RESEARCH ARTICLE

An explorative study towards the chemical synthesis of the immunoglobulin G1 Fc CH3 domain

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Monoclonal antibodies, fusion proteins including the immunoglobulin fragment c (Ig Fc) CH2-CH3 domains, and engineered antibodies are prominent representatives of an important class of drugs and drug candidates, which are referred to as biotherapeutics or biopharmaceuticals. These recombinant proteins are highly heterogeneous due to their glycosylation pattern. In addition, enzyme-independent reactions, like deamidation, dehydration, and oxidation of sensitive side chains, may contribute to their heterogeneity in a minor amount. To investigate the biological impact of a spontaneous chemical modification, especially if found to be recurrent in a biotherapeutic, it would be necessary to reproduce it in a homogeneous manner. Herein, we undertook an explorative study towards the chemical synthesis of the IgG1 Fc CH3 domain, which has been shown to undergo spontaneous changes like succinimide formation and methionine oxidation. We used Fmoc-solid-phase peptide synthesis (SPPS) and native chemical ligation (NCL) to test the accessibility of large fragments of the IgG1 Fc CH3 domain. In general, the incorporation of pseudoproline dipeptides improved the quality of the crude peptide precursors; however, sequences larger than 44 residues could not be achieved by standard stepwise elongation with Fmoc-SPPS. In contrast, the application of NCL with cysteine residues, which were either native or introduced ad hoc, allowed the assembly of the C-terminal IgG1 Fc CH3 sequence 371 to 450. The syntheses reported here show advantages and limitations of the chemical approaches chosen for the preparation of the synthetic IgG1 Fc CH3 domain and will guide future plans towards the synthesis of both the native and selectively modified full-length domain.

KEYWORDS
Fc CH3 domain, native chemical ligation, pseudoproline, solid phase peptide synthesis

1 | INTRODUCTION

Monoclonal antibodies (mAbs) and immunoglobulin fragment c-fusion proteins are the most prominent representatives of the rapidly emerging class of biopharmaceuticals, which are applied to treat life-threatening diseases like cancer and autoimmune disorders (eg, MabThera/Rituximab for B-cell lymphoma, Herceptin/Trastuzumab for breast cancer, Enbrel/Etanercept, and Remicade/Infliximab for rheumatoid arthritis).[1-3] Still in the context of cancer therapy, Ab engineering, including minibodies that are formed by the dimer of a single polypeptide chain reassembling the Ab V_H and V_L regions with the immunoglobulin fragment c CH3 domain,[4,5] has become a valuable approach, as it allows designing Ab-related molecules with tuned pharmacokinetic and immunogenic properties.[6,7]

The analytical and biological characterization of mAbs and, in general, of large biomolecules is a challenging but indispensable task.

Indeed, the quality of a protein-based drug may be affected by the existence of multiple variants displaying altered biophysical and biochemical properties; such chemical diversity mainly arises from post-translational modifications (PTMs), like glycosylation, and spontaneous degradation during the production and formulation processes.[8,9] To this regard, the most commonly encountered enzyme-independent modifications are methionine oxidation,[10-12] deamidation of asparagine coupled to succinimide (Snn), aspartate and isoaspartate (isoAsp) formation,[13-16] N-terminal glutamate or glutamine cyclization to pyroglutamate,[17-19] and arginine glycation or carbonylation.[20,21] Spontaneous degradation products due to pyroglutamate formation, methionine oxidation, and asparagine deamidation have been observed in therapeutic mAbs.[22] In particular, the IgG1 Fc domain contains two conserved and solvent-exposed methionines at positions 432 in the CH3 domain (Figure 1) and 256 in the CH2 domain, which have been shown to be susceptible to oxidation upon manufacturing and storage.[11] Moreover, it has been demonstrated by surface plasmon resonance that oxidation of Met-432 and Met-256 impacts the binding affinity to the human neonatal Fc receptor (FcRn),[23] protein A, and protein G,[24] which has been attributed to the alteration of the secondary structure surrounding the residues involved in the binding.[25]

Asparagine deamidation and related products (Asp, isoAsp, and Snn) have been detected in both the Fc and Fab regions of mAbs, causing alterations of their secondary structure, potency, and binding affinity, especially when occurring across the flexible complementarity-determining region,[26-28] thus having direct consequences on the Ab-mediated immune response. In the IgG1 Fc region, such PTM has been reported at Asn-319 within the sequence Leu-Asn-Gly-Lys in the CH2 domain, as well as at Asn-388, Asn-393, and Asn-438 within the CH3 domain[29-31] (Figure 1). The biological impact of deamidation has been studied in the Fab region of mAbs,[26-28] whereas its effect on the Fc region remains to date unclear. However, the results of an alanine-scan on human IgG1 have revealed that replacement of Asn-438 influences the binding affinity for FcRn.[32] Therefore, also the structural change caused by deamidation at Asn-438 might potentially affect the FcRn binding.

Thus, oxidation and deamidation events may be considered two of the most important sources of enzyme-independent PTMs in drugs based on mAbs and Fc-fusion proteins. Moreover, both changes may influence the Ig binding to FcRn, thus affecting the half-life of Igs and Fc-fusion proteins in the blood.[33-35] Although spontaneous PTMs are fortunately present only as minimal impurities, which may be hardly detectable even by highly sophisticated MS analysis,[36,37] nevertheless, the assessment of their structural and biological effects is of high significance. For this reason, it is often necessary to treat the protein chemically, in order to induce and investigate the desired PTM. For example, H_2O_2 treatments are usually performed to increase the amount of oxidized methionine. This, however, results in multiple combinations of reduced and oxidized methionine residues.[11,23,38] In contrast, chemical protein synthesis (CPS) based on the native chemical ligation (NCL) of synthetic fragment precursors would, in principle, allow obtaining only one species at the time by replacing only the desired methionine with methionine sulfoxide. CPS has been successfully applied for small and medium-size proteins,[39-43] whereas other approaches have been developed to overcome the protein-size limitation, which rely on the NCL between synthetic and recombinant fragment precursors (so-called expressed protein ligation[44-46]), or on the incorporation of unnatural amino acids (AAs) into a protein by genetic-code expansion.[47-49]

At the light of the important role played by the CH3 domain in the binding of IgG molecules to FcRn[50,51] as well as in the dimerization process of minibodies,[4] we undertook the present work to assess the accessibility of this domain by chemical synthesis, which, in turn, would make variants containing, for example, oxidized methionine or D-amino acids at the desired position also accessible. In particular, we focused on the preparation of IgG1 CH3 fragment precursors for two possible NCL strategies that rely on threonine-based[52,53] and cysteine-based[43,54] chemoselective reactions (Scheme 1). The C-terminal fragment precursor (either 1, 4, or 6) contains oxidized methionine at position 432. Problems encountered during the solid-phase peptide synthesis (SPPS) of the precursors and some solutions to them, as well as NCL attempts, are discussed below. Although the total chemical synthesis of the IgG1 Fc CH3 domain could not be achieved in this work, the conducted explorative study provides useful insights into the Fmoc-based SPPS of IgG1 Fc CH3 sequences, including C-terminally activated intermediates bearing the salicyladehyde ester (Sal) or a thioester.

2 | MATERIALS AND METHODS

2.1 | Chemicals

All protected AAs, Fmoc-Rink amide MBHA resin, N-(9-fluorenylmethyloxycarbonyloxy)-succinimide (Fmoc-OSu), N,N-dimethylformamide (DMF), 1-methyl-2-pyrrolidinone (NMP), dichloromethane (DCM), diethylether (Et₂O), 2,2,2-trifluoroethanol (TFE), and N,N-diisopropylethylamine (DIPEA) were purchased from Iris Biotech GmbH (Marktredwitz, Germany). Pseudoproline dipeptides, O-(7-azabenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium-hexafluorophosphate (HATU), H-Gly-2-Cl-trityl resin, H-Thr(tBu)-2-Cl-trityl resin, Fmoc-Gly-NovaSyn-TGT resin, Fmoc-Dbz-NovaSyn-TGR resin, 4-nitrophenylchloroformate, and disodiumhydrogenphosphate were from Merck (Darmstadt, Germany). 1-Hydroxybenzotriazole (HOBt), 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), N,N'-diisopropylcarbodiimide (DIC), trifluoroacetic acid (TFA), and piperidine were obtained from Biosolve (Valkenswaard, The Netherlands). HPLC-grade acetonitrile (ACN), thiophenol, 4-
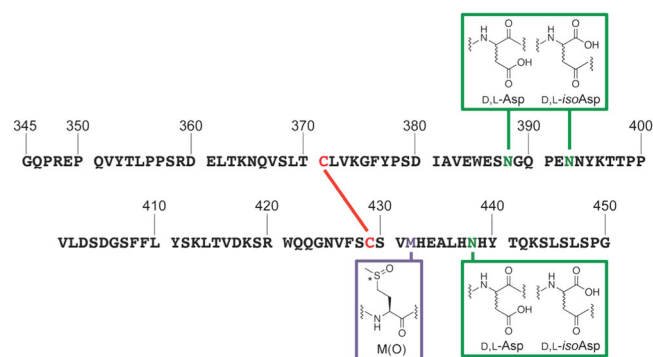
FIGURE 1 Sequence of the IgG1 Fc CH3 domain and detected, spontaneously occurring PTMs at Met and Asn sites
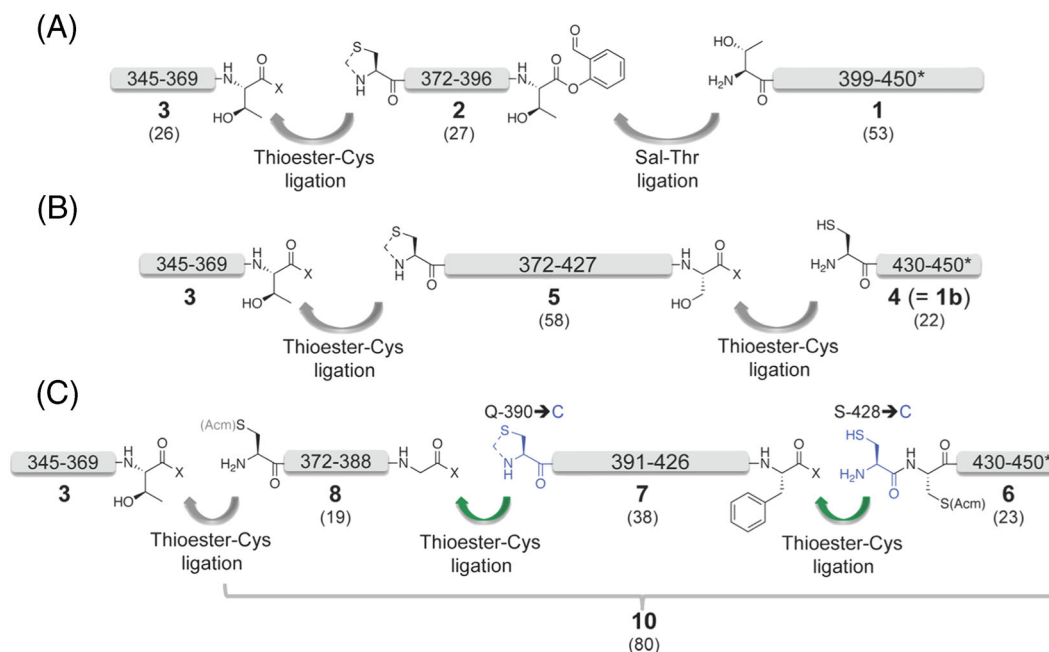
SCHEME 1 Proposed strategies for the synthesis of the IgG1 Fc CH3 domain based on two (A and B) or three (C) ligation points. The * in fragments **1**, **4**, and **6** indicates the presence of Met(O)-432. Sal, salicylaldehyde ester. Peptide **4** is also referred to as **1b** in the following schemes. The ligations indicated by green arrows were successfully performed in this work

mercaptophenylacetic acid (MPAA), triisopropylsilane (TIS), thioanisole (TIA), tris(2-carboxyethyl) phosphine hydrochloride (TCEP), guanidine hydrochloride (GuHCl), and salicylaldehyde were obtained from Sigma Aldrich (Vienna, Austria). HPLC-grade trifluoroacetic acid (TFA) was from Alfa-Aesar (Karlsruhe, Germany).

2.2 | Methods

Analytical RP-HPLC was performed using a Thermo Fisher Scientific Dionex UltiMate 3000 UHPLC system (Germering, Germany) and either a Syncronics C-18 column (100 Å, 5 μm, 250 × 4.6 mm, Thermo Fisher Scientific) at a flow rate of 1.5 mL/min or a Nucleosil C-18 column (100 Å, 5 μm, 250 × 4 mm, Macherey-Nagel, Düren, Germany) at a flow rate of 1 mL/min. Unless specifically stated, the first one was used. The UV detection was set at 220 nm. The elution system was (A) 0.06% (v/v) TFA in water, and (B) 0.05% (v/v) TFA in ACN. The crude products were dissolved in ACN/H_2O (10:90, v/v) containing 0.1% TFA. Analytical chromatograms were obtained with the following gradients: method A: 10% B for 5 minutes, 10% to 70% B in 40 minutes; method B: 20% B for 5 minutes, 20% to 70% B in 30 minutes; method C: 25% B for 5 minutes, 25% to 75% B in 40 minutes; method D: 20% B for 5 minutes, 20% to 60% B in 40 minutes. Mass spectra were recorded on an Autoflex Speed MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) by using α-cyano-4-hydroxycinnamic acid as matrix.

2.3 | Stepwise synthesis of [Met(O)-432]-IgG1-Fc 419-450 (1a^TRT) and Fmoc-[Met(O)-432]-IgG1-Fc 407-450 (1a^TGT+ψ)

Solid-phase peptide synthesis was automatically performed on a Syro-I (Biotage, Uppsala, Sweden) peptide synthesizer by using the Fmoc/tBu

strategy. Sequence assembly was carried out on H-Gly-2-Cl-trityl (loading: 0.63 mmol/g; 18-μmol scale) or Fmoc-Gly-NovaSyn-TGT resin (loading: 0.2 mmol/g; 8-μmol scale). The couplings of the protected AAs were carried out using AA/HOBt 1:1 (5 equivalents each), HBTU (4.9 equivalents), and DIPEA (10 equivalents), in DMF/ NMP (7:3, v/v). Each coupling was performed twice (2 × 45 minutes). The couplings of the pseudoproline dipeptides were performed manually by employing (AAψAA)/HOBt 1:1 (2 equivalents each), HBTU (1.9 equivalents), and DIPEA (4 equivalents), in DMF/NMP (7:3, v/v) for 1 hour. The completeness of the coupling was then checked by the ninhydrin test, and, when needed, the coupling was repeated under the same conditions to assure complete acylation. N^α-deprotection was obtained with a 3-minute treatment with 30% piperidine in DMF, followed by a 12-minute treatment with 15% piperidine in DMF. After the coupling of the last residue, the peptidyl-resin was washed with DMF, DCM, and Et$_2$O (three times each) and vacuum-dried overnight. The resin-bound peptide was treated with TFA/H_2O/TIA/ TIS/EDT (90:3:2:2:3, v/v) for 3 hours at room temperature (rt), then the peptide was precipitated with cold Et$_2$O, recovered by centrifugation, and washed three times with the same solvent to remove the residual scavengers. The crude products were characterized by analytical RP-HPLC and MALDI-TOF-MS.

2.4 | Synthesis of [Met(O)-432]-IgG1-Fc 398-450 (1) by chemoselective ligation

Peptide **1b** was assembled on the H-Gly-2-Cl-trityl resin (loading: 0.63 mmol/g; 18-μmol scale) by using the SPPS protocol described in the subsection 2.3. The crude product was characterized by analytical RP-HPLC using method A and by MALDI-TOF-MS. Peptide **1c** was synthetized with the commercially available Fmoc-Dbz-NovaSyn-TGR resin (loading: 0.2 mmol/g; 9-μmol scale). The Fmoc group of the Dbz

linker was cleaved with 20% piperidine in DMF for 20 minutes, then the resin was treated with 5 equivalents Fmoc-Ser(tBu)-OH, 5 equivalents HATU, and 10 equivalents DIPEA in DMF for 1 hour at rt. Then, the resin was washed with DMF, DCM, and Et$_2$O (three times each) and dried under vacuum. The new loading was determined by measurement of the UV absorbance of the dibenzofulvene-piperidine adduct at 301 nm. The peptide chain was assembled with the SPPS protocol described in the subsection 2.3 and the use of the pseudoproline dipeptides Fmoc-Lys(Boc)-Ser($\psi^{Me,Me}$pro)-OH, Fmoc-Tyr(tBu)-Ser($\psi^{Me,Me}$pro)-OH, and Fmoc-Asp(tBu)-Ser($\psi^{Me,Me}$pro)-OH. The coupling of Gly-424 was performed manually with Fmoc-Gly-OPfp/HOBt 1:1 (6 equivalents) for 1 hour in DMF at rt, and the progress of the reaction was monitored by the ninhydrin test. After coupling the last residue as Boc-AA, the resin was swollen in DCM for 30 minutes and treated with 5 equivalents p-nitrophenylchloroformate dissolved in DCM (50 mM) for 1 hour under nitrogen at rt. The solvent was sucked off, the resin was washed with DCM, DMF, and again DCM (three times each), and finally subjected to a 30-minute treatment with 0.5 M DIPEA in DMF at rt. After washing cycles with DMF, DCM, and Et$_2$O, the peptide was cleaved from the resin with TFA/H$_2$O/TIS (95:2.5:2.5, v/v) for 2.5 hours at rt, precipitated and successively washed with cold Et$_2$O, and finally dried under vacuum. The crude product was characterized by analytical RP-HPLC using method B and by MALDI-TOF-MS. Peptides **1b** and **1c** were dissolved in nitrogen-purged ligation buffer (0.2 M phosphate buffer at pH 7, 6 M GuHCl, 20 mM TCEP, and 1% thiophenol). The mixture was shaken for 2.5 hours, then acidified by using 0.5% TFA in water and lyophilized. The ligation product was isolated by analytical RP-HPLC using method D. The fraction containing the peak of interest was collected and lyophilized.

2.5 | Synthesis of [Thz-371, Thr-Sal-397]-IgG1-Fc 371-397 (2)

Peptide chain elongation was performed by Fmoc/tBu chemistry on H-Thr(tBu)-2-Cl-trityl resin (loading: 0.73 mmol/g; 18-μmol scale) as described in the subsection 2.3. The protected peptide was cleaved from the resin by using six 30-minute treatments with TFE/DCM (2:8, v/v). The combined filtrates resulting from each treatment were collected and concentrated in vacuum, and the fully protected peptide was precipitated with a cold mixture of Et$_2$O/hexane (2:8, v/v), recovered by centrifugation and vacuum-dried overnight. The solid was dissolved in DCM/DMF (9:1, v/v), and the resulting solution was cooled to 0°C before 8 equivalents DIC and 10 equivalents salicylaldehyde dimethyl acetal were added. The mixture was stirred first at 0°C for 3 hours, and then at rt for 16 hours. The solvent was evaporated, and the product was precipitated from cold Et$_2$O/hexane (2:8, v/v) and washed twice with the same mixture. The resulting white solid was then treated with a mixture of TFA/H$_2$O/TIA (95:2.5:2.5, v/v) for 2 hours at rt, and the deprotected peptide was recovered by centrifugation from cold Et$_2$O. The crude product was characterized by analytical RP-HPLC using method B and by MALDI-TOF-MS.

2.6 | Synthesis of [Thr-Dbz-370]-IgG1-Fc 345-370 (3a)

Mono-Fmoc-Dbz-OH was prepared starting from commercially available 3,4-diaminobenzoic acid, then coupled to Rink amide MBHA resin (loading: 0.45 mmol) as previously described.[55] Briefly 3,4-diaminobenzoic acid (0.5 g, 3.3 mmol) was suspended in 15-mL 0.1 M NaHCO$_3$ (aq.)/ACN (1:1), and Fmoc-OSu (1.1 g, 3.3 mmol) was added over 20 minutes. The reaction was stirred for 6 hours, then the pH was brought to 2 by adding HCl (aq.). The resulting white precipitate was filtered, washed with water, cold Et$_2$O, hexane, and dried under vacuum to afford the expected mono-Fmoc-Dbz-OH (0.6 g, 48%). The latter (0.04 g, 0.11 mmol) was dissolved in DMF, and the resulting solution was added to pre-swelled Rink amide MBHA resin (0.05 g, 0.022 mmol) together with HBTU (0.042 g, 0.11 mmol) and DIPEA (40 μl, 0.22 mmol) and stirred for 2 hours. The resin was washed three times with DCM and DMF and subjected to Fmoc deprotection of the linker with 20% piperidine in DMF for 20 minutes. Subsequently, the resin was treated with 5 equivalents Fmoc-Thr(tBu)-OH, 5 equivalents HATU and 10 equivalents DIPEA, for 1 hour at rt; the coupling was performed a second time to assure complete acylation. The loading was determined by measurement of the UV absorbance of the dibenzofulvene-piperidine adduct at 301 nm. The peptide chain was elongated by Fmoc/tBu chemistry as described in the subsection 2.3. The resin-bound peptide was then treated with a mixture of TFA/TIS/TIA (95:2.5:2.5, v/v) for 2.5 hours at rt, and the peptide was recovered by centrifugation from cold Et$_2$O. The crude product was characterized by analytical RP-HPLC using method B and by MALDI-TOF-MS.

2.7 | Synthesis of [Thz-371, Ser-Nbz-428]-IgG1-Fc 371-428 (5)

Fragment **5** was synthesized with the protocol that was used for **1c**. An additional pseudoproline dipeptide, Fmoc-Glu(tBu)-Ser ($\psi^{Me,Me}$pro)-OH, was used in the SPPS. The crude product was characterized by analytical RP-HPLC using method B and by MALDI-TOF-MS.

2.8 | Synthesis of [Cys(Acm)-371,429, Cys-390,428]-IgG1-Fc 371-450 by chemoselective ligation (10)

Peptides **6** and **8** were assembled on a H-Gly-2-Cl-trityl resin (loading: 0.63 mmol/g; 18-μmol scale) with the protocol described in the subsection 2.3. Peptide **6** was cleaved from the resin and deprotected by using a mixture of TFA/H$_2$O/TIA/TIS/EDT (90:3:2:2:3, v/v) for 3 hours at rt and then recovered by precipitation from cold Et$_2$O and centrifugation. The crude product was characterized by analytical RP-HPLC using method A and by MALDI-TOF-MS. For peptide **8**, the peptidyl-resin was treated for 30 minutes with a mixture of TFE/DCM (2:8, v/v), and the procedure was repeated six times. The combined filtrates resulting from each treatment were collected, concentrated in vacuum, and the fully protected peptide was precipitated with cold Et$_2$O. After centrifugation and overnight drying under vacuum, the solid was dissolved in DCM/DMF (7:3, v/v), and the resulting solution was cooled at 0°C before 3 equivalents DIC and 5 equivalents benzylmercaptan were added. The mixture was stirred at 0°C for 1 hour, and at rt for 16 hours. Afterwards, the solvent was evaporated, then the product was precipitated with cold Et$_2$O and washed twice with the same solvent. The resulting white solid was dried under vacuum and then treated with a mixture of TFA/H$_2$O/TIA (95:2.5:2.5, v/v) for 2 hours,

at rt, and the peptide was recovered by precipitation from cold Et_2O and centrifugation. The crude product was characterized by analytical RP-HPLC using method B and by MALDI-TOF-MS. Peptide **7a** was assembled on the commercially available Fmoc-Dbz-NovaSyn TGR resin (loading: 0.2 mmol/g; 9-μmol scale). The Fmoc group of the linker was cleaved with 20% piperidine in DMF for 20 minutes, and the resin was treated with 5 equivalents Fmoc-Phe-OH, 5 equivalents HATU, and 10 equivalents DIPEA for 1 hour at rt. The resin was then washed with DMF, DCM, and Et_2O (three times each). After drying under vacuum, the degree of substitution was determined by measurement of the UV absorbance of the dibenzofulvene-piperidine adduct at 301 nm. The peptide chain was assembled by Fmoc/tBu chemistry as described in the subsection 2.3, with the use of the pseudoproline dipeptides Fmoc-Lys(Boc)-Ser($\psi^{Me,Mepro}$)-OH, Fmoc-Tyr(tBu)-Ser($\psi^{Me,Mepro}$)-OH and Fmoc-Asp(tBu)-Ser($\psi^{Me,Mepro}$)-OH within the SPPS. The coupling of Gly-424 was performed manually with Fmoc-Gly-OPfp/HOBt 1:1 (6 equivalents) for 1 hour in DMF at rt, and the progress of the reaction was monitored by the ninhydrin test. Once the synthesis was completed, the resin was swollen in DCM for 30 minutes and treated with 5 equivalents p-nitrophenylchloroformate dissolved in DCM (50 mM) for 1 hour under nitrogen at rt. The solvent was sucked off, the resin was washed with DCM, DMF, and again DCM (three times each), and finally subjected to a 30-minute treatment with 0.5 M DIPEA in DMF at rt. After washing cycles with DMF, DCM, and Et_2O, the peptide was cleaved from the resin with TFA/H_2O/TIS (95.2.5:2.5, v/v) for 2.5 hours at rt, precipitated and successively washed with cold Et_2O, and finally dried under vacuum. The crude product was characterized by analytical RP-HPLC using method B and by MALDI-TOF-MS. Peptides **6** and **7a** were dissolved in nitrogen-purged ligation buffer (0.2 M phosphate buffer at pH 7, 6 M GuHCl, 90 mM TCEP, and 180 mM MPAA). The mixture was shaken for 2.5 hours, then 20 μL of a nitrogen-flushed 1.4 M methoxylamine solution containing 200 μM TCEP dissolved in 0.2 M Na_2HPO_4 and 6 M GuHCl were added, and the resulting mixture was acidified to pH 4 and stirred for 6 hours at rt. The mixture was then eluted through a column prepacked with Sephadex G-25 (NAP GE Healthcare) using 1 M GuHCl as elution system. After lyophilization, the solid and peptide **8** were dissolved in nitrogen-flushed ligation buffer (0.2 M phosphate buffer at pH 6.8, 6 M GuHCl containing 50 mM TCEP and 100 mM MPAA). After 2.5 hours, the reaction mixture was acidified by using 0.5% TFA in water and lyophilized. The ligation product was isolated by analytical RP-HPLC using method B. The fraction containing the peak of interest was collected, lyophilized, and characterized by analytical RP-HPLC using method B and by MALDI-TOF-MS.

# 3 | RESULTS AND DISCUSSION

3.1 | Ligation routes

Our work toward the chemical synthesis of the IgG1 Fc CH3 domain consisted of the preparation of fragment precursors that were designed on the base of the three ligation routes shown in Scheme 1.

In the first route, the 53-residue long fragment precursor **1** should be ligated to the 27-residue long fragment **2** by salicylaldehyde ester (Sal)-Thr ligation: the chemoselective reaction between the C-terminal Sal of **2** and the N-terminal Thr of **1** in pyridine/AcOH forms an N-acyl oxazolidine intermediate that can be cleaved by acidic hydrolysis to afford the native peptide bond at the ligation site[52,53] (Scheme 1A). The resulting ligation product should be subjected to thiazolidine (Thz) ring opening, followed by Cys-thioester ligation[43] with the 26-residue long fragment **3**. The second route exploits the presence of native Cys-429 and Cys-371 and the sequential NCL between the precursors **3**, **4** and **5** (Scheme 1B). Both routes involve the synthesis of one large precursor (either **1** with 53 residues or **5** with 58 residues), which may represent a hurdle for the synthesis of the precursor as well as for the ligation reaction. Moreover, the presence of a C-terminal threonine at the ligation site may slow down the reaction rate, as shown by a systematic study conducted by Dawson and coworkers[42] on all 20 proteinogenic AAs, which classified threonine as one of the slowest ligation sites after valine, isoleucine, and proline. Then, we envisioned a third route that contemplates the Cys-thioester ligation of four fragments, the largest one containing 38 residues (**7**), at two fast-reacting (Gly-389 and Phe-427) and one slow-reacting (Thr-370) C-terminal thioester (Scheme 1C). This, however, requires the temporary replacement of two native residues, Gln-390 and Ser-428, with Cys, and the reconversion of the two non-native residues to the native ones with suitable methods (see section 3.7).

3.2 | Attempts of stepwise synthesis of [Met(O)-432]-IgG1-Fc 398-450 (1)

Fragment **1** contains the C-terminal part of the IgG1 Fc CH3 domain, covering 53 residues and including the PTM site Met(O)-432. At first, we proved the feasibility of this fragment to be assembled by stepwise SPPS with Fmoc/tBu chemistry. We started the synthesis on a poly-styrene-divinylbenzene-2-chlorotrityl resin preloaded with glycine (0.63 mmol/g) (Scheme 2A). Met-432 was inserted as Met(O) to take into account the PTM. We checked the growing chain after 22 and 32 cycles: unfortunately, the homogeneity of the crude product dropped from ~80% to ~20% (**1a**^{TRT} in Scheme 2A). Thus, we decided to repeat the synthesis of **1** by choosing a polar resin[56,57] with low loading (Fmoc-Gly-NovaSyn TGT resin, based on low cross-linked hydroxyethylpolystyrene-polyethylene glycol, with a loading of 0.2 mmol/g). However, these two parameters (polarity and low loading of the solid support) were not sufficient to accomplish the synthesis of the desired 53-residue long fragment, as the growing chain was highly inhomogeneous after 44 cycles. Neither the addition of 2% DBU to the conventional 20% piperidine in DMF,[58] nor the use of HATU in place of HBTU for the activation of selected AAs improved the synthesis (data not shown).

Considering the presence of quite regularly distributed Thr and Ser residues in the sequence, we envisioned the possibility to increase the homogeneity of fragment **1** by incorporation of pseudoproline dipeptides, which are well known to reduce the on-resin self-association propensity of the growing peptide and, consequently, to improve the quality of the crude product.[59-61] Accordingly, by using the combination of a polar and low-loaded resin with the employment of the
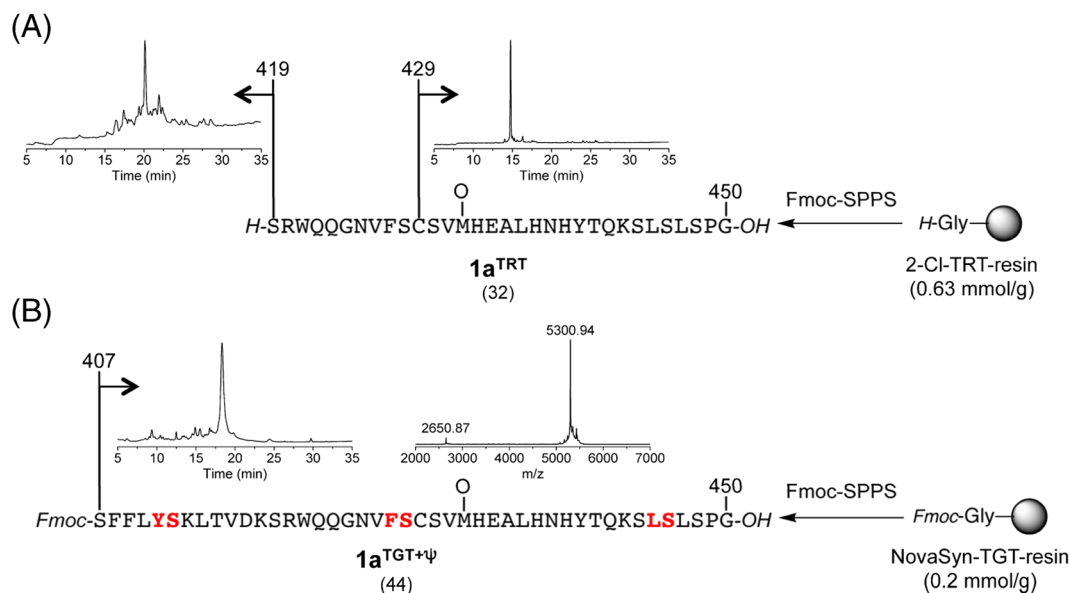
SCHEME 2 Attempts of stepwise synthesis of [Met(O)-432]-IgG1-Fc 398–450 (**1**). A, Assembly attempt on polystyrene-divinylbenzene 2-chlorotrityl resin preloaded with glycine (0.63 mmol/g). The RP-HPLC profiles of the crude peptide acids with free *N*-terminus at cycle 22 (till Cys-429) and 32 (till Ser-419, **1a^TRT**) were obtained by using method A. B, Assembly attempt on the polar and low-loaded resin NovaSyn TGT (0.2 mmol/g Fmoc-glycine) by using three pseudoproline dipeptides (in red). The RP-HPLC profile of the Fmoc-protected crude peptide acid at cycle 44 (till Ser-407, **1a^TGT+ψ**) was obtained by using method C with the Nucleosil C-18 column (MALDI-TOF-MS peaks for M + H⁺ and [M + 2H⁺]/2. $M_{calc.}$ for $C_{242}H_{352}N_{62}O_{69}S_2$: 5298.01 Da)

pseudoproline dipeptides Leu-445-ψSer-446, Phe-427-ψSer-428, and Tyr-411-ψSer-412, the growing chain was ~60% homogeneous after 44 couplings (**1a^TGT+ψ** in Scheme 2B). Nevertheless, despite this encouraging result, the attempt to elongate this fragment with additional nine residues to obtain the desired precursor **1** failed (data not shown).
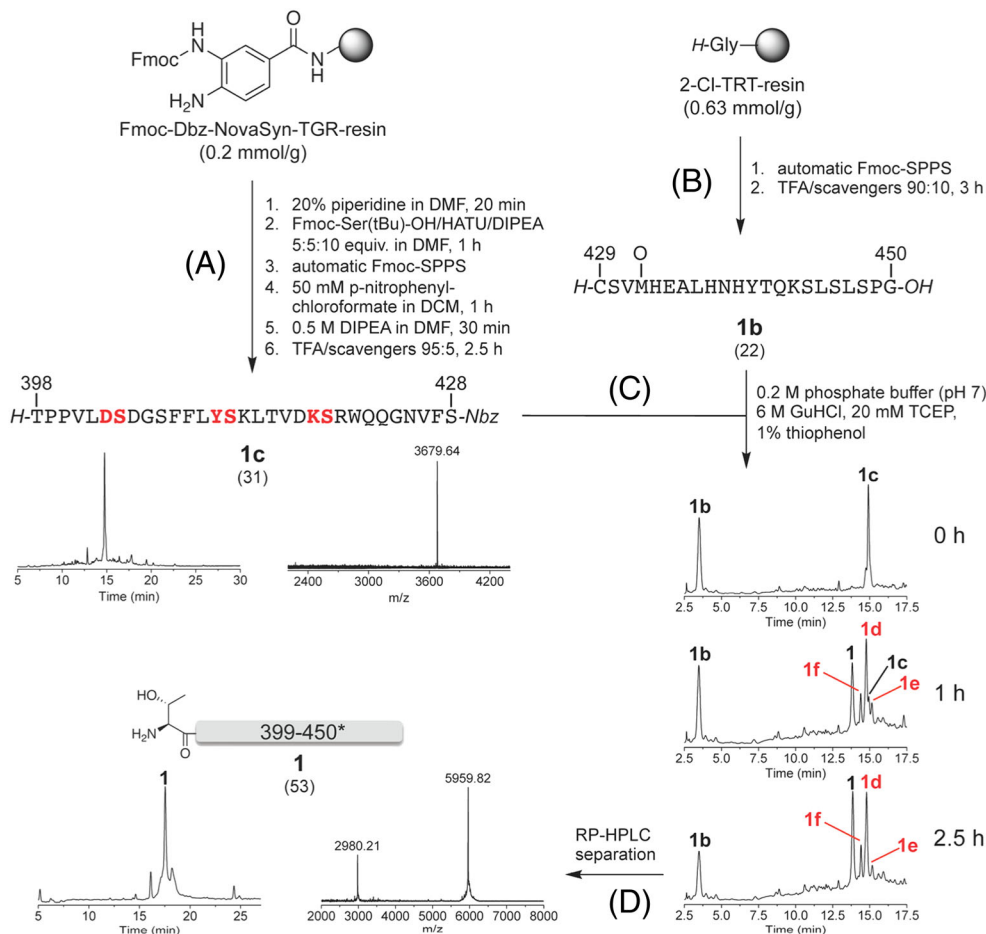
3.3 | Synthesis of [Met(O)-432]-IgG1-Fc 398-450 (1) by chemoselective ligation

Given the difficulties encountered in the standard stepwise Fmoc-SPPS of the fragment precursor **1**, we considered the chemoselective ligation approach to accomplish the 53-residue long fragment by using the natural Cys-429 as ligation point between the two fragments **1b** (= **4** in Scheme 1) and **1c** (Scheme 3). As shown in Scheme 2A, the C-terminal fragment **1b** could be successfully prepared by standard Fmoc-SPPS on the polystyrene-divinylbenzene 2-chlorotrityl resin preloaded with glycine (0.63 mmol/g). For the synthesis of the peptide thioester **1c**, we chose the *N*-acyl-urea approach,[55] in which the peptide is synthesized on a diaminobenzoic (Dbz) acid linker by Fmoc-SPPS, followed by treatment with p-nitrophenylchloroformate and, then, a tertiary base to afford the corresponding *N*-acyl-benzimidazolinone (Nbz). The latter can be either used directly for the NCL[62,63] or subjected to thiol exchange reaction providing access to the corresponding peptide thioester.[64,65] Thus, we accomplished the synthesis of **1c** starting from the low-loaded Fmoc-Dbz NovaSyn TGR resin (0.2 mmol/g). The first residue, Fmoc-Ser(tBu)-OH, was attached manually by using HATU/DIPEA. Then, the peptide chain was elongated by automated SPPS using the HBTU/HOBt/DIPEA coupling protocol, except for Gly-424 that was coupled manually using

Fmoc-Gly-OPfp (Pfp = pentafluorophenyl) to reduce the formation of branched by-products arising from the double acylation of the Dbz moiety. Also, in this case, we used pseudoproline dipeptides (Lys-418-ψSer-419, Tyr-411-ψSer-412, and Asp-403-ψSer-404), as the IgG1 Fc CH3 sequence 398-428 resulted to be otherwise very difficult to assemble (data not shown). After the coupling of the last residue as Nα-Boc-protected AA, the C-terminal Dbz group was converted into the Nbz group, and TFA cleavage gave the desired C-terminal Nbz peptide **1c** with satisfactory homogeneity (~60%) (Scheme 3A).

Fragments **1b** and **1c** were used for the NCL without any further purification (Scheme 3C). After 1 hour, a significant amount of the desired ligation product **1** could be detected; however, also a large amount of the acid arising from the hydrolysis of **1c** was formed (**1d**). Moreover, two other by-products were identified, having a mass difference of −18 Da (**1e**) and +41 Da (**1f**) with respect to the acid **1d** (Scheme 3C): the first one (**1e**) might arise from an intramolecular cyclization of the thioester precursor **1c** or of the corresponding phenyl thioester formed in situ. The second one (**1f**) might be the result of the reaction of **1c** or of the corresponding phenyl thioester formed in situ with guanidine.[66]

After 2.5 hours, the C-terminal Nbz peptide **1c** was completely consumed, and no further changes were observed with respect to the previous control (Scheme 3C). Therefore, the reaction mixture was acidified, and the HPLC fraction containing the ligation product **1** was collected and lyophilized. The mass spectrum confirmed the successful separation of the desired ligation product **1** from all impurities present in the NCL mixture. However, an additional peak at slightly higher retention time appeared in the HPLC profile, whose identity could not be assessed by mass spectrometry.

SCHEME 3 Synthesis of [Met(O)-432]-IgG1-Fc 398–450 (**1**) by chemoselective ligation. A, Fragment **1c** was prepared on a Fmoc-Dbz NovaSyn TGR resin by using three pseudoproline dipeptides (in red). The RP-HPLC profile of the crude product was obtained with method B (MALDI-TOF-MS peak for M + H^{+}. $M_{calc.}$ for $C_{169}H_{245}N_{43}O_{50}$: 3679.10 Da). B, Fragment **1b** was assembled on a 2-chlorotrityl resin preloaded with glycine (the RP-HPLC profile is shown in Scheme 2A). C, Ligation of **1c** and **1b** was followed by RP-HPLC over 2.5 h. The peaks labelled with **1d-f** are degradation products of **1c** due to hydrolysis (**1d**: MALDI-TOF-MS peak for M + H^{+}: 3521.35 Da. $M_{calc.}$ for $C_{161}H_{240}N_{40}O_{49}$: 3519.95 Da), intramolecular aminolysis (**1e**: MALDI-TOF-MS peak for M + H^{+}: 3503.52 Da. $M_{calc.}$ for $C_{161}H_{238}N_{40}O_{48}$: 3501.93 Da), and N-acylation of guanidine with the Nbz-peptide (**1f**: MALDI-TOF-MS peak for M + H^{+}: 3562.72 Da. $M_{calc.}$ for $C_{162}H_{243}N_{43}O_{48}$: 3561.0 Da). The HPLC runs were performed by using method B. D, The ligation product **1** was separated from the impurities by HPLC fraction collection. The corresponding RP-HPLC profile was obtained by using method D (MALDI-TOF-MS peaks for M + H^{+} and [M + 2H[+]]/2. $M_{calc.}$ for $C_{265}H_{401}N_{71}O_{82}S_2$: 5957.71 Da)

3.4 | Synthesis of [Thz-371, Thr-Sal-397]-IgG1-Fc 371-397 (2)

Initially, we attempted to prepare fragment **2** by on-resin phenolysis of the corresponding C-terminal Nbz peptide with salicylaldehyde dimethyl acetal.[52,67] However, despite successful elongation of the peptide chain on the Fmoc-Dbz Rink amide MBHA resin (0.45 mmol/g), both the activation of the Dbz linker with p-nitrophenylchloroformate and the subsequent on-resin phenolysis were poorly efficient, probably due to the sterically hindered C-terminal Thr(tBu)-397. Therefore, we looked for alternative solutions. Owing to the fact that the presence of a tryptophan residue in the sequence excluded the use of any protocol involving aldehyde generation by means of post-SPPS oxidative processes,[68] the esterification in solution was chosen. The peptide was assembled on the H-Thr(tBu)-chlorotrityl resin (0.73 mmol/g) and released from the solid support under mildly acidic conditions (Scheme 4). The fully protected

peptide acid was esterified with salicylaldehyde dimethyl acetal by using DIC as condensation agent, followed by TFA treatment for the removal of the side-chain protecting groups. HPLC and MS analysis showed that the desired peptide could be obtained with low homogeneity (~30% for the major HPLC peak in Scheme 4). Indeed, some impurities due to deleted sequences and double coupling of salicylaldehyde were present (MS in Scheme 4). We decided not to purify the crude product for further use in the ligation, as solubility tests of **2** under the conditions reported for Sal-Ser/Thr ligation (at least 1 mM in pyridine/AcOH[52,53,69]) were unsatisfactory (good solubility of **2** was obtained in pure AcOH, which, however, would not allow for efficient ligation). Another concern for the use of **2** in the ligation strategy was related to the possibility of 5(4H)-oxazolone-mediated epimerization of the C-terminal threonine during the esterification step of the fully protected fragment (Scheme 4). To this regard, it would have been of benefit to incorporate the C-terminal threonine as pseudoproline,[70] or to couple it as aldehyde-protected
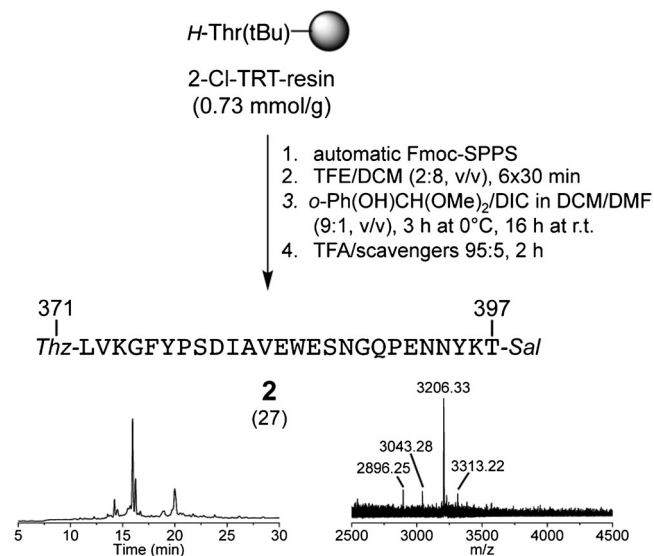
SCHEME 4 Synthesis of [Thz-371, Thr-Sal-397]-IgG1-Fc 371–397 (**2**) by esterification in solution. The RP-HPLC profile of the crude product was obtained with method B (MALDI-TOF-MS peak for M + H$^+$. M$_{calc.}$ for C$_{146}$H$_{206}$N$_{34}$O$_{46}$S$_1$: 3205.53 Da. The masses at 2896.25, 3043.28, and 3313.22 Da were attributed to impurities with ΔFY, ΔY, or condensation with an additional salicylaldehyde)

amino salicylaldehyde ester to the fully protected peptide by using the Sakakibara conditions.[71,72]

## 3.5 | Synthesis of [Thr-Dbz-370]-IgG1-Fc 345-370 (3a)

For the synthesis of the C-terminal Nbz peptide **3**, we first functionalized the Rink amide MBHA resin (0.45 mmol/g) with mono-Fmoc-diaminobenzoic acid synthetized from commercially available 3,4-diaminobenzoic acid. The first residue, Fmoc-Thr(tBu)-OH, was coupled manually with HATU/DIPEA, followed by spectrophotometric determination of the Fmoc group, which confirmed the success of the coupling. The peptide chain was then elongated by automated SPPS with the HBTU/HOBt/DIPEA coupling protocol. After the assembly was completed, we attempted to convert the Dbz group into the Nbz group. However, the conversion was incomplete, probably due to the sterically hindered C-terminal Thr(tBu)-370. Thus, we decided to cleave the peptide from the resin in the Dbz form **3a**, which is more stable than the Nbz form **3** but may be orthogonally converted into a benzotriazole moiety with NaNO$_2$ and then displaced by the thiol additive in the ligation buffer to afford the peptide thioester in situ.[73] The TFA-cleaved product **3a** showed satisfactory homogeneity (~60%) (Scheme 5).

3.6 | Synthesis of [Thz-371, Ser-Nbz-428]-IgG1-Fc 371-428 (5)

Fragment **5** was prepared by following the same protocol used for the synthesis of fragment **1c** (Scheme 3). To maintain a periodic distribution of the pseudoprolines, an additional pseudoproline dipeptide (Glu-386-ψSer-387) was incorporated. After activation of the Dbz
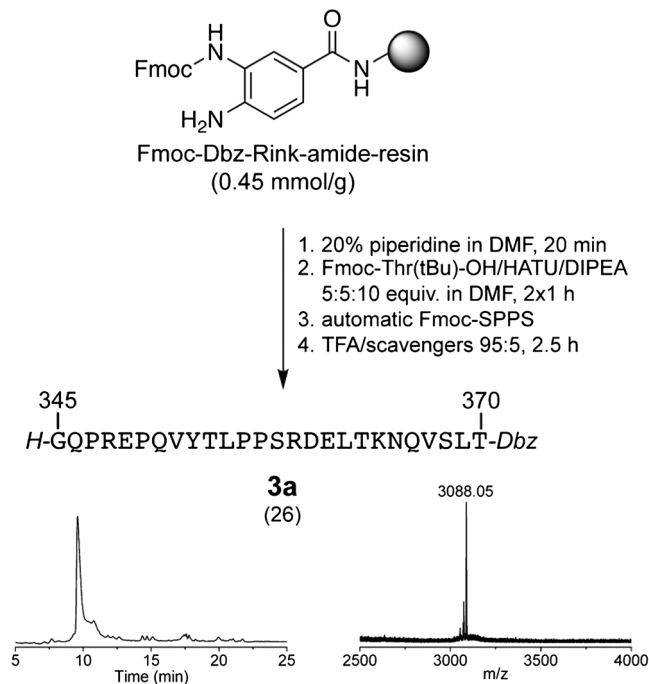
SCHEME 5 Synthesis of [Thr-Dbz-370]-IgG1-Fc 345-370 (**3a**). The RP-HPLC profile of the crude product was obtained with method B (MALDI-TOF-MS peak for M + H$^+$. M$_{calc.}$ for C$_{135}$H$_{216}$N$_{40}$O$_{43}$: 3087.47 Da)
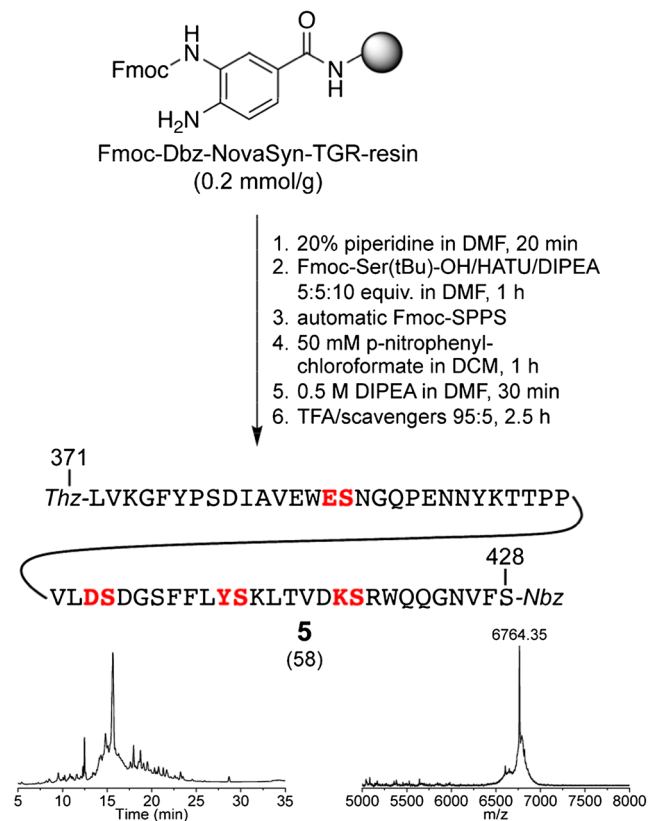
SCHEME 6 Synthesis of [Thz-371, Ser-Nbz-428]-IgG1-Fc 371-428 (**5**). The fragment was prepared on a Fmoc-Dbz NovaSyn TGR resin by using four pseudoproline dipeptides (in red). The RP-HPLC profile of the crude product was obtained with method B (MALDI-TOF-MS peak for M + H$^+$. M$_{calc.}$ for C$_{308}$H$_{445}$N$_{77}$O$_{94}$S$_1$: 6762.50 Da)

linker and TFA cleavage, the desired 58-residue long C-terminal Nbz peptide was obtained with poor homogeneity (~25%), as shown in Scheme 6. In an attempt to purify **5** by HPLC, we encountered some difficulties that were associated with the tendency of the C-terminal Nbz moiety to hydrolyze as well as with the poor solubility of the crude peptide in the HPLC elution system (TFA/ACN/H_2O).
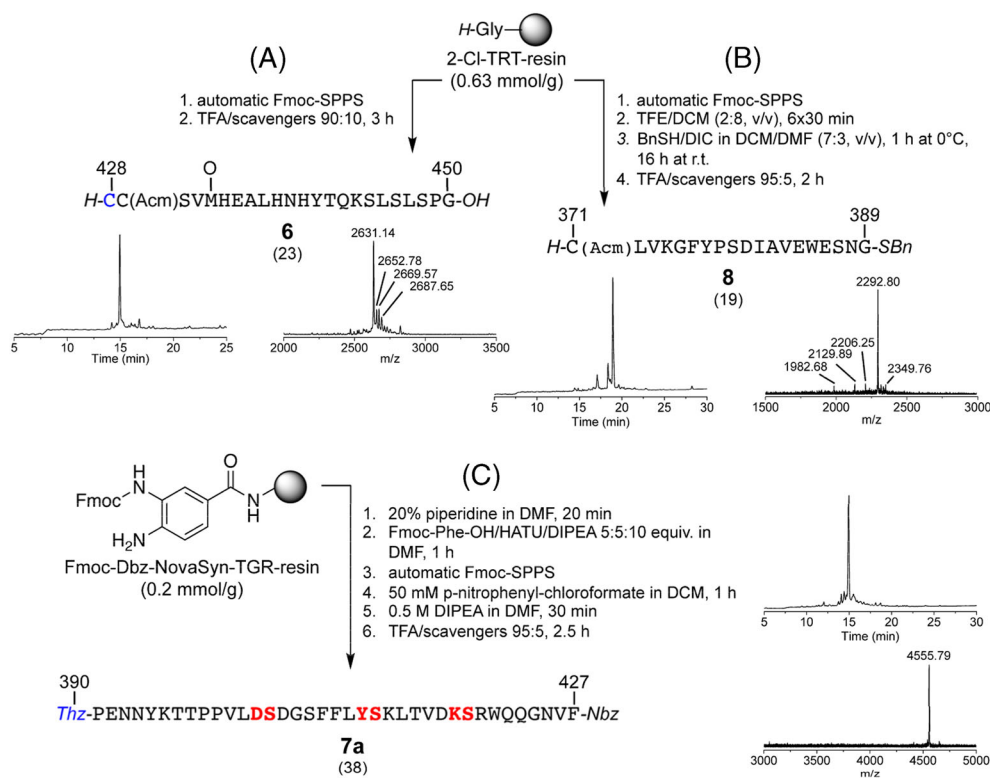
3.7 | Assembly of [Cys(Acm)-371,429, Cys-390,428]-IgG1-Fc 371-450 (10)

Although known strategies addressing the synthesis of C-terminal salicylaldehyde peptide esters offer a solution to the epimerization risk of fragment **2**,[71,72] its very poor solubility in pyridine/AcOH prompted us to explore a way to perform the assembly of the IgG1 fragment 371 to 450 in aqueous solution by Cys-based NCL. However, this requires the presence of cysteine residues at suitable ligation sites, which is not the case for the native Cys-371 and Cys-429 (see Scheme 3 for ligation at Cys-429: although the ligation product was quickly built, concurrent side-reactions leading to thioester degradation were also fast). Therefore, Ser-428 and Gln-390 were substituted with cysteine, in order to exploit highly ligation-favourable sites at Phe-427 and Gly-389[42] and also to have the possibility to achieve the assembly of the native IgG1 CH3

domain in the future (Scheme 1C). Indeed, desulfurization/deselenization protocols allow performing NCL also in the absence of native cysteine residues. In this specific case, selenocysteine[54] and γ-mercapto-glutamine[74] would be suitable precursors for Ser-428 and Gln-390. With this in mind, we incorporated the native Cys-429 and Cys-371 in the Acm-protected form.

For the synthesis of [Cys(Acm)-371,429, Cys-390,428]-IgG1-Fc 371–450 (**10**), we prepared three new fragments (**6–8**) as shown in Scheme 7. Fragment **6** was obtained with ~60% homogeneity. Fragment **7a** (the N-terminally Thz-protected version of **7**) was obtained by Fmoc-SPPS on low-loaded Fmoc-Dbz NovaSyn TGR resin that was preloaded manually with Fmoc-Phe-OH. Three pseudoproline dipeptides were incorporated (Lys-418-ψSer-419, Tyr-411-ψSer-412, and Asp-403-ψSer-404). Fragment **8** was prepared by C-terminal thioesterification of the fully protected peptide acid in solution, and its homogeneity was ~60%.

Then, we proceeded with the ligation of the three peptides, starting with **6** and **7a** that were mixed together in phosphate buffer (pH 7) containing 6 M GuHCl (Scheme 8A). MPAA was added for in situ conversion of the N-acyl-Nbz moiety into an aryl thioester. After 2-hour reaction, methoxylamine was added at pH 4 and left react for 6 hours to convert the N-terminal thiazolidine into cysteine and deliver peptide **9**. Some impurities were present, which arose from


SCHEME 7 Synthesis of the fragment precursors **6**, **7a**, and **8** for the NCL-mediated assembly of [Cys(Acm)-371,429, Cys-390,428]-IgG1-Fc 371-450 (**10**). A, Fragment **6** was prepared on a 2-chlorotrityl resin preloaded with glycine. The RP-HPLC profile of the crude product was obtained with method A (MALDI-TOF-MS peak for M + H^+. $M_{calc.}$ for $C_{110}H_{173}N_{33}O_{36}S_3$: 2630.00 Da. The other annotated masses correspond to sodium and potassium adducts and plus tBu group. Lower masses were also detected, indicating the presence of deleted sequences). B, Fragment **8** was prepared on a 2-chlorotrityl resin preloaded with glycine. The RP-HPLC profile of the crude product was obtained with method B (MALDI-TOF-MS peak for M + H^+. $M_{calc.}$ for $C_{106}H_{151}N_{23}O_{30}S_2$: 2291.65 Da. The other annotated masses indicate the presence of deleted sequences and a tBu group). C, Fragment **7a** was prepared on a Fmoc-Dbz NovaSyn TGR resin by using three pseudoproline dipeptides (in red). The RP-HPLC profile of the crude product was obtained with method B (MALDI-TOF-MS peak for M + H^+. $M_{calc.}$ for $C_{207}H_{299}N_{53}O_{62}S_1$: 4554.07 Da)
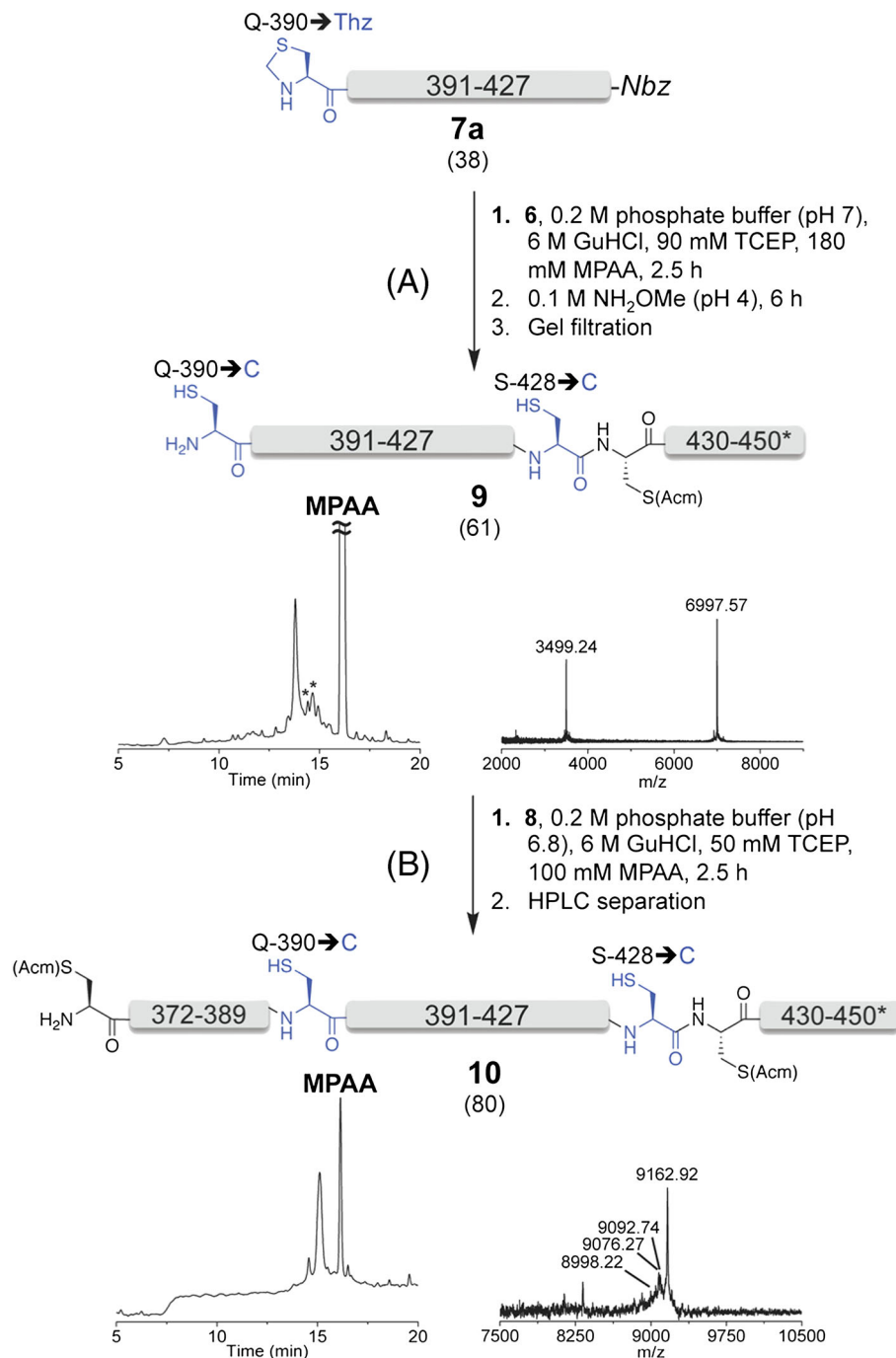
SCHEME 8 Synthesis of [Cys(Acm)-371,429, Cys-390,428]-IgG1-Fc 371-450 (**10**) by tandem chemoselective ligation. A, Fragments **6** and **7a** were ligated in the presence of MPAA. The ligation product **9** was obtained after treatment with NH_2OMe. The RP-HPLC profile of the crude product was obtained with method B. The mass spectrum of the major HPLC peak is shown (MALDI-TOF-MS peak for M + H^{+}. $M_{calc.}$ for $C_{308}H_{465}N_{83}O_{96}S_4$: 6994.89 Da). The HPLC peaks labelled with * were attributed to the N-terminally deprotected peptide acid deriving from hydrolysis of peptide **7a** (MALDI-TOF-MS peak for M + H^{+} = 4384.76 Da. $M_{calc.}$ for $C_{198}H_{294}N_{50}O_{61}S_1$: 4382.91 Da) and its C-terminal methoxylamide derivative (MALDI-TOF-MS peak for M + H^{+} = 4414.20 Da. $M_{calc.}$ for $C_{199}H_{297}N_{51}O_{61}S_1$: 4411.96 Da). B, The second ligation between **9** and **8** was performed after removal of excess NH_2OMe by gel filtration. Also, in this case, MPAA was added to convert the benzyl thioester into an aryl thioester in situ. After 2.5 h, the ligation product **10** was isolated by RP-HPLC. The RP-HPLC profile of the collected fraction was obtained with method B (MALDI-TOF-MS peak for M + H^{+}. $M_{calc.}$ for $C_{407}H_{608}N_{106}O_{126}S_5$: 9162.34 Da. The annotated lower masses indicate the presence of deleted sequences)

the hydrolysis and methoxylaminolysis of the residual amount of unreacted peptide **7a**. Before performing the second ligation with the peptide thioester **8**, the mixture was eluted through a pre-packed gel-filtration column to remove the excess of methoxylamine that, otherwise, would have reacted with **8**. The eluate was

lyophilized, re-dissolved in the ligation cocktail at pH 6.8 in the presence of MPAA, and left react with the peptide thioester **8** till full consumption of the latter (Scheme 8B). The ligation product **10** was finally isolated by RP-HPLC; however, some MPAA as well as some impurities due to deleted sequences were still present.

Secondary structure of the IgG1 Fc CH3 domain
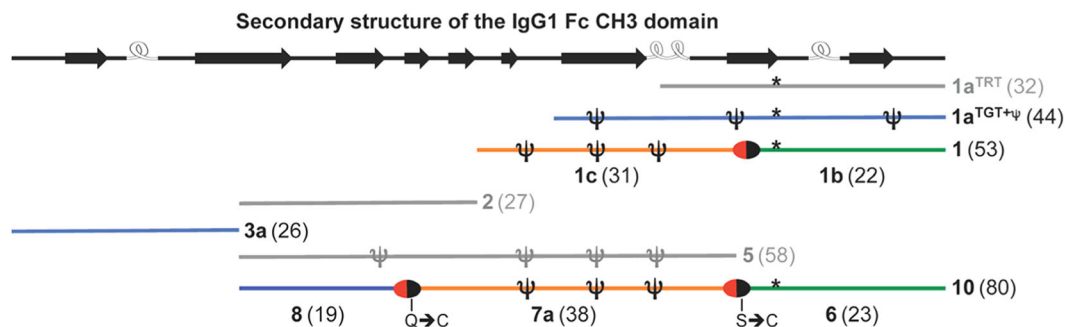
FIGURE 2 Summary of the synthetic IgG1 Fc CH3 fragments of this study. All fragment precursors were obtained with homogeneity of ~60% as crude products, except those colored in grey that were less homogeneous. The red/black ovals represent the ligation sites. The * represents Met(O)-432. On the top, the secondary structure (β-strands and helical turns) composition of the IgG1 Fc CH3 domain is shown (based on the crystal structure with PDB ID 1OQO)

4 | CONCLUSIONS

In this work, we evaluated the synthesis of different fragments of the IgG1 Fc CH3 domain with regard to peptide-chain length and preparation of C-terminal thioesters or thioester precursors. Stepwise elongation from the C-terminal Gly-450 by Fmoc-SPPS became challenging after Asn-425. However, the simultaneous use of pseudoproline dipeptides and a polar, low-loaded resin allowed reaching Ser-407 (fragment 1a^TGT+ψ with 44 residues). Unfortunately, this fragment could not be elongated further, but it was possible to assemble the C-terminal fragment 398 to 450 (1) by using NCL between an Nbz-activated peptide (1c, 398-428) and an N-terminal Cys-peptide (1b, 429-450). These results suggest that the C-terminal region, once reached a length of more than 22 residues, starts to aggregate, thus preventing an efficient stepwise elongation. The presence of β-sheet breakers like pseudoprolines and the use of low-loaded, highly swelling resins partially reduced the formation of aggregates, but this was not sufficient to obtain sequences larger than 44 residues, as also shown for the 58-residue long fragment 371 to 428 (5) (Figure 2). However, optimization of the protocol for the stepwise Fmoc-SPPS might still be possible by the application of polyethylene glycol resins like ChemMatrix,[75] backbone-amide protecting groups like Dmb (dimethoxybenzyl)[76,77] and Hmb (hydroxymethoxybenzyl),[78] or the O-acyl isopeptide method.[79-83] Moreover, heat-assisted SPPS has been recently very successful in the synthesis of very difficult sequences.[84-91]

We did not investigate the accessibility of the N-terminal fragment 345 to 397 by stepwise elongation, but rather prepared the two shorter fragments 2 and 3. Unfortunately, the Sal-activated peptide 2 was poorly soluble in the ligation solvent (pyridine/AcOH). Thus, the assembly route to obtain the IgG1 Fc CH3 sequence was redesigned on the base of only Cys-based NCL. To test the feasibility of this route, besides using the native Cys-371, we decided to incorporate two non-native Cys residues in place of Ser-428 and Gln-390. The choice of the ligation points at these sites was dictated by the following reasons: (1) the length of the four fragment precursors was between 19 and 38; (2) the presence of the non-native Cys-428 allows the ligation with a fast-reacting C-terminal glycine thioester; (3) the ligation at the native Cys-429 was not ideal due to fast

degradation of the thioester precursor, which led us move the ligation site at the non-native Cys-428; and (4) in future, the use of Sec-428 and γ-mercapto-Gln-390 would allow obtaining the native Ser-428 and Gln-390 residues upon deselenization/desulfurization. Three of these fragments were ligated in a tandem reaction to give the sequence 371–450 (10). Although this tandem ligation looks promising, the third ligation might be challenging, due to the presence of threonine at the ligation site (Scheme 1). We conducted preliminary ligation experiments between 3a (Scheme 5) and the C-terminal free acid of 8 (Scheme 7B). Peptide 3a contains the Dbz moiety at the C-terminal Thr-370. As the Dbz peptide precursor 3a could not be converted into the Nbz form by using 4-nitrophenylchloroformate in DCM, we repeated the reaction in DMF: indeed, Brik and coworkers reported that the use of DMF instead of DCM could convert Dbz into Nbz on a peptide bearing a C-terminal leucine.[63] Unfortunately, in our case, the use of DMF did not solve the problem, and no Nbz peptide was formed (probably, because of the presence of the β-branched threonine). Thus, we activated the Dbz peptide 3a by converting it into an N-acyl-benzotriazole peptide by using NaNO₂ at pH~3, as previously shown by Liu and coworkers.[73] For the Acm cleavage from the C-terminal free acid of 8, we used the Pd-catalyzed reaction reported by Brik and coworkers.[92] Both reactions were successful, but, unfortunately, no ligation product was detected after 18-hour reaction. We did not try to change further the protocol, as the slow ligation rate of the C-terminal threonine is likely to prevent an efficient synthesis. An alternative would be to move the third ligation site between Gly-375 and Phe-376, which would be advantageous for the preparation of the C-terminal Nbz peptide precursor as well as for the ligation rate. However, β-mercapto-Phe must be used, whose synthesis and use in NCL have been previously reported.[93] Another way would be the stepwise synthesis of the segment 345 to 389, which would avoid a third ligation.

In conclusion, this explorative study has shed light on the behaviour of different fragments of the IgG1 Fc CH3 domain during the standard Fmoc-SPPS as well as NCL, which will be useful to plan future syntheses. Once completed the CPS of the CH3 domain, it will be necessary to test, if the synthetic bis(cysteinyl)-polypeptide chain can undergo oxidative folding and build the expected non-covalent homodimer.

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