Structure-based discovery of small molecules improving stability of human broadly-neutralizing anti-HIV antibody 2F5 in plant suspension cells

Manoj K. Mandal1 | Thales Kronenberger2 | Marie I. Zulka1 | Björn Windshügel2,3 | Andreas Schiermeyer1

1 Fraunhofer Institute for Molecular Biotechnology and Applied Ecology IME, Aachen, Germany
2 Fraunhofer Institute for Translational Medicine and Pharmacology ITMP, Discovery Research ScreeningPort, Hamburg, Germany
3 Department of Life Sciences and Chemistry, Jacobs University Bremen, Bremen, Germany

Abstract

The production of biopharmaceuticals in engineered plant-based systems is a promising technology that has proven its suitability for the production of various recombinant glyco–proteins that are currently undergoing clinical trials. However, compared to mammalian cell lines, the productivity of plant-based systems still requires further improvement. A major obstacle is the proteolytic degradation of recombinant target proteins by endogenous plant proteases mainly from the subtilisin family of serine proteases. In the present study, the authors screened for putative small molecule inhibitors for subtilases that are secreted from tobacco BY-2 suspension cells using an in silico approach. The effectiveness of the substances identified in this screen was subsequently tested in degradation assays using the human broadly-neutralizing anti-HIV monoclonal antibody 2F5 (mAb2F5) and spent BY-2 culture medium as a model system. Among 16 putative inhibitors identified by in silico studies, three naphthalene sulfonic acid derivatives showed inhibitory activity in in vitro degradation assays and are similar to or even more effective than phenylmethylsulfonyl fluoride (PMSF), a classical inhibitor of serine proteases, which served as positive control.

KEYWORDS
biopharmaceuticals, molecular farming, protease inhibitor, protein modeling, virtual screening

1 | INTRODUCTION

The production of biopharmaceuticals in plant-based systems, commonly referred to as molecular farming, has made significant progress during the last decade.[1] Several plant-derived biopharmaceuticals are currently evaluated in clinical trials and one product for the treatment of Gaucher’s disease has already obtained market approval.[2] Plants and plant cell lines have been engineered to optimize the glycosylation profile to match N-glycosylation patterns usually found on human proteins. It is therefore possible to produce authentic human glycoproteins (e.g., enzymes, antibodies) for the treatment of various conditions in plant-based systems.[3] One major obstacle for the broader application of plant molecular farming is its moderate productivity compared to mammalian cells. The lower productivity is attributed to the presence of various endogenous proteases that degrade recombinant proteins.[4,5] Among the diverse proteases secreted from plant cells, members of the subtilase family are especially abundant.[6,7] Accord-
ing to the MEROPS classification, subtilases belong to the S8A family of serine proteases. A couple of secreted subtilases have been identified from tobacco suspension cells and their encoding genes have been cloned. Inhibitor studies using intercellular washing fluid of Nicotiana benthamiana (Domin) leaf samples or conditioned tobacco BY-2 cell culture medium demonstrated the involvement of serine protease in the degradation process of recombinant human antibodies. The subtilase family in plants is diverse, comprising 56 members in both Arabidopsis thaliana (L.) Heynh. and N. benthamiana. Currently it is not known which particular members of the subtilase family interfere with the production of recombinant proteins, and therefore a broad inhibition is desirable. As the interference of a given small molecule inhibitor with the enzymatic activity differs among members of the subtilase family, broad inhibition might require the use of multiple inhibitors that are applied simultaneously. Therefore, a screen for novel types of subtilase inhibitors is required to suppress unwanted proteolysis of recombinant proteins.

In the present study we used an in silico approach to screen for potential subtilase small molecule inhibitors. Taking advantage of the known three-dimensional structures of SBT3 from Solanum lycopersicum, we generated structural models of two selected subtilases from tobacco that are particularly abundant in spent BY-2 culture medium. Using a structure-based virtual screening approach, putative small molecule inhibitors were identified. In subsequent biochemical degradation assays, three out of sixteen candidate molecules were proven to effectively stabilize the mAb2F5 in conditioned culture medium of tobacco BY-2 cells.

2 | EXPERIMENTAL SECTION

2.1 | Three-dimensional modeling of tobacco subtilases

Sequences for subtilisin-like proteases SBT1.1 (GenBank ID: ABQ58079) and SBT1.5 (GenBank ID: AIX97848) from Nicotiana tabacum were retrieved from the UniProt database. NtSBT1.1 and NtSBT1.5 sequences were initially edited by removing the first 23 amino acids, which were annotated as signal peptides. Since SBTs were known zymogens, sequences were further trimmed to obtain the mature active form, by removing the propeptides spanning the regions from amino acid 24 to His110 and His106, respectively. Three-dimensional protein structures were generated by homology modeling using the Modeller (v9.1) program. SBT3 from S. lycopersicum (PDB ID: 316S chain A, resolution 2.5 Å and 3174-chain A, resolution 2.6 Å) were chosen as modeling template, due to the highest sequence similarity (NtSBT1.1: 57%, NtSBT1.5: 55%). The model quality was assessed using MolProbity.

The protein models were prepared by adding hydrogen and adjusting ionization states of amino acid side chains using H++ with standard options. Afterwards, the models were further refined with a minimization procedure involving gradually releasing atom constraints using SYBYL-X 2.0 (Certara Inc.).

2.2 | In silico screening for potential inhibitors

Compounds to be docked were downloaded as SMILES from the ZINC database. Three-dimensional structures were generated using Molecular Operating Environment (MOE) version 2015.1001 (Chemical Computing Group Inc.), followed by minimization using Amber10:EHT force field with implicit solvation model (R-Field).

Molecular docking was carried out using GOLD version 5.4.1 (Cambridge Crystallographic Data Centre) and the GoldScore scoring function. Compounds were docked into a sphere of 7.5 Å radius defined around the catalytic residues. For each compound, 15 docking runs were carried out. The early termination option was switched off and pyramidal nitrogen atoms in ligands were allowed to flip. The results were evaluated according to the docking scores, the ability of the ligand to interact with the catalytic triad, the number of hydrogen bonds using the GOLDMine program, followed by visual inspection of the docking poses.

2.3 | Plant cell cultivation

Suspension cells of Nicotiana tabacum cv. BY-2 were maintained in Murashige and Skoog (MS) medium (Duchefa, Netherlands) at 26°C in the dark on an orbital shaker (Adolf Kühner AG, Switzerland) at 140 rpm. By completion of a cultivation cycle at day 7, fresh MS-medium was seeded with a 4% v/v inoculum from the previous culture.

Transgenic tobacco BY-2 cell lines expressing mAb 2F5 were generated as described and screened by Western blot analysis. The selected cell line, BY-2 2F5-DsRed #26 was maintained in MS medium for regular maintenance on a weekly subculturing scheme. The cells were cultured in the dark with constant orbital agitation of 140 rpm. Prior to inhibitor studies the cells were transferred to D11b culture medium to facilitate optimal antibody secretion.

2.4 | In vitro degradation assays

Test compounds (Table 1) were purchased from Vitas-M Laboratory (Hong Kong), TCI (Germany), Enamine (Latvia), AppliChem (Germany), or Merck (Germany) and dissolved in DMSO (Merck) at 10 mM.

The broadly neutralizing human anti-HIV gp41 monoclonal antibody 2F5 (Pollymun, Austria) was used as a model protein. 1 µg of mAb 2F5 was spiked into spent BY-2 culture medium from day 7, supplemented with 10 mM MES, pH 5.8, 2 mM L-cysteine, 12 mM methyl-beta-cyclodextrin (final volume: 25 µl). For screening purposes, inhibitors were added at a final concentration of 1 mM. Samples were incubated at 26°C for three days and the reactions stopped by the addition of LDS sample buffer with reducing agent (Thermo Fisher Scientific, Germany) and subsequent heat treatment at 95°C for 10 min. Aliquots were subsequently analyzed by immunoblot. The mixtures were separated by polyacrylamide gel electrophoresis using NuPAGE 4%-12% Bis-Tris mini gels (Thermo Fisher Scientific).
The gels were subsequently blotted onto nitrocellulose membranes using the Semi-Dry device (BioRad, Germany). The mAb 2F5 chains were detected on the membrane by incubation with HRP-labeled goat anti-human IgG (H+L) affinity-purified antibodies (Dianova, Germany). HRP activity was revealed using the SuperSignal West Pico PLUS chemiluminescent substrate (Thermo Fisher, Germany) and the light signal detected with a CCD camera using the UVP ChemStudio PLUS imaging system (Analytik Jena, Germany).

3 | RESULTS

3.1 | Modeling of tobacco subtilases and virtual screening for inhibitors

The sequences of NtSBT1.1 and NtSBT1.5 share high sequence similarity with S. lycopersicum SB Tb3 (57% and 55%, respectively). Using a standard homology modeling approach, we generated structural models for both enzymes (Figure S1). Analysis of the stereochemical quality of the models using a Ramachandran plot revealed seven (1.1%, NtSBT1.1) and six (0.9%, NtSBT1.5) residues located in the unfavorable region. All of these are located in the loop regions.

In order to identify putative NtSBT1.1 and NtSBT1.5 inhibitors, a structure-based approach was utilized. This involved virtual screening of a library of commercially available compounds by docking all molecules into the active site of both structural models using the molecular docking program GOLD. Initially, we prioritized the compounds based on the docking scores. All compounds with a score above 30 were retained. Subsequently, the top-ranked docking poses were visually inspected with respect to the binding modes. Only molecules for which the docking poses showed formation of polar interactions with one of the catalytic residues were considered as potential hits. A table with the docking scores of all compounds selected for experimental testing is provided in the Supporting Information (Table S1).

Eventually, ten compounds were selected for experimental validation of which eight were purchasable and soluble in DMSO (Figure 1A).

3.2 | In vitro degradation assays of mAb 2F5

The broadly neutralizing monoclonal anti-HIV antibody 2F5 has been proven unstable in spent culture medium from tobacco BY-2 suspension cells. Therefore, the spiking of intact, recombinantly produced, mAb2F5 into spent culture medium from BY-2 cells, provides a

| Name                                      | CAS     | ZINC ID     | Vendor   | Order number |
|-------------------------------------------|---------|-------------|----------|--------------|
| a) Identified substances from screen      |         |             |          |              |
| 2-Aminobenzene-sulfonic acid             | 88-21-1 | ZINC1530395 | Vitas-M  | STL163408    |
| 2-Amino-5-(1-methylethyl) benzenesulfonic acid | 369609-63-2 | ZINC2350614 | Vitas-M  | STK529947    |
| 8-Amino-1-naphthalenesulfonic acid       | 82-75-7 | ZINC1688378 | TCI      | A0350        |
| 5-Hydroxy-1-naphthalenesulfonic acid     | 117-59-9 | ZINC1700210 | Vitas-M  | STK370832    |
| 4-Amino-1-naphthalenesulfonic acid       | 84-86-6 | ZINC1555329 | Vitas-M  | STK379926    |
| 5-Amino-1-naphthalenesulfonic acid       | 84-89-9 | ZINC1683633 | Enamine  | EN300-123988 |
| 4-Amino-5-hydroxy-1-naphthalenesulfonic acid | 83-64-7 | ZINC1683638 | TCI      | A0369        |
| 5-Amino-4-hydroxy-1-naphthalenesulfonic acid | 571-79-9 | ZINC5933703 | Vitas-M  | STL426736    |
| 4,5-Diamino-1-naphtalenesulfonic acid    | 6362-18-1 | ZINC2266329 | Vitas-M  | STK525462    |
| 3-Amino-1,5-naphtaledisulfonic acid      | 131-27-1 | ZINC1683641 | Vitas-M  | STK426739    |
| 8-mercapto-5-quinolone-sulfonic acid     | 5825-36-5 | ZINC2566827 | Vitas-M  | STK741645    |
| 6-Methyl-1H-benzimidazole2-sulfonic acid | 106135-27-7 | ZINC6023309 | Vitas-M  | STK213105    |
| 6-Amino-9H-purine-8-sulfonic acid       | 696638-47-8 | ZINC2486839 | Vitas-M  | STK523846    |
| 6-Amino-2,3-dihydro-2-oxo-1H-benzimidazole-5-sulfonic acid | 330991-27-0 | ZINC2765676 | Vitas-M  | STK667413    |
| 6-Amino-9-(phenylmethyl)-9H-purine-8-sulfonic acid | 704874-89-5 | ZINC3656490 | Vitas-M  | STK132191    |
| 1,2-Dihydro-2-oxobenz[cde]indole-6,8-disulfonic acid | 301851-27-4 | ZINC1803012 | Vitas-M  | STK238422    |
| b) Standard serine protease inhibitors   |         |             |          |              |
| Phenylmethylsulfonyl fluoride (PMSF)      | 329-98-6 | ZINC8220691 | AppliChem | A0999        |
| 4-(2-Aminoethyl) benzenesulfonyl fluoride (AEBSF) | 34284-75-8 | ZINC8034834 | AppliChem | A1421        |
| 6-Amino-2,3-dihydro-2-oxo-1H-benzimidazole-5-sulfonic acid | 330991-27-0 | ZINC2765676 | Vitas-M  | STK667413    |

The broadly neutralizing monoclonal anti-HIV antibody 2F5 has been proven unstable in spent culture medium from tobacco BY-2 suspension cells. Therefore, the spiking of intact, recombinantly produced, mAb2F5 into spent culture medium from BY-2 cells, provides a
simple assay to identify protease inhibitors. In a first screening round, compounds shown in Figure 1A were tested in a final concentration of 1 mM. Among those molecules (Figure 2, substances labeled blue), 4,5-diamino-1-naphtalenesulfonic acid (CAS 6362-18-1) was identified as a potent inhibitor of mAb2F5 degradation and performed similar compared to the established protease inhibitor PMSF. Notably, AEB SF, TPCK, and antipain were not effective in degradation inhibition. In contrast, none of the other tested compounds revealed any significant inhibition. In order to carry out preliminary structure-activity relationship studies and to validate the scaffold as generally suited for protease inhibition, structurally similar compounds were selected from the ZINC database. Altogether eight compounds were selected (Fig-
FIGURE 3 Potential binding modes of active and inactive compounds within the structural model of SBT1.5 using molecular docking. Representative docking poses of CAS 6362-18-1 (carbon atoms in green) and CAS 84-89-9 (carbon atoms in pink) are shown as capped sticks. The molecular surface of SBT1.5 in proximity to the docked ligands is shown together with the catalytic triad as well as Ser219

ure 1B) and tested at final concentrations of 1 mM. Of these additional compounds (Figure 2, substances labeled in orange), 5-amino-4-hydroxy-1-naphtalenesulfonic acid (CAS 571-79-9) and 4-amino-5-hydroxy-1-naphtalenesulfonic acid (CAS 83-64-7) effectively inhibited degradation of the mAb2F5 heavy chain. Docking of these analogues into the active site of SBT1.5 showed different binding modes for the inactive compounds compared to CAS 6362-18-1 (Figure 3). In contrast, the two additional active substances CAS 571-79-9 and CAS 83-64-7 share an almost identical binding mode with CAS 6362-18-1.

Furthermore, the three effective substances identified by our screening procedure as well as PMSF were used to explore the minimum effective concentration for mAb2F5 stabilization. An in vitro spiking experiment was performed using inhibitor concentrations of 500 µM, 100 µM, and 10 µM, respectively. For all four substances, only the highest concentration (500 µM) was effective to prevent the degradation of the mAb2F5 heavy chain as shown in Figure 4. Among the tested substances 4-amino-5-hydroxy-1-naphtalenesulfonic acid (CAS 83-64-7) and 5-amino-4-hydroxy-1-naphtalenesulfonic acid (CAS 571-79-9) acid seem to be most effective as no sign of heavy chain degradation is visible while for PMSF and 4,5-diamino-1-naphtalenesulfonic acid (CAS 6362-18-1) at 500 µM concentration, weak signs of heavy chain degradation could be observed.

3.3 In vivo effects of inhibitor application

For a first evaluation of the in vivo effects, we added compound CAS 83-64-7 at a final concentration of 500 µM to a transgenic BY-2 cell culture that secretes mAb2F5 into the culture medium. Parallel BY-2 cell cultures were supplemented with CAS 83-64-7 either once on day 6, twice (days 5 and 6), three times (days 4, 5, and 6) or four times (days 0, 4, 5, and 6). The cultures were harvested on day 7 and the integrity of the 2F5 antibody in the culture supernatant was assessed by Western blot analysis (Figure 5A). In the culture that received four applications of CAS 83-64-7, an almost complete absence of the 40 kDa degradation band was observed. To evaluate any potential negative effects of the substance on cell growth, the final packed cell volume (PCV) of the different cultures was determined. With increasing frequency of CAS 83-64-7 application to the cell culture, the PCV dropped from 52.3% in the control fermentation without inhibitor addition to 40.9% in the fermentation that received four applications of CAS 83-64-7 (Figure 5B).

4 DISCUSSION

The application of in silico approaches for the identification of novel bioactive substances has become a standard approach in various research areas.[26–28] Here we combined protein modeling of two subtilases with a virtual screen for novel enzyme inhibitors. This led to the identification of a novel compound that proved to be effective in our biochemical assay and prevented the degradation of mAb2F5 heavy chain in conditioned BY-2 culture medium. A hit expansion approach using a similarity search procedure further identified two even more potent compounds.

Our docking studies suggest that the 4,5-dual substituted naphthalene sulfonic acid scaffold seems to be particularly suited to closely interact with the catalytic serine residue within the active site of the target enzymes. The presence of a hydroxyl or amino group in position 4 and 5 is essential for the compound’s function. Structurally similar compounds with only one substitution, for example, 5-amino-1-naphtalene sulfonic acid (CAS 84-89-9) did not show inhibitory potential in the in vitro degradation assay (Figure 2). This finding is supported by
In vivo application of inhibitor. Parallel cultures of transgenic BY-2 cells producing mAb 2F5 were supplemented with CAS 83-64-7. Inhibitor was applied once (day 6), twice (days 5 and 6), three times (days 4, 5, and 6), or four times (days 0, 4, 5, 6) at final concentrations of 500 µM. Equal volumes (10 µl) of culture supernatants were subjected to SDS-PAGE and immunoblotting. The antibody chains were detected using an anti-human IgG (H+L) antibody and ECL (A). The intact heavy chain is marked with a filled arrow head and the heavy chain degradation product is marked with an open arrow head. The packed cell volume (PCV) from each culture was determined and is shown for all treatment groups (B).

The three molecules that we have identified as potent inhibitors have previously not been analyzed for their potential to inhibit serine proteases. Rather, they have a long history as reactants for the synthesis of azo dyes in organic chemistry.[29]

A striking difference regarding their inhibitory potential has also been observed for the classical serine protease inhibitors PMSF and AEBSF (Figure 2). While their structure is rather similar, with the AEBSF molecule possessing an ethylamino group in the para position (Figure 1), only PMSF functions as an effective inhibitor in our biochemical degradation assay. Given these strong differences in inhibitor functions of structurally similar molecules, it is advisable to screen multiple different inhibitors to safeguard a target protein in a given biological matrix. In this respect, it is of particular interest to note the striking difference between PMSF and AEBSF as these inhibitors are often regarded as being equally effective and AEBSF is generally advertised as being preferable due to its lower toxicity compared to PMSF.[30]

To assess the implementation of the identified inhibitors in a production process, we analyzed the effect of CAS 83-64-7 on mAb 2F5 secretion from a transgenic tobacco BY-2 cell line. In the application scheme that involved four additions of this compound over a seven day culture period (Figure 5A), a nearly complete disappearance of the 40 kDa heavy chain degradation band could be observed, indicating successful in vivo inhibition of subtilisin-like proteases involved in the 2F5 heavy chain degradation process. As a side effect we noticed that the final PCV of the BY-2 cultures gradually declined, as the application frequency of CAS 83-64-7 increased (Figure 5B). Therefore, it is likely that CAS 83-64-7 does not only interfere with the activity of secreted subtilisin-like proteases that act on recombinant mAb 2F5 but also – upon uptake into the cell – with other members of the subtilase family that possess roles in cell homeostasis. To gain a better understanding of these in vivo effects, the identification of the extra- and intracellular target proteases of CAS 83-64-7 is required. For the identification of those proteases an activity-based protein profiling approach could be used as recently described for the identification of apoplastic subtilisin-like proteases from N. benthamiana leaves.[16] Once the target proteases for CAS 83-64-7 have been identified it might be possible to optimise the inhibitor structure so that this molecule targets preferably those proteases involved in mAb degradation while preserving the activity of other proteases that are not involved in the degradation of recombinant proteins.

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CONFLICT OF INTEREST
The authors declare no financial or commercial conflict of interest.
DATA AVAILABILITY STATEMENT
Data available on request from the authors

ORCID
Andreas Schiermeyer https://orcid.org/0000-0002-2057-4111

REFERENCES

1. Schiermeyer, A. (2020). Optimizing product quality in molecular farming. Current Opinion in Biotechnology, 61, 15–20. https://doi.org/10.1016/j.copbio.2019.08.012.

2. Zimran, A., Durán, G., Giraldo, P., Rosenbaum, H., Giona, F., Petakov, M., Terreros-Muñoz, E., Solorio-Meza, S. E., Cooper, P. A., Varughese, S., Alon, S., & Chertkoff, R. (2019). Long-term efficacy and safety results of taliglucerase alfa through 5 years in adult treatment-naïve patients with Gaucher disease. Blood Cells, Molecules and Diseases, 78, 14–21. https://doi.org/10.1016/j.jbcmd.2016.07.002.

3. Montero-Morales, L., & Steinkellner, H. (2018). Advanced plant-based glycan engineering. Frontiers in Bioengineering and Biotechnology, 6, 81. https://doi.org/10.3389/fbioe.2018.00081.

4. Jutras, P. V., Dodds, I., & van der Hoorn, R. A. L. (2020). Proteases of Nicotiana benthamiana: An emerging battle for molecular farming. Current Opinion in Biotechnology, 61, 60–65. https://doi.org/10.1016/j.copbio.2019.10.006.

5. Grosse-Holz, F., Kelly, S., Blaskowski, S., Kaschani, F., Kaiser, M., & van der Hoorn, R. A. L. (2018). The transcriptome, extracellular proteome and active secretome of agroinfiltrated Nicotiana benthamiana uncover a large, diverse protease repertoire. Plant Biotechnology Journal, 16, 1068–1084. https://doi.org/10.1111/pbi.12852.

6. Hoernstein, S. N. W., Fode, B., Wiedemann, G., Lang, D., Niederkrüger, H., Berg, B., Schaaf, A., Frischmuth, T., Schlosser, A., Decker, E. L., & Reski, R. (2018). Host cell proteome of Physcomitrella patens harbors proteases and protease inhibitors under bioproduction conditions. Journal of Proteome Research, 17, 3749–3760. https://doi.org/10.1021/acs.jproteome.8b00423.

7. Santos, R. B., Chandrasekar, B., Mandal, M. K., Kaschani, F., Kaiser, M., Both, L., van der Hoorn, R. A. L., Schiermeyer, A., & Abranches, R. (2018). Low protease content in Medicago truncatula cell cultures facilitates recombinant protein production. Biotechnology Journal, 13, e1800050. https://doi.org/10.1002/biot.201800050.

8. Rawlings, N. D. (2020). Twenty-five years of nomenclature and classification of proteolytic enzymes. Biochim Biophys Acta. Proteins and Proteomics, 2020, 1688, 140345. https://doi.org/10.1016/j.bbapap.2019.140345.

9. Rawlings, N. D., Barrett, A. J., & Bateman, A. (2012). MEROPS: The database of proteolytic enzymes, their substrates and inhibitors. Nucleic Acids Research, 40, D343–D350. https://doi.org/10.1093/nar/gkr987.

10. Schaller, A., Stintzi, A., & Graff, L. (2012). Subtilases – versatile tools for protein turnover, plant development, and interactions with the environment. Physiologia Plantarum, 145, 52–66. https://doi.org/10.1111/j.1399-3054.2011.01529.x.

11. Millar, D. J., Whitelegge, J. P., Bindschedler, L. V., Rayon, C., Bouvet, A. M., Rossignol, M., Borderies, G., & Bolwell, G. P. (2009). The cell wall and active secretome of agroinfiltrated Nicotiana tabacum cv. Bright Yellow 2 (BY-2) suspension cells. Biotechnology Journal, 9, 1065–1073. https://doi.org/10.1002/biot.201300424.

12. Schaller, A., Stintzi, A., & Graff, L. (2014). The human anti-HIV antibodies 2F5, 2G12, and PG9 differ in their susceptibility to proteolytic degradation: Down-regulation of endogenous serine and cysteine proteinase activities could improve antibody production in plant-based expression platforms. Biotechnology Journal, 2014, 9, 493–500. https://doi.org/10.1002/biot.201300207.

13. Mandal, M. K., Fischer, R., Schillberg, S., & Schiermeyer, A. (2014). Inhibition of protease activity by antisense RNA improves recombinant protein production in Nicotiana tabacum cv. Bright Yellow 2 (BY-2)
ing approaches. Molecules (Basel, Switzerland), 25, 4723, https://doi.org/10.3390/molecules25204723

27. Patel, L., Shukla, T., Huang, X., Ussery, D. W., & Wang, S. (2020). Machine learning methods in drug discovery. Molecules (Basel, Switzerland), 25, 5277, https://doi.org/10.3390/molecules25225277

28. da Silva Rocha, S. F. L., Olanda, C. G., Fokoue, H. H., & Sant’Anna, C. M. R. (2019). Virtual screening techniques in drug discovery: Review and recent applications. Current Topics in Medicinal Chemistry, 19, 1751-1767, https://doi.org/10.2174/1568026619666190816101948

29. Freeman, H. S., Whaley, W. M., Esancy, M. K., Esancy, J. F., Freeman Harold S., Whaley Wilson M., Esancy Michelle K. & Esancy James F. (1986). Anomalous behavior of aminohydroxynaphthalenesulfonic acids during diazo coupling. Dyes and Pigments, 7, 215-230. https://doi.org/10.1016/0143-7208(86)85010-0.

30. Mintz, G. R. (1993). An irreversible serine protease inhibitor. BioPharm (Eugene, Oreg.), 6, 34.

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