verify the idea that "if the guest molecule is accommodated in the cyclodextrin cavity, then the hydrogen atoms located in the interior of the cavity, i.e. C3-H and C5-H, will be significantly shielded by the guest," whereas "the hydrogen atoms on the outer surface, i.e. C2-H, C4-H and C6-H of the glucose unit, will not be affected by the formation of the inclusion complex" (Casu and Rava 1966). It was interesting to note that, at that time, only X-ray diffraction data proved that the incorporated guest was located really in the interior of the ring molecule (Takeo and Kuge 1969, 1970; Szejtli 1982).

Professor Casu also was the first to show that the acid dissociation constants of substituted benzoic acids included in cyclodextrins gave a linear correlation with Hammett's substituent constants (Fig. 3.7). The stability of the complex became higher with the increase of the electron donor character of the substituents of the included guest (Casu and Rava 1966). One year later, Professor Cramer published similar conclusions (Cramer and Hettler 1967; Cramer et al. 1967). Professor Casu, studying the interactions between dyes and α-cyclodextrin (Fig. 3.8), showed that complex formation with cyclodextrin altered the original UV absorption spectrum of the guest molecule (Casu and Rava 1966). This result was in agreement with the previous conclusions reported by Cramer's group (Cramer 1952, 1954; Cramer and

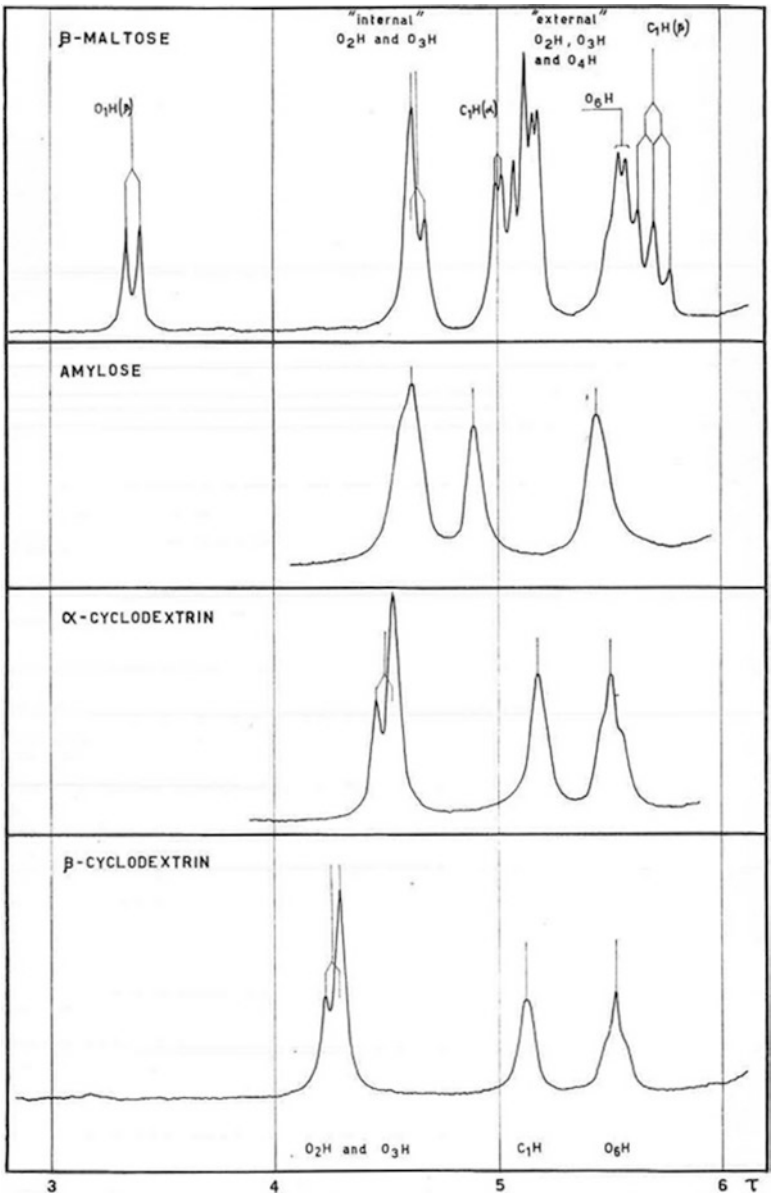


Fig. 3.6 The first NMR spectra of β-maltose, α-cyclodextrin, and β-cyclodextrin in dimethyl sulfoxide at 28 °C performed by Professor Casu in 1966 using a 100 MHz spectrometer. (Source: Ronzoni Institute archives)

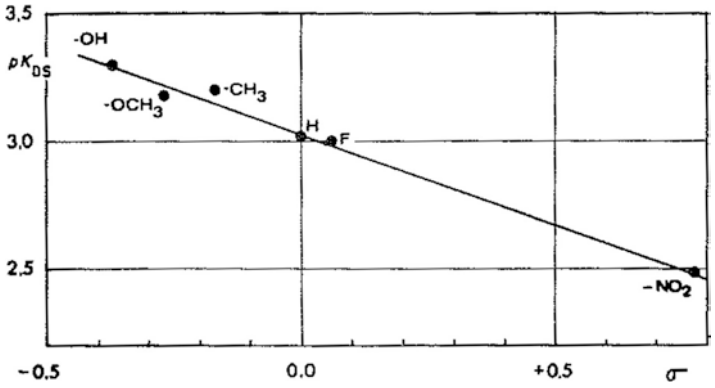


Fig. 3.7 Relationship between the dissociation constants of substituted benzoic acids included in α-cyclodextrin and the Hammett's substituent constants proposed by Professor Casu in 1966. (Source: Ronzoni Institute archives)

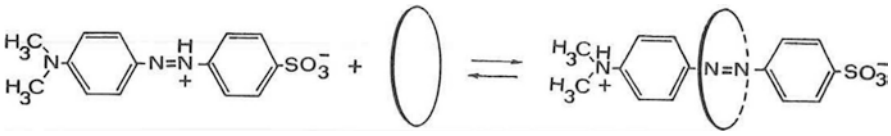


Fig. 3.8 Inclusion complex between methyl orange dye and α-cyclodextrin proposed by Professor Casu in 1966. (Source: Ronzoni Institute archives)

Henglein 1956) and by French's group (Thoma and French 1959, 1960; James et al. 1959). The intensity at the absorption maximum was considerably changed with the increase of α-cyclodextrin concentration. Professor Casu suggested that molecular encapsulation was "an interesting tool to remove dyes from aqueous solution" (Casu and Rava 1966).

In 1967, Professor Casu published the first perspective view of 2,6-di-O-methylated cyclodextrin (Casu et al. 1967). When deuterium replaced a hydrogen-bonding proton, the resulting D-bond was weaker than the original H-bond if this H-bond was strong, while the contrary was valid for weak H-bonds. The data showed that the NMR signals of the corresponding hydroxyl groups occurred at field higher than that of the O_2-H/O_3-H signals of amylose. The existence of an intramolecular H-bond was strongly substantiated by the concentration and solvent independence of the NMR and IR OH absorptions. However, this internal H-bond was not particularly strong. If it was regarded as somewhat intermediate between the "strong" and the "weak" H-bond, no substantial preferences of H of D were expected for the oxygen at C-3 of di-O-methylated cyclodextrins. Professor concluded that "more accurate results will certainly shed light on this facet in the near future when NMR instruments with higher resolution and sensitivity will be available" (Casu et al. 1967).

Later, Professor Casu prepared cyclodextrin derivatives such as methylated (Casu 1968a; Casu et al. 1968c) and acetylated (Casu et al. 1970) products. This work was further supported by a grant from the US Department of Agriculture. The

results showed that partial methylation increased solubility, due to the disruption of the hydrogen bond system. The solubility of a derivative containing seven methyl groups increased, e.g., from 1.8 g/100 mL to 6.7 g/100 mL at 25 °C. Later, Professor Casu studied inclusion properties of methylated cycloamyloses, demonstrating that the inclusion complexes of methylated derivatives were more stable than the corresponding complexes with the parent cyclodextrins (Casu et al. 1968c, 1974a, b, 1979). All these results were presented at an invited plenary lecture at the 7th International Symposium on Carbohydrate Chemistry (Bratislava, August 5–9, 1974). During this conference, Professor Casu met Professor Szejtli for the first time. They then spent a "pleasant evening" discussing these methylated compounds.

A year later, Professor Szejtli invited Professor Casu and Professor Reggiani (Director of the G. Ronzoni Institute) to Budapest for a seminar to present their results on methylated cyclodextrins. The following years saw the emergence of a fruitful and friendly collaboration between their two groups (Crini 2014). The experimental protocols for methylated cycloamyloses, detailed by Professor Casu, were repeated in the 1980s by Professor Szejtli (Szejtli et al. 1980; Szejtli 1982). Since then, these derivatives have had a tremendous development.

In 1979, methylated cyclodextrins were used as versatile complexing agents for the complexation of some n-alkanes and dyes (Casu et al. 1979). Professor Casu's latest work on cyclodextrins was carried out in the late 1980s, publishing four important articles on the interactions of cyclodextrins with glycolipids (Casu et al. 1988, 1990, 1992; Ahmed et al. 1994).

3.3.3 Professor Casu: A Pioneer in the Dissemination of the Knowledge of Cyclodextrins

Professor Casu was among the first to publish general notes and reviews on cyclodextrins for a broader public (Crini 2014; Crini et al. 2018). He is distinguished for his important contribution to the dissemination of knowledge about these substances (Casu 1966, 1968a, b, 1981, 1980, 1981, 1982, 1986, 1987; Casu and Rava 1966; Casu et al. 1974a, b, 1979, 1988).

3.3.4 Professor Casu: A Pioneer of Glycosaminoglycan Research

In 1969, Professor Casu visited the McGill University Department of Chemistry at Montréal, for 1 year as a guest of Professor Perlin. This laboratory was prominent in pure and applied carbohydrate chemistry (Crini and Torri 2017). Professor Casu pursued his studies on the structure of carbohydrates using NMR techniques (Perlin and Casu 1969; Perlin et al. 1970a). His sound collaboration with Professor Perlin

was compiled in an excellent monograph entitled *Spectroscopic Methods* published by Academic Press in 1982 (Perlin and Casu 1982). During this stage in Canada, Professor Casu was also introduced to biologically active natural substances such as heparin (Perlin et al. 1969, 1970b).

After this experience, Professor Casu returned to Milan and abandoned his research on cyclodextrins in the mid-1970s, although he continued to publish several articles on cyclodextrins (Crini and Torri 2017). Indeed, he focused his work on heparin, which earned him greater international visibility for his contribution to the chemistry and biochemistry of glycosaminoglycans (Casu 1993, 1994, 2005; Casu and Torri 1999; Petitou et al. 2003; Harenberg and Casu 2009; Guerrini et al. 2009).

Professor Casu is also recognized as one of the pioneers in glycobiology and glycochemistry and in the dissemination of the knowledge of heparin (Casu 1985b, 1986, 1989, 1993, 1994, 2005; Casu and Torri 1999; Casu and Lindahl 2001; Casu et al. 2001, 2015; Casu and Naggi 2003; Petitou et al. 2003; Harenberg and Casu 2009; Guerrini et al. 2009). He introduced and experimentally validated the concept that the unusual conformational properties of iduronic acid residues are determinant in protein binding and associated biological activities of glycosaminoglycans (Casu et al. 1986).

The 1980s saw the emergence of numerous scientific research collaborations on heparin that almost always led to interpersonal relationships between Professor Casu and eminent scientists working in this field (Torri and Cassinelli 2018). We mention a few of them because it is impossible to establish an exhaustive: e.g., the collaborations with R. Rosenberg, D. Atkins, J. Scott, E. Conrad, and J. Vercellotti, the one with the NIBSC of London in the person of E. Johnson, and mostly the *Connection Française* with J. Choay, D. Ferro, G. Gatti, J. Fareed, M. Petitou, and P. Sinaÿ.

Finally, we would like to mention the one that marked subsequent significant developments in the non-anticoagulant activities of heparin and was based on the deep mutual esteem in which Professor Casu and Professor Ulf Lindahl held each other (Torri and Cassinelli 2018). Under his direction, the Ronzoni Institute's research units have acquired a high reputation in this field, contributing, among other things, to the study of the binding mechanism of heparin to antithrombin, to the development of heparin-based drugs and new active derivatives (Casu and Lindahl 2001; Casu et al. 2001; Casu and Naggi 2003), and to the analytical insights (Guerrini et al. 2008, 2010).

The following years saw the emergence of fruitful collaborations with J. Harenberg, R. Sasisekharan, I. Vlodavsky and G.R. Sanderson, among others. G.R. Sanderson, J.R. Vercellotti, R. Laine, M. Iacomini, M. Hricovíni, G. Crini (Fig. 3.9), E.A. Yates, G. Sassaky, and C.K. Larive spent an enriching sabbatical year with Professor Casu. More than a hundred foreign researchers have been trained by Professor Casu through numerous International and European research projects that he has coordinated.

Fig. 3.9 Professor Casu with Dr. Crini at an Italian-French seminar on polysaccharides (Milan, Italy, 2010), organized by Dr. Torri, celebrating 15 years of friendly collaboration between the two laboratories. (Credit image: G. Crini)

3.4 Conclusion

Professor Benito Casu was a giant among carbohydrate chemists and biochemists, a pioneer in the dissemination of the knowledge of cyclodextrins during more than 30 years (Table 3.1), a pioneer of glycosaminoglycan research during 40 years, a gifted and dedicated teacher, and a warm, friendly, and generous communicator. Gentleman Professor Benito Casu will remain a mentor and a guide for all the researchers involved in the carbohydrate field.

We conclude with two citations of Professor Benito Casu: (1) "Cyclodextrins have been a source of fascination for over a hundred years as the heart of these molecules is easy to penetrate although they are hard to crack (Milano, 1993); 2) "*La ricerca intesa come strumento di conoscenza e non come oggetto di competizione e strumento di potere*," i.e., "Research as a tool of knowledge and not as a matter of competition and power tool" (Montalcini, Elogio dell' imperfezione).

Table 3.1 Recap of the main results of Professor Casu on cyclodextrins

Year	Result
1964	Infrared spectroscopy is a powerful method to study the conformations of cycloamyloses The first infrared spectra of four different hydrates of cyclohexaamylose/α-cyclodextrin Cyclic oligomers of amylose can occur in a variety of crystalline forms: eight crystalline modifications of cyclohexaamylose are published Spectral differences between amorphous amylose, cycloamyloses, and amylodextrin are limited to small frequency displacement of some bands The first suggestion on the fact that the glucopyranose rings in a cyclodextrin molecules possess the C1 chair conformation from spectroscopic data
1965	An infrared spectrophotometric procedure is developed for determining water in carbohydrates Proton nuclear magnetic resonance is also a powerful method to study the conformations of cycloamyloses The first NMR spectra of α-cyclodextrin and β-cyclodextrin NMR data demonstrate that (i) the D-glucopyranose units in cyclodextrins and in maltose possess the C1 chair conformation; (ii) β-cyclodextrin possesses a perfect rigid structure; (iii) the secondary hydrogen bond belt in α-cyclodextrin is incomplete; and (iv) the primary and secondary hydroxyl groups have a similar conformation in both the dissolved and the crystalline state The first suggestion on the existence in water of hydrogen bonds between the secondary hydroxyl groups
1966	The hydrogen bonds in β-cyclodextrin are stronger than in α-cyclodextrin Temperature strongly affects the presence of hydrogen bonds The complete oxidation of all primary hydroxyl functions of α- and β-cyclodextrins is reported Using chemical experiments and NMR data, the existence in water of hydrogen bonds between the secondary hydroxyl groups is demonstrated Intramolecular hydrogen bonding renders the secondary hydroxyl groups in cyclodextrins more resistant to hydrogen exchange The chemical shift and splitting of C_1-H signals of cyclodextrins and amylose in DMSO strongly substantiate the C1 chair conformation C1 chair units are consistent with strong intramolecular H-bonding between O_2-H and O_3-H hydroxyls The strongest intra H-bonding of β-cyclodextrin compared to α-cyclodextrin can arise either from different values of the glycosidic angles or from a slightly different rotation of the glucose units about the glycosidic bond The acid dissociation constant of p-substituted benzoic acids complexed by cyclodextrins give a linear correlation with Hammett's substituent constants The stability of the complex becomes higher with the increase of the electron donor character of the substituents of the included molecule

(continued)

Table 3.1 (continued)

Year	Result
1967	The isotopic hydrogen-deuterium exchange provides valuable information on the conformation of glucose and polyglucoses in solution The O_2-H and O_3-H protons of β-cyclodextrin are less exchangeable than those of α-cyclodextrin and consistently more resistant to exchange than those of maltose and amylose The coupling constants are dependent essentially on the conformation of the glucopyranose units; the chemical shifts are influenced by the magnetic anisotropy of the C-O and C-C bonds of the adjacent units A new method is proposed for methylation of cyclodextrins in solution using DMF or DMF-DMSO mixture in the presence of BaO The first perspective view of 2,6-di-O-methylated cyclodextrin is published The first evidence that O-methylation does not appreciably modify the C1 conformation of the D-glucopyranose units of cyclodextrins and amylose The first suggestion that ^{1}H-NMR spectroscopy is applicable to quantitative analysis of concentrated solutions of mixtures of cyclodextrins in dimethyl sulfoxide
1968	A detailed study using NMR spectroscopy on the effect of temperature on the hydrogen bonds On raising the temperature, the hydroxyl signals shift to higher fields, which is indicative of a weakening of the hydrogen bonds; at the same time, the signals for the anomeric protons remain practically unchanged A detailed study of hydrogen-deuterium exchange on the hydroxyl groups in dimethyl sulfoxide by NMR spectroscopy demonstrates that the equilibrium constant for the secondary hydroxyls is 0.75 in α-cyclodextrin and 0.65 in β-cyclodextrin, both much less than the corresponding value for amylose, i.e., 0.85: this also confirms that intramolecular hydrogen bonding renders the secondary hydroxyl groups in cyclodextrins more resistant to hydrogen exchange The degree of substitution of partially methylated cyclodextrins is determined using NMR spectroscopy First detailed discussion of the conformation of O-methylated cyclodextrins and amylose
1970	The spin-spin coupling constants of the ring protons confirmed the C1 conformation for the D-glucopyranose units in cyclodextrin molecules First detailed discussion of the conformation of acetylated cyclodextrins β-cyclodextrin triacetate has a quasi-eclipsed chain conformation
1974	The inclusion complexes of methylated cyclodextrins are more stable than the corresponding with the parent cyclodextrin Methyl groups introduced during the modification of cyclodextrins by methylation are not expected to obstruct the macrocycle cavities
1979	Methylated cyclodextrins form crystalline complexes with homologous n-alkanes, the stability of which depends on the size and shape of the guest molecule Methylated cyclodextrins are used as stationary phases
1982	The analysis of coupling between ^{13}C and ^{1}H across the glycosidic bridges appears the most promising approach for evaluating inter-residue torsional angles High-resolution NMR spectra of solid samples: these spectra are of obvious interest for direct comparison with spectra in solution

(continued)

Table 3.1 (continued)

Year	Result
1988	A comprehensive study of the interaction of cyclodextrins with neutral glycolipids Cyclodextrin molecules are able to form inclusion complexes with long hydrophobic chains of surfactant molecules The NMR spectra of cyclodextrin change in the presence of the glycolipid not only with shifts of the H-3 and H-5 signals, those usually affected by formation of inclusion complexes, but also with involvement of other signals, e.g., shift of H-2 and H-4 groups
1990	The first demonstration that α-cyclodextrin can disrupt the microaggregates of alkylglycosides in aqueous solutions: the NMR parameters sensitively reflect the interaction of cyclodextrins with these amphiphilic molecules Cyclodextrins can control the aggregation and surfactant properties of amphiphilic substances
1992	Further evidence that cyclodextrins can disrupt micellar aggregates of glycolipids are obtained through ultrafiltration experiments
1994	Confirmation that micellar aggregates of glycolipid can be disrupted by the formation of inclusion complexes only with α-cyclodextrin β-cyclodextrin gives weaker complexes with glycolipid but does not produce any significant disaggregation effects Fully methylated β-cyclodextrin and hydroxyethyl-β-cyclodextrin are also ineffective

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